Protein Refolding Methods for Biomanufacturing

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Thesis submitted for the degree of Doctor of Philosophy

Institute of Cell and Molecular Biosciences

University of Newcastle upon Tyne

March 2008
Abstract

Interferon alpha 2b (IFN-α2b) is a soluble cytokine and a member of the type I interferon (IFN) family of which there are fourteen members. IFN-α2b is used in the treatment of hairy cell leukaemia and both hepatitis B and C. IFN-α2b actions are mediated through specific receptor binding which initiates a series of signalling cascades leading to the transcription of genes carrying the promoters containing the interferon stimulated response element (ISRE). Due to these actions the IFNs as a whole are an ideal family of proteins to be investigated further with the possibility of the production of life saving drug products. Cobra Biomanufacturing have a keen interest in IFN-α2b especially due to its beneficial effects. This project is therefore an investigation of the IFN-α2b molecule from expression to final purified product. The refolding of the IFN-α2b was the main concern of this project due to it being the 'bottleneck' for many protein based processes. The investigation of natively folded IFN-α2b led to the conclusion that IFN-α2b is a stable protein which remains folded under reducing conditions. The addition of guanidine-HCl or urea to the native protein reveals differences in the success of unfolding. These differences were also witnessed during the solubilisation of the IFN-α2b inclusion bodies. The refolding is only successful when the inclusion bodies have been solubilised in guanidine-HCl. Due to the differing refolding results gained, which depended on the denaturant used to solubilise the inclusion bodies, the solubilised inclusion bodies themselves were investigated. This revealed partial structure present in the urea solubilised inclusion bodies; however this was confirmed as being not native. The implications of this for biomanufacturing are that if the starting conditions are not correct even as early as the solubilisation of the inclusion bodies then the following process will result in incorrectly folded product.
The work in this thesis was carried out in the Institute of Cell and Molecular Biosciences, University of Newcastle upon Tyne, during the period of October 2004 – October 2007. The work is original except where acknowledged by reference.

No part of this work is being, or has been submitted for a degree, diploma or any other qualification at this or any other university.
Acknowledgements

I would like to thank my supervisor Jeremy Lakey for your guidance, constant input and most importantly your patience. I will never look at another gin and tonic without seeing it as a fluorescence experiment!

Thank you, to the members of the lab (as I remember it), Andrei, Anton, Beth, David, Helen and Liz it has been a rollercoaster ride but I think we've all emerged unscathed. Helen you are wonder woman, your efforts do not go unnoticed. Thanks to Anton and Liz who became my therapy group for the last 12 months, I miss craft group. Beth, my partner in crime, thanks for joining me in the hysterical laughing/crying fits and always listening whether you had the choice or not, it's been a laugh but I don't miss sharing a bench with you! I wish everyone all the best for the future.

Thank you to NIBSC for putting up with me for a few weeks especially Tony and Paula, who probably felt like they were on mastermind most of the time but answered each and every one of my endless questions.

Thank you to Cobra for paying my bills for three years.

To my grandparents, thank you for the science gene (especially Grandpa, the carotenoid king), I will do my best to keep the Biochemistry flag flying.

Kerrie you have become a great friend and I thank you for the advice and general girly giddiness (especially concerning weddings). Holly you have remained a good friend throughout and as someone who had a very rough time it is comforting to see you happy on the other side. Thanks to Sam who has kept me busy throughout whether it be nights out, make-up parties or just coffee, I look forward to seeing/working with you next year.

To my friends from home who as non-scientists remain constantly impressed with my work, you've always made me smile and have continuously entertained me with many a wedding saga. At last I join you all in the commercial world.
Dedication

For Mum, Dad, Robert, George, Bonnie, Bess, Meg and Andrew

Thank you for your constant support……I needed it.

Andrew you are my star!
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- Aims
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A$_{280}$</td>
<td>Absorbance at 280 nm</td>
</tr>
<tr>
<td>A$_{600}$</td>
<td>Absorbance at 600 nm</td>
</tr>
<tr>
<td>AEBSF</td>
<td>4-(2-aminoethyl) benzenesulphonyl fluoride-HCl</td>
</tr>
<tr>
<td>ANS</td>
<td>8-Anilino-1-naphthalenesulphonate</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CD</td>
<td>Circular Dichroism</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-cholamidopropyl)dimethylamminino]-1-propanesulfonate</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>Da</td>
<td>Daltons</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EMCV</td>
<td>Encephalomyocarditis virus</td>
</tr>
<tr>
<td>g</td>
<td>Relative Centrifugal Force</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IFN-α</td>
<td>Interferon alpha</td>
</tr>
<tr>
<td>IFN-αs</td>
<td>Interferon alpha family</td>
</tr>
<tr>
<td>IFN-α2b</td>
<td>Interferon alpha 2b</td>
</tr>
<tr>
<td>Kan</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani media</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-assisted laser desorption/ionization</td>
</tr>
<tr>
<td>MRW</td>
<td>Mean Residue Weight</td>
</tr>
<tr>
<td>MWM</td>
<td>Molecular Weight Marker</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular Weight Cut Off</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>ON</td>
<td>Overnight</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline.</td>
</tr>
</tbody>
</table>
RPMI  Roswell Park Memorial Institute 1640  
rpm  Revolutions per minute  
sa  straight away, upon immediate addition  
SDS-PAGE  Sodium dodecyl sulphate-Polyacrylamide Gel Electrophoresis  
SN  Supernatant  
TCEP  Tris(2-carboxylethyl)phosphine hydrochloride  
TEMED  N, N, N', N' – Tetramethylethylenediamine  
Tris  Tris(Hydroxymethyl)aminomethane  
UV  Ultra violet  
$\lambda_{\text{Bary}}$  Barycentric mean  
$\lambda_{\text{max}}$  Maximum emission wavelength
Chapter 1
Chapter 1

Introduction

1.1: Interferon

Discovery
Interferon (IFN) was discovered by Isaacs and Lindermann in 1957. They found that chick chorioallanotic membranes incubated with heat activated influenza virus stimulated the production of Interferon, giving fresh membranes resistance to infection by virus (Isaacs and Lindermann, 1957). The interferons were then discovered to be produced by many cells and tissues. Initial biochemical experiments and clinical trials were carried out using simply crude IFN samples from infected white blood cells (Cantell et al., 1981). This method led to problems with blocked antibody synthesis, antibacterial activities and inhibited cell growth however there was no evidence of whether this was due to IFN itself (Pestka, 2000). IFN was purified to homogeneity in 1978 (Rubinstein et al., 1978; Rubinstein et al., 1979; Stein et al., 1980; Friesen et al., 1981; Rubinstein et al., 1981). The IFN was produced by incubating human white blood cells with Newcastle virus. Purification by reverse-phase HPLC and gel filtration chromatography followed. The purification resulted in the discovery and separation of the different types of IFN.

Multiple types of interferon
The production of interferon and the subsequent purification allowed the many types of interferon to be isolated and investigated. There are now at least 10 mammalian interferon species of which seven are found in humans.
The interferons have now been found to be proteins consisting of a family of distinct polypeptides which show homology in both the amino acid sequence and three dimensional structure (Pfeffer et al., 1998). A new nomenclature was therefore devised where the species of IFN were individually named rather than named by their source, for example IFN-α had been named leukocyte IFN, IFN-β had previously been known as fibroblast IFN and IFN-γ was known as immune IFN. Table 1.1 details the IFN proteins.

The interferons are divided into three separate classes, type I (IFN α/β), type II (IFN γ) and type III (IFN λ). As well as IFN α and β other members of the IFN type I family include IFN, epsilon (ε) (Hardy, et al., 2004) and Kappa (κ) (LaFleur et al., 2001), these are found in the human genome while IFN omega (ω) (Adolf, 1987) can be found in some mammalian genomes and primate

<table>
<thead>
<tr>
<th>Type</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td>IFN-α</td>
</tr>
<tr>
<td></td>
<td>IFN-β</td>
</tr>
<tr>
<td></td>
<td>IFN-ε</td>
</tr>
<tr>
<td></td>
<td>IFN-κ</td>
</tr>
<tr>
<td></td>
<td>IFN-ω</td>
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<td></td>
<td>IFN-ν</td>
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<td></td>
<td>IFN-ζ</td>
</tr>
<tr>
<td></td>
<td>IFN-τ</td>
</tr>
<tr>
<td></td>
<td>IFN-δ</td>
</tr>
<tr>
<td>Type II</td>
<td>IFN-γ</td>
</tr>
<tr>
<td>Type III</td>
<td>IFN λ1 (IL-28A)</td>
</tr>
<tr>
<td></td>
<td>IFN λ2 (IL-28B)</td>
</tr>
<tr>
<td></td>
<td>IFN λ3 (IL-29)</td>
</tr>
</tbody>
</table>

Table 1.1: The interferons. The interferons are separated into three classes depending on their sequence, receptor specificity, chromosomal location, physiochemical properties and structure.
interferons. IFN, tau (τ) (Roberts et al., 1992) and delta (δ) (LaFevre et al., 1998) are involved in reproductive events unique to pigs. IFN-zeta (ζ) (Oritani and Tomiyama, 2004) and nu (ν) are only present in rodent and feline genomes (Pestka et al., 2004). There is only one human IFN-β and IFN-ω, but a family of human IFN-α exist. The IFNs are grouped into the three different types due to their sequence, receptor specificity, chromosomal location, physiochemical properties and structure (Samarajiwa et al., 2006).

**Type I interferons**

The human type I IFNs are encoded by a multigene family clustered on chromosome 9. This gene consists of 13 nonallelic IFN-α genes and a single functional IFN-β, ε, κ and ω gene. With the exception of IFN-κ all the type I IFN-α2b genes are intronless (Bogdan et al., 2004). The type I proteins have a closely related amino acid sequence but display different biological activities due to the interaction with their cell surface receptors (Samarajiwa et al., 2006). IFN-β acts as a dimer and only has one disulphide bond (Foster and Finter, 1998). IFN-β only has 19-23 % homology to the IFN-α protein sequence (Weissmann and Weber, 1986). IFN-ω has multiple genes however only one is functional and the protein is similar to the IFN-α group by 55-60 % homology (Adolf, 1995). IFN-κ by 27-32 % to IFN-α, 34 % similarity to IFN-β, 28 % to IFN-ω and ε. IFN-ε is most similar to IFN-β (Hardy et al., 2004). IFN-ζ sometimes named limitin, has 25-28% identity to IFN-α, 21 % identity to IFN-beta and 30% identity to IFN-ω. IFN-ζ has higher antiviral activity compared to the IFN-αs (Kawamoto et al., 2004). IFN-δ has 27 % homology to IFN-β and 42 % homology to IFN-α2 (Lefevre and Boulay, 1993).

**Type II interferons**

Type II IFNs are made up solely of IFN-γ. IFN-γ exists as a tetramer and binds to a different cell receptor than the type I named the interferon gamma receptor (IFNGR) made up of two subunits IFNGR1 and IFNGR2 (Soh et al., 1994). IFN-γ is synthesised by T-cells and natural killer cells as part of the inflammatory response. Synthesis by T-cells is stimulated by antigens and stimulation by IL-2 causes production by natural killer cells (Sen and Lengyel, 1992).
**Type III interferons**

The type III IFNs consist of three members; IL-28A, -28B and -29 and are also called IFN-like cytokines. They are similar to the type I IFNs due to sequence and antiviral and signalling activities (Zhou et al., 2007). They activate the same signal transducers and activators of transcription proteins (STAT) upon receptor binding and induce genes that contain the IFN-stimulated response elements (ISRE) in the promoter.

**Interferons and their actions**

Interferons are now defined as a family of soluble cytokine proteins with antiviral activity that are secreted from cells in response to a variety of stimuli (Sen and Lengyel, 1992). As well as antiviral activity IFN-α and IFN-β have antiproliferative functions on a wide variety of immunologically important cells such as T-cells, natural killer cells, monocytes, macrophages and dendritic cells therefore inducing cytotoxic effects (Ortaldo et al., 1983). These effects increase expression of tumour associated surface antigens and major histocompatibility complex (MHC) class I antigens, induce proapoptotic genes and proteins and repression of antiapoptotic genes and proteins (Greiner et al., 1987). Exposure of cells to viruses and double-stranded RNAs, a side product of viral replication, primarily induces the production of IFN-α and IFN-β. Low levels of IFNs can be detected in human tissue even without induction (Sen and Lengyel, 1992). IFNs bind to the specific cell receptors as the first step in their biological activity. All type I IFNs bind two cellular receptors, Interferon type I receptor I (IFNαR1) and Interferon type I receptor II (IFNαR2), binding of the three IFNs; IFN-κ, IFN-ε and IFN-γ has not yet been determined. IFN-α and IFN-β compete for the same receptor whereas IFN-γ binds to a different receptor (Sen and Lengyel, 1992). Upon binding of the IFNs to their receptors multiple signal transduction pathways are initiated leading to the activation of many genes (Sen and Lengyel, 1992).
Figure 1.1: Ribbon diagram of the crystal structure of IFN-α2b and IFN-β. The five alpha helices are coloured grey and the cysteine residues are coloured red. IFN-α2b, PDB no. 1RH2 (Radhakrishnan et al., 1996). IFN-β, PDB no. 1AU1 (Karpusas et al., 1997). Drawn using Rasmol.
Receptors

All type I IFNs bind a cellular receptor made up of two chains, Interferon type I receptor I (IFNaR1) and Interferon type I receptor II (IFNaR2) (Mogensen et al., 1999). IFNaR2 is the major ligand binding component of the type 1 receptor. The binding is species specific (Sen and Lengyel, 1992) and the various IFNs interact with the receptor differently creating the different responses. IFN-α and IFN-β compete for the same receptor whereas IFN-γ and the type III IFNs bind to their own specific receptor (Sen and Lengyel, 1992). Upon binding of the IFNs to their receptors multiple signal transduction pathways are initiated leading to the activation of many genes (Darnell et al., 1994).

Signal transduction

Binding of IFN to the cell surface receptors promotes oligomerization of the two receptor chains; IFNaR1 and IFNaR2. The IFN receptor chains lack intrinsic tyrosine kinase activity, but due to the association with Janus Activated Kinase 1 (JAK-1) through its own tyrosine-specific kinases results in transphosphorylation, JAKs are only activated on ligand binding. This activation and transphosphorylation of JAKs causes tyrosine residues present on the receptor chains to become phosphorylated and become in turn docking sites for cytoplasmic signal transducers and activators of transcription proteins (STAT). Both STAT-1 and 2 are involved with IFN Type I signalling. These STAT proteins form heterodimers and with the association of IFN-regulatory factor 9 (IRF-9) leads to the translocation of the STAT dimmers to the nucleus. It is within the nucleus where they are able to bind to the IRSE stimulating expression of IFN induced genes.
Figure 1.2: IFN-α signalling pathway. IFNAR1 and 2 are the two different receptor chains associated with IFN Type I signalling. P = phosphorylation, IRF-9 = IFN regulatory factor 9. Both STAT 1 and 2 are involved with Type I signalling along with JAK and specific tyrosine kinases (TYK). Phosphorylation of the STAT molecules leads to their translocation to the nucleus causing expression of IFN induced proteins by activation of the Interferon stimulated response element (ISRE).

The result of the IFN induced pathway is the up regulation of IFN-α2b-inducible proteins for example 2'-5'-oligoadenylate synthetase (2'-5' OAS) or the RNA-dependent protein kinase (PKR). 2'-5' OAS is the necessary cofactor for activation of RNase L which can in turn degrade viral mRNA and inhibit viral protein synthesis. PKR a protein kinase which phosphorylates and inactivates eIF-2 involved in viral mRNA translation and MHC class I expression is increased stimulating cell-mediated immunity (Meager, 2006).
Interferon alpha

The human α-interferons have now been found to be encoded by a family of at least 14 genes. From these genes 13 proteins are expressed, IFNA1 and IFNA13 are exactly the same so 12 different proteins are actually expressed (Peskta et al., 2004). The family of IFN-α genes has 70 % homology between them (Samarajiwa et al., 2006). Human IFN-α is heterogeneous and several bands exhibiting antiviral activity range in molecular weight from 15000 to 21000 Da by SDS-PAGE (Pestka, 2000). All the IFN-α gene products are made up of 165-166 amino acids and they all contain two conserved disulphides between the cysteine (Cys) residues Cys1-Cys98 and Cys29-Cys138 (Wetzel, 1981). Each IFN-α subtype behaves differently through their antiviral and antiproliferative actions however they do share between 76% and 99% homology at the protein level and 79 out of the 166 amino acids are conserved (Kontsek, 1994). The only IFN-αs to be used therapeutically are IFN-α2a, IFN-α2b and IFN-αc and so the remaining IFN-αs are said to be an untapped source of opportunity (Pestka, 2000). It is not known why the body produces so many variants.

The increased interest in the interferons has lead to their high resolution protein structures. The three dimensional structures of the major type I interferons were solved during the mid-nineties. The tertiary structure of IFN-α2a was detailed by Klaus et al., (1997) by heteronuclear nuclear magnetic resonance (NMR) along with human IFN-α2b by X-ray crystallography (Radhakrishnan et al., 1996) and both human and murine IFN-β by Karpusas et al., (1997) and Senda et al., (1995) respectively.

IFN-α2b

The only two IFN-αs which are mass produced in Escherichia coli (E. coli) by recombinant means and used as drugs are IFN-α2b and IFN-α2a. IFN-α2a was the first investigated in 1981 due to its ability to shrink tumours (Gresser and Bourali, 1969), IFN-α2b followed closely that year. In 1996 Radhakrishnan et al., reported the crystal structure of recombinant human zinc IFN-α2b by X-ray crystallography. This form of IFN-α2b unexpectedly existed as a dimer (Radhakrishnan et al., 1996).
Each monomer of IFN-α2b is composed of five α helices labelled A-E linked by one overhand loop (figure 1.1), the AB loop, and three short segments, the BC, CD and DE loops. All type I IFNs exist in this form also. The five core α-helices range in length from 13-24 amino acids. Each helix is straight except for helix B which contains a bend centered around the residue Thr-69 (Radhakrishnan et al., 1996). The long connection between helices A and B (the AB loop) is usually described in three sections, AB1: residues 22-33, AB2: residues 34-39 and AB3: residues 40-51 (Radhakrishnan et al., 1996). Residues 26-29 and 30-33 within the AB loop form two turns of 3\(^{10}\) helix. Cys-29 links 3\(^{10}\) helix to helix E through a disulphide bond with Cys-138 (Radhakrishnan et al., 1996). This 3\(^{10}\) helix redirects the mainchain by 90° to run parallel to helix D where specific interactions take place with the sidechains of the residues present in helix D. In contrast to the AB loop the BC loop consists of two residues and the DE loop contains four residues. The largest structural difference between IFN-α and IFN-β has been shown to be the AB loop, as it contains more residues in IFN-α2b than murine IFN-β (Radhakrishnan et al., 1996). Based on the structure of human IFN-α2b some of the main aromatic residues, Phe-36, Tyr-122 and Tyr-129 are found within the molecular core. (Radhakrishnan et al., 1996). The active form of IFN-α2b has always been thought of as the monomer due to the small concentrations required for in vivo activity (Radhakrishnan et al., 1996). As Radhakrishnan et al., (1996) crystallised IFN-α2b as a zinc dimer they also reported that the addition of 1 mM zinc to a gel filtration column did not shift the equilibrium in favour of dimer. Suggestions for the unexpected dimer are that it acts as a storage system of the molecule or could be involved with the recruitment of the receptor molecules (Radhakrishnan et al., 1996).

In 1986 IFN-α2a and IFN-α2b were approved for treatment of hairy cell leukaemia by the U.S. Food and Drug Administration. Subsequently IFN-β1a and IFN-β1b were approved for treatment relapsing multiple sclerosis. IFN-α is now used in the treatment of hairy cell leukaemia, malignant melanoma, follicular lymphoma, condylomata acuminata (genital warts) (Brockmeyer et al., 2006), AIDS-related Kaposi sarcoma (de Wit et al., 1988) and hepatitis B
and C (Yotsuyanagi and Koike, 2007), IFN-γ for malignant osteoporosis. IFN-α is also given alongside other drugs for treatment of bladder (Nagabhushan et al., 2007) and renal cancers (Motzer et al., 2002).

Consensus interferon and pegylation
Consensus Interferon (C-IFN) is a genetically engineered interferon with unusually high biologically activity. It is obtained by scanning IFN-α subtypes and assigning the most frequently observed amino acids to each position creating a new DNA sequence termed consensus interferon (Aloton, 1983; Klein et al., 1988; Hu et al., 1995).

There are two pegylated interferons available, the first is a linear PEG chain linked to IFN-α2b and the second consists of a branched PEG chain linked to IFN-α2a. These are usually administered with ribavirin (Foster, 2004). Pegylation of therapeutic proteins is a well established method for reducing protein immunogenicity and, as IFN-α2b treatment usually requires a high amount of injections, pegylation has proved successful in IFN-α2b treatment (Jabbour et al., 2007).

IFN-α2b expression, purification and refolding
There have been many successful attempts to express, purify and refold IFN-α2b to give high yields. As there are many IFNs all of which are being investigated there is a diverse range of methods used to purify and refold them, ultimately they all have the same goal; to produce as much pure IFN-α2b to be used in treatment of viral diseases. The method used by Srivastava et al., (2005) has been the most consulted throughout this project. The IFN-α2b is expressed in both BL21 (DE3) and DH5α E. coli cells as inclusion bodies under the same heat inducible promoter used during this project. The IFN-α2b is then refolded followed by purification by Q Sepharose™ ion exchange chromatography. It is common practice to refold proteins, not necessarily just IFN-α2b before they are purified. As the refolding and purification of IFN-α2b will ultimately be subject to scale-up some considerations should be taken into consideration along with time restraints. Valente et al., (2006) expressed IFN-α2b as inclusion bodies in JM109 (DE3)
and refolding consisted of a two step dialysis. The first removed the guanidine-HCl which had been used to solubilise the inclusion bodies and the second involved dialysis into 0.4 M urea, this was carried out over 9 days, whilst the biological activity was monitored. The yield was 30%, however the 9 day refolding is not suitable for an industrial setting. Beldarrain et al., (2001) incorporated metal ion catalysed air oxidation using copper sulphate for the refold, this resulted in a 12 % yield. Swaminathan and Khanna, (1999) added L-arginine to the refold mixture, this mixture was left for 24-36 hours before dialysis to remove the L-arginine. The IFN-α2b was purified following refolding by affinity chromatography on a dye-ligand matrix, Green-1 A6XL was used. The elution takes place with increasing NaCl, resulting in a yield of 82 %. Neves et al., 2004 mutated Cys-1 and Cys-98 into serine residues and expressed it as a polyhistidine-tagged protein. The disulphide Cys-1 and Cys-98 is not required for biological activity (Weissmann and Weber, 1986). They concluded that the his-tagged IFN-α2b expressed was more stable.

As well as bacteria IFN-α2b has been expressed as a soluble protein in Pichia pastoris (Shi et al., 2007). The IFN-α2b was purified by Q Sepharose™ ion exchange, concentrated and applied to a Superdex™ 75 gel filtration column giving a 64 % yield (Shi et al., 2007). A second method of expressing IFN-α2b as a soluble protein was as a GST-fusion protein (Rabhi-Essafi et al., 2007). The IFN-α2b was purified by affinity chromatography, thrombin cleaved, followed by size exclusion chromatography.

1.2: Protein folding

The three-dimensional structure of a protein is determined by its amino acid sequence as most purified proteins can refold spontaneously in vitro after being unfolded (Creighton, 1990). Early work by Anfinsen (1973) on denaturation and renaturation of Ribonuclease A concluded that the amino acid sequence determined the three-dimensional structure of proteins and ultimately their function. The work further determined that proteins will fold spontaneously.
The folding reaction

The folding reaction for single domain proteins is assumed to be a one step, two state, first order reaction, shown in equation 1.1.

\[
\text{Denatured or unfolded (D) } \xleftrightarrow{\text{Folding}} \text{ Native or folded (N)} \\
\text{Unfolding}
\]

Multi-domain proteins fold and unfold in a step-wise manner.

The native state

The conformations during folding are driven by the restrictions of rotation of the main peptide chain $\Psi$ and $\Phi$ angles and the non-covalent interactions such as hydrogen bonds, van der Waals, salt bridges and hydrophobic interactions. The peptide chain angles are hindered by the side chains of the adjacent molecules allowing only one specific structure to be native (Ramachandran et al., 1963). The non-covalent interactions allow stability of the protein molecule to be attained.

Both the native and the denatured forms of proteins are stable. The native form is the most stable of the conformations but, not necessarily the one with the lowest free energy. A similarity in all water soluble proteins is the presence of the hydrophilic side chains on the exterior of the three dimensional structure and the hydrophobic side chains buried in the core (Perutz et al., 1965). Different folded proteins have different degrees of flexibility giving information about their free energy of folding and unfolding, the most flexibility being on the outside of the molecule relative to the inside (Creighton, 1990).

The unfolded state

The extreme unfolded state of a protein is incorrectly termed the "random coil" and is achieved under highly denaturing conditions such as high
concentrations of guanidine-HCl or urea (Tanford et al., 1967). The random coil is defined by Creighton (1990) as a structure in which the rotation about each bond of the backbone and side chains is independent of that of bonds anywhere in the sequence. The restriction from the covalent bonding of these residues will not simply disappear if the protein is unfolded however there are many possibilities for these different conformations to exist. The radius of gyration calculated for unfolded proteins by Wilkins et al., (1999) and further experimental evidence of proteins unfolded states do support one another (Ding et al., 2005). The unfolded structures of proteins denatured by temperature and pH are currently being investigated as they do not always follow random coil conformations but retain structure especially compared to guanidine-HCl and urea denatured proteins.

**Intermediate states**

The reaction pathway can involve intermediates with structures very similar to the denatured or the native state;

\[
\begin{align*}
D & \leftrightarrow I & I & \leftrightarrow N
\end{align*}
\]

There is not necessarily just one intermediate (I), there can be several some having a more native (N) structure and some having a more denatured (D) structure (Richards, 1992). The formation of these intermediates has allowed pathways of folding and unfolding to be monitored and broken down into their separate components (Kim and Baldwin, 1982). The rate of each reaction within the folding and unfolding can provide information about which are the rate limiting steps.

Some intermediates exist as stable conformations suggesting that proteins have a third conformational state (Uversky and Ptitsyn, 1996). The observation of the molten globule state confirms that these intermediate structures do exist (Ptitsyn, 1994). Intermediates are usually termed kinetic or equilibrium intermediates and are a common way of studying folding pathways (Ptitsyn, 1994). Ptitsyn, (1994) details the formation of these intermediates. An initial intermediate forms within a few milliseconds of
folding which has native secondary structure and binds 8-anilino-1-naphthalene-sulphonate (ANS) (Semisotnov et al., 1987). The second intermediate forms within 1s closely followed by the native state. Even in the two state reaction shown in equation 1.2 intermediates can form and be monitored by techniques such as far and near UV circular dichroism, binding of ANS and intrinsic fluorescence. Even though intermediates are formed during folding they are not necessarily on the pathway to the native protein, and may require a return to the denatured state to carry on successful refolding (Fersht, 1999).

During protein folding and unfolding the interactions within the protein molecule are broken along with energy barriers which must be overcome. If the activation energy of a specific energy barrier is much higher than the rest this step will be rate limiting and considered the transition state of the reaction.

**Molten globule**
Under certain conditions during folding and unfolding, proteins can have a structure which is neither folded nor unfolded. The dimensions of this form are usually larger than the folded state but smaller than the unfolded state and contain secondary structure similar to that of the native along with the hydrophobic core, however it is not as closely packed (Ptitsyn, 1987). This structure which occurs as an intermediate in protein refolding has been termed the molten globule (Ptitsyn, 1990). The molten globule has been described as a stable intermediate formed during secondary structure formation and therefore occurs during the early stages of folding (Ptitsyn, 1990). The molten globule state has many common features;

1. The dimensions are larger than the native state but smaller than the denatured state (Zerovnik and Pain, 1987).
2. The content of secondary structure is similar to that of the native protein (Baum et al., 1989).
3. Interconversions with the native state is slow but rapid with the unfolded state (Ptitsyn et al., 1990).
The term compact intermediate (CI) is sometimes used to describe the molten globule (Kim and Baldwin, 1990). The molten globule state can be identified and analysed due to its stability. Methods such as gel filtration chromatography (Zerovnik and Pain, 1987) and ANS binding have been used to investigate the molten globule state (Semisotnov et al., 1987).

**Stability of the native state**

In a two step unfolding transition the equilibrium constant is able to be calculated and therefore the free energy of folding (detailed in chapter 3, section 3.2.4). The following equation is used;

\[ \Delta G = -RT \ln K \]  

(1.3)

\( \Delta G \) = Gibbs free energy, \( R \) = gas constant, \( T \) = temperature (Kelvin), \( K \) = equilibrium constant.

The native state is only slightly more stable than the unfolded state usually between 1-15 kcal/mol (Daggett and Fersht, 2000).

**Thermodynamics of protein folding**

The free energy change upon unfolding (\( \Delta G_{\text{fold}} \)) can be described in terms of the enthalpic and entropic contributions by the following equation.

\[ \Delta G = \Delta H - T\Delta S \]  

(1.4)

Where \( \Delta G \) = Gibbs free energy, \( \Delta H \) = Enthalpy, \( T \) = Temperature, \( \Delta S \) = Entropy.

The enthalpy is the change in bond energies and the entropy is the change in randomness of a reaction. The formation of hydrophobic interactions is driven by a change in entropy i.e. the water molecules become ordered around the hydrophobic residues restricting their free motion therefore lowering the entropy.
Protein stability

The intramolecular forces within a protein molecule keeping it stable are a collection of van der Waals interactions, charged interactions, polar interactions and hydrophobic interactions. The van der Waals interactions are assumed to exist within the protein molecule itself but alongside the water molecules also, this is similar for the polar interactions such as hydrogen bonds. These interactions can also exist in the unfolded state so are not usually considered to be of major importance to the proteins stability (Privalov, 1992). Equally the charged groups are small in number and so must only compensate for a small amount of stabilisation. The remaining interaction is that of hydrophobic residues, usually clustered together in the core of the protein. It is assumed that the major factor in stabilising the protein molecule comes from this hydrophobic force. However based on this assumption the positive entropy should exceed the negative entropy of the peptide chain becoming ordered. An increase in entropy is however observed with increasing temperature which in turn denatures the protein creating disorder, which should indicate an increase in the hydrophobic effect, this however is not the case. The conclusion from this is that the stability of the protein molecule cannot be considered to rely on hydrophobic interactions only; there must be other factors involved.

Thermal denaturation

Heat denaturation

As discussed, if the temperature increases then the entropy increases overcoming the enthalpy of the interactions holding the protein structure together. Temperature induced denaturation at high temperatures involves heat absorption and an increase in the heat capacity of the protein therefore an increase in the enthalpy (Privalov, 1990). The enthalpy of the unfolded state increases with temperature as the ordered water molecules around the hydrophobic residues move away, the water molecules therefore form fewer hydrogen bonds with each other. If the enthalpy increases with temperature then the entropy must also increase for disorder and with the reduced order of the water molecules the hydrophobic residues have more freedom. The
temperature and the entropy of the unfolded state must exceed the enthalpy (see equation 1.4) for $\Delta G$ to become negative favouring the reaction.

**Cold denaturation**

From equation 1.4 decreasing the temperature should create order however, when the protein is in its native state that is when it is assumed to be at its most ordered (Privalov, 1990). So why would decreasing the temperature improve on this? During cold denaturation the water molecules become more ordered leading to a decrease in entropy. The enthalpy is negative due to heat release. With the increase in order it is strange that cold temperatures lead to denaturation and so it is important to have experimental evidence that this is actually the case, which is difficult due to the freezing of the aqueous solution (Privalov, 1990). This experimental evidence is provided by calorimetric data confirming that cold denaturation is an exothermic process (Cho et al., 1982).

**1.3: Protein refolding in biotechnology**

Protein refolding of denatured proteins has become of great importance to the biotechnology industry. This is due to folding of a protein leading to either product or useless misfolded protein. The mass production of proteins in biotechnology does however rely on a trial-and-error method of refolding proteins (Tsumoto et al., 2003).
Inclusion bodies

Inclusion bodies are dense electron-refractile particles of aggregated protein found in the cytoplasm and the periplasmic space of *E. coli* (Bowden, 1991). The size of inclusion bodies varies between 0.17-1.3 μm and have a density...
of ~1.3 g/ml, higher than cellular components (Taylor, 1986). Expression in *E. coli* can result in the protein being produced as inclusion bodies. Inclusion bodies are misfolded, insoluble, polypeptide aggregates (Rudolph *et al.*, 1996). Large-scale production of proteins usually begins with expression in bacterial systems, most commonly *E. coli*. *E. coli* has become a widely used host in industry for the production of many therapeutic proteins. As the proteins are expressed in a foreign host under forced conditions the products are not often produced in their natural forms and inclusion bodies are the result. Expression of proteins in the form of inclusion bodies is usually deemed undesirable however it can be advantageous as the separation of the inclusion bodies from other cellular debris is quite easy and successful. This separation usually involves repeated centrifugation whilst washing the inclusion bodies with specific buffers.

There are many advantages of expressing a protein as inclusion bodies.

1. Expression levels are high, 30% of cellular protein in some cases.
2. Proteins are protected from proteolysis by host cell enzymes.
3. Initial purification which does not include the risk of denaturation.
4. Easy separation from cells and other soluble contaminants using techniques such as centrifugation.
5. If the expression product is toxic to the host, the inclusion body form can prevent this.

(De Bernardez Clark, 2001; Singh and Panda, 2005).
Once the stages of harvesting, centrifugation, solubilisation and purification have been developed there still remains a need to turn the protein into its native state to make it active. The challenge lies in that there is no universal method for refolding proteins (Middelberg, 2002). Protein refolding is a well researched academic subject and there is no shortage of detailed reviews on where to begin when refolding a protein (Panda, 2003; De Bernardez Clark 2001). The confusion starts with the vast quantity of methods and combinations of techniques to choose from. Much of the research into refolding proteins involves the expression and purification of small amounts of protein for biophysical analysis or studies on the folding pathways of individual proteins. The methods used are usually made up of many unnecessary purification stages leading to poor yields (De Bernardez Clark, 2001). Due to the importance of protein refolding to industry the scale up issues must be acknowledged and poor yields are not an option.

The aggregation which occurs on inclusion body formation of recombinant proteins is probably due to the highly oxidizing environment of the bacterial cell and the limiting amount of chaperones and foldases (Lorimer, 1996;
Growth temperature and the pH of the media can also stimulate inclusion body formation (Chalmers et al., 1990; Strandberg and Enfors, 1991). During the high level of expression the chances of exposed hydrophobic surfaces on the recombinant protein to come into contact with each other increases and the protein aggregates.

**Solubilisation**

Once inclusion bodies have been isolated the preparation for refolding begins immediately with solubilisation. The two most commonly used denaturing agents are urea and guanidine-HCl.

![Figure 1.5: Chemical structure of urea and guanidine. The figure illustrates how these two commonly used denaturants have very similar chemical structures. Drawn using ChemWindow™ 6.](image)

Urea and guanidine-HCl are considered to break the hydrogen bonds within the protein molecule and the water molecules either by interacting directly with the protein molecule or with the surrounding solute molecules. Urea is usually preferred as the denaturant in an industrial setting due to the corrosive properties of guanidine-HCl, however this is not always the most efficient as urea has the potential to spontaneously produce cyanate which can react with the protein and carbamylate the amino groups (Cejka et al., 1968). The disruption of protein structure by urea and guanidine-HCl is still very much debated. These two denaturants are similar in structure however guanidine-HCl is documented as the best possibly due to the high salt concentration present, however it can depend on the protein (Greene and Pace, 1974). Their effects could be displayed by binding directly or indirectly to the protein.
molecules or by altering the environment by reacting with the water molecules (Bennion and Daggett, 2003).

Denaturation should cause the complete disruption of the protein structure as it is difficult to refold a protein which is only partially unfolded (De Bernardez Clark, 2001). Detergents can also be used to denature inclusion bodies, an example being sodium dodecyl sulphate (SDS). However detergents can interfere with downstream purification stages (De Bernardez Clark, 2001) and it is difficult to prove the removal of detergents once the process has ended. Reducing agents such as dithiothreitol (DTT) and β-mercaptoethanol may also be added on solubilisation as they will prevent any disulphide bond formation (Fischer et al., 1993). Ethylenediaminetetraacetic acid (EDTA) can also be added to prevent air-catalyzed oxidation of the cysteine residues (Singh and Panda, 2005). Inclusion bodies have been shown to solubilise in extremes of pH alongside low concentrations of denaturant (Patra et al., 2000) however this can also irreversibly modify protein molecules, examples being deamidation and alkaline desulphuration of cysteine residues (Thatcher and Hitchcock, 1994). The choice of solubilisation buffer can be of great importance to a process due to the high cost involved of the denaturants but also by ensuring maximum yield throughout.

**Simple renaturation**

After solubilisation, refolding is usually carried out by removing the high concentrations of denaturants and the reducing agent, if any. This may be carried out by dilution, diafiltration or gel filtration chromatography. The technique used depends heavily on the protein (De Bernardez Clark, 2001). Additives can be added to the refolding buffers to aid refolding and decrease the formation of aggregates. All these methods shall be discussed individually.

**Dilution**

Dilution of the solubilised protein directly into the refold buffer is the most commonly used method in small-scale refolding due to its simplicity (Li et al., 2004). If the protein is to be exposed to low denaturant concentrations the
initial shock can result in aggregation and so protein concentrations are an important factor to consider when choosing dilution, Lilie et al., (1998) and Jungbauer and Karr, (2007) suggest 10-100 μg/ml. Staged or pulsed dilution into intermediate concentrations of denaturants can also be used. Unfortunately staged dilution is both time and buffer consuming and therefore not suitable for large scale production (Li et al., 2004). Pulsed dilution consists of aliquots of protein being added to the renaturation buffer at different time intervals in order to reduce the need for large volumes. However pulsed dilution must be stopped once the denaturant and protein concentrations become critical (Lilie et al., 1998).

Dialysis
Dialysis exposes the protein to intermediate denaturant concentrations and usually involved long periods of time of exposure to these concentrations. This can be beneficial in the refolding as partially folded intermediates may be formed at these reduced denaturant concentrations. However this intermediate state can increase the chances of aggregation, it is very much protein specific (Rudolph and Lilie, 1996). Being a slow process dialysis is often replaced by diafiltration in industry. The principle is the same, small molecules pass through a membrane and the protein of interest is retained whilst the buffer containing the denaturant passes through the membrane. A new buffer is added to the retained protein resulting in buffer exchange. The process of diafiltration is driven by pressure and so does not depend on the rate of diffusion therefore making it quicker than dialysis.

Refolding using chromatography
Refolding using chromatography can be a long process due to slow flow rates but also expensive especially if pre-packed columns are used, however it is being investigated more as an alternative to non-chromatographic methods due to better quality proteins being produced (Swietnicki, 2006)

Buffer exchange
Chromatography has been used to successfully refold proteins especially size exclusion or gel filtration chromatography (or size exclusion, SEC). SEC has
been used due to it being a good buffer exchange system, essentially it is a batch dilution method which creates a bottleneck in the process and problems with scale-up, Lanckriet and Middelberg, (2004) investigated the possibility of continuous SEC chromatography to reduce the time constraints. They found it be more beneficial in reducing process time while still remaining a successful stage.

The refolding is based on the principle that the column is pre-equilibrated in the refolding buffer, the sample applied in high denaturant and the protein eventually elutes in the refolding buffer. The column provides separation of the protein refolding and any aggregates due to them being separated by their sizes, but also provides time for the protein to refold and possibly gives the aggregates a second opportunity to refold (De Bernardez Clark et al., 1999). Change in the buffer composition can also be coupled to a change in pH. The application of crude samples to the gel filtration column can result in problems as the resin will require rigorous and harsh cleaning protocols possibly compromising the stability and consistency of the chromatography.

Matrix assisted protein refolding
A second way of ensuring the folded protein does not come into contact with aggregates or aggregate-prone intermediates is to attach the protein to a support. Examples of these being ion exchange chromatography, hydrophobic interaction and affinity chromatography requiring a tag or fusion protein capable of binding to the resin. The detachment would work with the chromatography conditions and allow the protein to be released from the resin, folded.

Hydrophobic interaction chromatography
Hydrophobic interaction chromatography (HIC) involves the proteins being loaded at high salt concentrations which coincides well with the high guanidine-HCl concentrations used for solubilisation. Refolding and elution takes place by a reduction in the salt concentration. During migration through the column the protein will encounter several steps of adsorption and desorption resulting in the formation of the intermediates which ultimately lead
to the native protein. Consensus IFN-α2b has been refolded successfully using HIC (Wang et al., 2006a). The method used a decreasing guanidine-HCl gradient alongside an increasing PEG gradient to elute the protein. Li et al., (2004) found that increasing hydrophobicity of the HIC media had a negative effect on the refolding, however with the incorporation of glycerol in the running buffer the correct refolding could be achieved. The apparent need for the chaperone (glycerol and PEG in these cases) could be because of the strong interaction of the protein with the HIC media. The chaperone can reduce this effect allowing the protein to refold rather than becoming irreversibly bound.

**Ion exchange chromatography**

Ion exchange methods of refolding proteins tend to involve a dual gradient elution to avoid the high concentrations of salt usually required to elute the protein. Li et al., (2004) successfully purified and refolded lysozyme using a decreasing urea gradient alongside an increasing pH gradient. Liu et al., (2007a) also successfully refolded and purified TGase from *Streptomyces fradiae* using ion exchange chromatography to high activity. Ion exchange has been coupled with expanded bed chromatography by Biazus et al., (2006). Expanded bed chromatography is used for more crude protein samples, as the name suggests as the expanded bed expands the surface area available to the protein molecules increases.

**Affinity chromatography**

Affinity chromatography makes use of the improved expression of some proteins when tagged. Immobilised metal ion affinity chromatography (IMAC) purifies polyhistidine-tagged proteins. The matrix is made up of divalent metal ions to which the polyhistidine-tag is attracted. Elution is carried out with increasing concentrations of imidazole or decreasing the pH. The refolding can be carried out while the protein is still attached as the matrices are compatible with high denaturant concentrations. Before elution the denaturant concentration can be reduced to refold the protein. The concentration of protein must be monitored as high protein concentrations can aggregate at the top of the column reducing the elution success. The tag also
must not interfere with the proteins refolding. Rogl et al., (1998) successfully refolded two polyhistidine-tag membrane proteins on a Ni-column by reducing the denaturant and increasing the detergent concentration.

**Pressure**

Pressure has been used to treat proteins which have a tendency to aggregate easily during refolding. Hydrostatic pressures of 150-200 MPa (1.5-2 kbar) are applied to the samples. The aggregates produced either upon denaturation with guanidine-HCl or urea or just simply the inclusion bodies at concentrations of 8.7 mg/ml results in high recovery of the native protein. Katoh and Katoh, (2000); Gorovits and Horowitz, (1998) investigated the effect of high pressure on the aggregation of the enzyme rhodonase. The enzyme begins to aggregate at 3.9 M urea after denaturation. An increase in pressure of 2 kbar resulted in an increase in refold yield. Interestingly though was the ability to reverse the formation of the aggregates using this high pressure. The formation of the aggregates was induced and monitored by light scattering. The pressure was applied, released and applied a second time. The formation of aggregation was monitored and a decrease in the light scattering was observed. They state that the formation of large irreversible aggregates cannot be improved mostly due to experimental design with too long incubation times under reduced pressure. High pressure has also been used to solubilise inclusion bodies with and without guanidine-HCl. St. John et al., (1999) solubilised β-lactamase inclusion bodies incubated at 200 MPa (2 kbar) for 48 hours. Their results show a recovery of soluble β-lactamase using just pressure however coupled with low concentrations of guanidine-HCl (≤ 1.2 M) the recovery of soluble β-lactamase improved.

**Aggregation**

The protein concentration also requires careful monitoring to prevent aggregation (Li et al., 2004). The process of folding of a protein has been described as being a directed process (Levinthal, 1968) proceeding through intermediate states (Privalov, 1996). The intermediate structure is unstable and less soluble causing aggregation and misfolding (Tsumoto et al., 2003).
Figure 1.6: Schematic diagram of refolding of a protein molecule. $U = \text{unfolded}$, $I = \text{Intermediate}$, $N = \text{Native}$ (Tsumoto et al., 2003). Formation of aggregates is the most common reason for low yields during protein folding, to minimise aggregation many different types of additives and/or chaperones can be added during the refolding process.

Aggregation has been quoted as being ‘the single most important constraint in the use of prokaryotes as expression vehicles for recombinant proteins’ (Thatcher et al., 1994). The folding buffer used must favour the formation of the native state while minimizing aggregation of the intermediates (Armstrong et al., 1999). Goldberg et al., (1991) showed that turkey egg white lysozyme formed aggregates that were not necessarily caused by incorrect disulphide bonds but non-specific interactions between the peptide chains. The rate of aggregation formation has been found to be second or third order whereas correct refolding is first order; therefore aggregation competes successfully with refolding (Hevehan and De Bernadez-Clark, 1997).

**Formation of disulphide bonds**

If the protein contains disulphide bonds then a controlled redox environment must be set up to ensure their correct formation. This redox environment can be set up using many additives, examples are reduced and oxidised glutathione (GSH/GSSH), cysteine and cystine, cysteamine and cystamine (De Bernardez Clark et al., 1999). Metal ions have been added to initiate oxidation of the cysteines using the oxygen present in the air (Ahmed et al., 1975) due to its simplicity and cost effectiveness.
Additives
Additives can be introduced into the refold to reduce chance of aggregation formation. A common additive is L-arginine. L-arginine reduces the possibility of aggregation by interacting with amino acid side chains, which is also a property of guanidine-HCl of which they share structural similarities (Arakawa et al., 2007). However Liu et al., (2007b) showed that L-arginine did reduce precipitation of consensus IFN but increased the formation of soluble oligomers. They found that increasing the L-arginine concentration decreased the refolding yield. Detergents are a popular choice of additive to refolding mixture, the choice of the detergent used such as CHAPS, SDS and Triton X-100 is usually down to efficiency and cost. Another technique is to chemically modify the protein to make it more soluble (Thatcher et al., 1994). A summary of all the available additives can be seen in table 1.2.

<table>
<thead>
<tr>
<th>Additive</th>
<th>Recommended Concentration</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Denaturants</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guanidine-HCl</td>
<td>≤ 1 M</td>
<td>Schulenburg et al., 2007</td>
</tr>
<tr>
<td>Urea</td>
<td>≤ 2 M</td>
<td>Futami et al., 2000</td>
</tr>
<tr>
<td>L-arginine</td>
<td>0.4-0.8 M</td>
<td>Liu et al., 2007b</td>
</tr>
<tr>
<td><strong>Salts</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.5 M</td>
<td>Futami et al., 2000</td>
</tr>
<tr>
<td><strong>Sugars</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>20-40 %</td>
<td>Dong et al., 2004</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.4 M</td>
<td>Sijwali et al., 2001</td>
</tr>
<tr>
<td>Glucose</td>
<td>1 M</td>
<td>Divsalar et al., 2006</td>
</tr>
<tr>
<td><strong>Detergents and surfactants</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHAPS</td>
<td>10-80 mM</td>
<td>Wetlaufer and Xie, 1995</td>
</tr>
<tr>
<td>Tween</td>
<td>0.001-%-1 %</td>
<td>Wells et al., 1994</td>
</tr>
<tr>
<td>SDS</td>
<td>1 mM</td>
<td>Xu and Keiderling, 2006</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>10 mM</td>
<td>Zardeneta and Horowitz, 1994</td>
</tr>
<tr>
<td>Polyethylene glycol</td>
<td>0.1-0.4 g/l</td>
<td>Wang et al., 2006a</td>
</tr>
<tr>
<td><strong>Alcohols</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n-pentanol</td>
<td>1-10 mM</td>
<td>Wetlaufer and Xie, 1995</td>
</tr>
<tr>
<td>n-hexanol</td>
<td>0.1-10 mM</td>
<td>Wetlaufer and Xie, 1995</td>
</tr>
</tbody>
</table>

Table 1.2: Commonly used additives to enhance refolding yields by reducing aggregation.
Chaperone assisted refolding

Chaperone assisted refolding is more commonly used during small scale refolding; the reason for this is purely availability and cost of the chaperones as they are usually added at an equimolar concentration to protein (Vallejo and Rinas 2004). Molecular chaperones mediate the correct assembly of proteins without forming any part of the final structure. They do this by binding and stabilising the folding intermediates (Ulrich, 1996). These chaperones exist naturally within the cell, aiding protein folding after formation on the ribosome. Many of them double as heat shock proteins also, due to their formation when the cell is under stress in order to protect other proteins. An example being Hsp 70 (also called DnaK in E.coli). In vivo the unfolded polypeptide chain is passed to Hsp 70 which exposes its hydrophobic pocket enabling it to bind to exposed hydrophobic regions in the protein. Hsp70 is able to do this by binding ATP, once bound the hydrolysis of ATP occurs and the peptide is released. The folding of some proteins requires chaperonins as well as Hsp70. In eukaryotes the chaperonins are called TCiP consisting of eight Hsp60 subunits. GroEL the TCiP homolog in bacteria consists of fourteen subunits (Lodish et al., 2001). The refolding of maltodextrin glucosidase was shown to be improved by the addition of the chaperonins GroEL and GroES (Paul et al., 2007). They also appeared to work well in conjugation with other additives such as polyol osmolytes and glycerol.

During protein folding catalysts can be added such as prolyl-peptidyl isomerase (PPI) which catalyses the cis/trans isomerisation of proline residues or protein disulphide isomerase (PDI) which forms the correct disulphide bonds (De Bernardez-Clark et al., 1999). These behave differently to the molecular chaperones as they are catalysts, therefore altering the rate of reaction whereas molecular chaperones do not (Ulrich, 1996). PDI is found in vivo during folding in the endoplasmic reticulum and promotes reshuffling of the incorrect disulphide bond formation (Gething and Sambrook, 1992). PPI activity is found in the cytosol and can be used to accelerate the isomerisation within proline rich proteins (Gething and Sambrook, 1992).
Screening kits
As there is no universal refolding buffer (Vincentelli et al., 2004) it has become clear that a way of screening proteins with many different buffers would be more efficient and less time consuming. It has become more popular to begin refolding of a protein with a screening kit much like the kits used for protein crystalisation. These kits usually incorporate all the additives discussed above, Novexin Ltd and Merck have a large range of kits available.

1.4: Investigation of protein folding
Once folding has taken place analysis has to be carried out to ensure the correct conformation has been achieved. Functional assays can be of great importance in elucidating whether the protein has reached its optimum activity, important if the protein has therapeutic implications. However biophysical techniques can also be used to analyse the folded and unfolded conformations of proteins. It is easier if both the folded and unfolded characteristics are known, to which the refolded can be compared however it is not always necessary. Luckily due to proteins being made up of chiral molecules, having partial double bond character and a very unique native and denatured 3-dimensional structure these characteristics can be exploited giving structural information. The two most utilised biophysical techniques used throughout this project are circular dichroism and fluorescence both of which shall be further discussed individually.

Spectroscopy
Electromagnetic waves are made up of two components, an electric field and a magnetic field; these two components oscillate at right angles to each other in the direction of propagation. Electromagnetic radiation is made up of many different types depending on the frequency, these components can be seen in figure 1.7 as the electromagnetic spectrum.
Figure 1.7: The electromagnetic spectrum. Areas used for protein structure investigation are the ultraviolet, visible and infrared.

The regions of ultraviolet, visible and infrared (IR) wavelengths are commonly used in analysis of proteins. Electromagnetic radiation behaves as two separate waves; an electric and a magnetic wave at right angles to each other. The electric field can be polarised and this property makes it suitable to be used in analysis of protein molecules.
Figure 1.8: Schematic diagram of the electric field (E) and magnetic field (M) oscillating to make up linearly polarised light (\( \lambda = \) wavelength). For circularly polarised light, the plane polarised light, made up of two components can change direction but not magnitude.

**Circular Dichroism**

In linearly polarised light the electric field is polarised in one plane, in one direction and only the magnitude changes (figure 1.8). For circularly polarised light, the plane polarised light, made up of two components can change direction but not magnitude. Therefore the light can rotate in a clockwise (right-handed) or anti-clockwise (left-handed) manner. Two linearly polarised beams of light are circularly polarised in either a right or left handed manner due to them being out of phase with each other. Circular Dichroism (CD) spectroscopy measures the difference in the absorption of left-handed compared to right-handed polarized light (Johnson, 1985).

\[
\Delta A = A_L - A_R
\]  

(1.5)

Where \( \Delta A \) = the difference in absorbance of left and right circularly polarised light (\( \Delta L \) and \( \Delta R \)).
This absorbance can be incorporated into Beers law;

\[ A = \varepsilon c l \]  
(1.6)

Where \( A = \) Absorbance, \( \varepsilon = \) Extinction coefficient, \( c = \) Molar concentration and \( l = \) pathlength (cm).

Using CD data Beers law becomes

\[ \Delta A = (\varepsilon_L - \varepsilon_R) c l \]  
(1.7)

Where \( \Delta A = \) Absorbance, \( \varepsilon = \) Extinction coefficient for right and left circularly polarised light, \( c = \) Molar concentration and \( l = \) Pathlength (cm).

Therefore the molar CD is calculated using

\[ \Delta \varepsilon = \varepsilon_L - \varepsilon_R \]  
(1.8)

\( \Delta \varepsilon = \) The difference in extinction coefficient of the left and right handed circularly polarised light, \( \varepsilon_L = \) Extinction coefficient of left handed polarised light and \( \varepsilon_R = \) Extinction coefficient of right handed polarised light.

If the absorption of both right and left handed light was equal no difference would be seen, figure 1.9A. \( \Delta \varepsilon \) is plotted against wavelength producing the CD spectrum. \( \Delta \varepsilon \) for far and near UV CD has the units Mol\(^{-1}\) cm\(^{-1}\). In the far UV the concentration used is that of the amino acid residues and in the near UV it refers to protein molar concentration.
Figure 1.9: The absorption of polarised light. A: If the right and left handed polarized light is absorbed to the same extent then the result is plane polarised light. B: If the right and left handed components are absorbed to different extents then the result is elliptically polarised light. (Adapted from Kelly et al., 2005)

Circularly polarised light can be used on molecules which are asymmetric (chiral). Amino acids all have a chiral centre due to the four different groups bonded to the α-carbon atom. CD can be used to measure the optical activity of proteins in solution (Schmid, 1997). Most common is the measurement of the secondary structure of the protein molecule. This is due to α-helices, β-sheets, β-turns and random coils all producing specific signals in the far UV region.

Far UV CD

The far-UV region (170-250 nm) gives information about the peptide bond and is used to investigate secondary structure of protein molecules. Figure 1.10 illustrates the various types of secondary structure which can be measured.
Figure 1.10: Representation of the different secondary structures and their far UV signals. Solid line shows α-helix, long dashed line shows β-sheet, dotted line shows β-turn, crossed dashed line shows 3_{10}-helix or poly (Pro) II helix and the short dashed line shows irregular structure (Kelly et al., 2005).

Measurements on conventional CD machines are not usually extended below 180 nm however with the introduction of synchrotron radiation CD (SRCD) more reliable and informative measurements can be made. Using this technique measurements at 160 nm have been achieved (Wallace, 2000).

Near UV CD

The near-UV region (250-320 nm) is dominated by the aromatic residues (260-320 nm) and disulphide bonds (260 nm). Tryptophan (Trp) shows a peak at 290 nm, tyrosine between 275-282 nm and phenylalanine between 255-270 nm (Kelly et al., 2005). The near UV spectrum can give information about which aromatic residues are present and their environments.
Thermal stability of protein can be measured using CD by following the changes in the spectrum with increasing temperature. A wavelength is chosen at which to monitor the changes and the entire spectrum can be followed over a range of temperatures. It is only when the unfolding is reversible that this becomes a direct relation to the conformational stability of the protein. If the protein aggregates as it unfolds the melting temperature indicates the kinetics of aggregation of the protein.

**Fluorescence**

Fluorescence is the absorption of a photon of radiation (from appropriate light source) to promote an electron from the ground state to an excited state. This electron returns to a lower energy state by emitting a second photon of a longer wavelength. This phenomenon is represented by the Jablonski diagram in figure 1.11.

![Jablonski diagram](image)

**Figure 1.11: Jablonski diagram.** The Jablonski diagram illustrates the energy levels encountered when absorption and emission takes place. The first, second and third electronic states are represented by $S_0$, $S_1$ and $S_2$ respectively.
In a singlet excited state the electron in the highest energy state is paired with a second electron in a lower energy state. These two electrons have opposite spin orientations. The consequence of the electrons returning to ground state is fluorescence. In a triplet state the electrons are unpaired, and are in the same spin orientation. Opposite spin orientation is required for return to the ground state. The return of the electron to the singlet ground state is phosphofluorescence and has a much longer lifetime of milliseconds to seconds whereas fluorescence has a lifetime of 10 nsec (Lakowicz, 1983).

For fluorescence to take place the electrons have to be excited at a specific wavelength. This absorption of light enables the transformation of the electron to a higher vibrational level, either S₁ or S₂. The excited electron relaxes down the vibrational levels to the first excited state, S₁0. The electron then returns to ground state emitted light at a longer wavelength. Non-radiative relaxation can also occur instead of fluorescence or intersystem crossing. This is a result of excited molecules transferring their energy and the result is heat or quenching (Lakowicz, 1983).

Substances which display fluorescence usually possess delocalized electrons present in double bonds. Emission spectra vary widely and are dependent on many factors such as chemical structure of the fluorophore and the environment in which the fluorophore is present (Lakowicz, 1983).
Figure 1.12: The chemical structures of tyrosine, phenylalanine and tryptophan. All three influence the protein absorption of light and the fluorescence emission due to their aromatic groups, drawn using ChemWindow™ 6.

Proteins that contain aromatic amino acids (figure 1.12) can be used to assay intrinsic fluorescence (Middelberg, 2002). Protein fluorescence is generally excited at 280 nm or longer wavelengths. The absorption at 280 nm is due to the two amino acids, tyrosine and tryptophan. Above 295 nm the absorption and subsequent fluorescence is due to solely tryptophan and so tryptophan fluorescence can be selectively monitored. Phenylalanine has a weak absorption at 250 nm. The emission maximum for tyrosine in water occurs at 303 nm and is relatively insensitive to solvent polarity. However, tryptophan whose emission maximum in water is 348 nm is highly sensitive to solvent polarity (Lakowicz, 1983).

In proteins that contain all three aromatic amino acids the observed fluorescence is usually due to the tryptophan residues as the emission is
usually greater than for tyrosine and phenylalanine. There is also energy transfer between the three residues. As tyrosine and tryptophan absorb at 280 nm, the wavelength at which phenylalanine emits fluorescence, the fluorescence emitted by phenylalanine is quenched. In a folded protein the residues may be closer together therefore increasing the level of energy transfer. As the protein unfolds the residues may move apart and the emission from the quenched residues can be seen. In proteins that contain only tryptophan and tyrosine the fluorescence from tyrosine is not usually seen due to energy transfer, however on unfolding the residues move apart and energy transfer becomes less efficient (Schmid, 1997).

1.5: Aims

Cobra Biomanufacturing PLC
Cobra Biomanufacturing PLC is the industrial partner in this BBSRC CASE award. Cobra Biomanufacturing PLC is a contract manufacturer providing customers with process development, scale-up, assay development and GMP manufacture, services required to take biopharmaceuticals from discovery, through development to commercialisation. Cobra have established leading capabilities in the manufacture of microbially derived biopharmaceuticals, peptide pharmaceuticals and DNA medicines. One of the projects Cobra is concerned with is the expression, purification, refolding and manufacture of Interferon-a2b (IFN-a2b).

Aims
The aim of this project is to improve the refolding strategy of IFN-a2b for Cobra Biomanufacturing. Initially biophysical characterisation studies of the natively folded IFN-a2b standard provided by Cobra Biomanufacturing were carried out. The IFN-a2b molecule consists of a five alpha helical bundle containing two disulphide bonds. The techniques used for analysis of IFN-a2bs biophysical characteristics was a collection of those considered previously; both far and near UV CD and intrinsic fluorescence. The findings from these initial studies provided a final point of reference at which expressed and refolded IFN-a2b was measured against. As discussed IFN-
α2b has two disulphide bonds and so their influence on IFN-α2b’s thermal and denaturant stability was also investigated. The ability of common denaturants to disrupt the IFN-α2b structure was analysed which subsequently influenced the choice of solubilisation method used on the IFN-α2b inclusion bodies.

The expression of IFN-α2b in E. coli shall provide the material for refolding studies to be carried out. The IFN-α2b was expressed as inclusion bodies using the plasmid provided. Washing and solubilisation of the inclusion bodies was optimised resulting in a relatively pure inclusion body. Purification of the solubilised inclusion bodies was required and was investigated by exploiting IFN-α2b structural features. Ion exchange was unsuccessful however gel filtration resulted in an efficient method of purifying the solubilised IFN-α2b. Recent literature of tried and tested methods were also consulted. Once soluble inclusion bodies were acquired the refolding trials took place. These were based on a combination of information gained from the biophysical characterisation studies and published literature. Once a refolding strategy was formed, optimisation including further purification or polishing steps were considered.

Due to the unique nature of inclusion bodies and interest in denaturation of proteins, especially where possible structure exists, basic biophysical studies were carried out on the IFN-α2b solubilised inclusion bodies. Many studies of denatured proteins are carried out on thermally denatured peptides using specialised techniques such as nuclear magnetic resonance (NMR) and Fourier transform infrared spectroscopy (FTIR). In this study only the techniques readily available were utilised providing evidence of potential structure present in the IFN-α2b inclusion bodies however non-native, when treated with denaturants.
Chapter 2

Methods

All the reagents and suppliers used can be found in Appendix 1.

2.1: General methods

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out to analyse the folded IFN-α2b standard and the refolded samples of IFN-α2b. This technique relies on the ability of SDS to denature and provide the protein molecules with a large negative charge. The samples are placed in the wells of the gel and subjected to an electric field in which they are attracted to the bottom of the gel, the anode. The acrylamide provides a barrier of pores allowing smaller molecules to move faster than larger molecules therefore separating the protein mixtures by size. The higher the percentage acrylamide the more resolved are smaller protein molecules.

SDS-PAGE analysis was carried out on 12 %, 15 % and 17 % acrylamide gels in accordance to Laemmli (1970) using the Mini Protean II Electrophoresis Cell (BioRad). Details of the buffers and solutions used throughout can be found in table 2.1 and 2.2.

Figure 2.1: Precision Plus Protein™ Dual colour Standard (Bio-Rad) used for all SDS-PAGE analysis.
<table>
<thead>
<tr>
<th>Component Type</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lower Buffer</strong></td>
<td>1.5 M Tris-HCl 14 mM SDS pH 8.8</td>
</tr>
<tr>
<td><strong>Upper Buffer</strong></td>
<td>0.5 M Tris-HCl 14 mM SDS pH 6.8</td>
</tr>
<tr>
<td><strong>Running buffer</strong></td>
<td>0.125 M Tris-HCl 0.96 M Glycine 0.5 % (w/v) SDS</td>
</tr>
<tr>
<td><strong>2X Sample buffer</strong></td>
<td>0.125 M Tris-HCl 2 % (w/v) SDS 0.01 % (w/v) bromophenol blue 15 % (v/v) glycerol 5 mM Na₂EDTA</td>
</tr>
</tbody>
</table>

**Table 2.1: Buffer and solution composition for SDS-PAGE**

<table>
<thead>
<tr>
<th>Component Type</th>
<th>Water (ml)</th>
<th>Lower buffer (ml)</th>
<th>40 % Acrylamide (ml)</th>
<th>APS (μl)</th>
<th>TEMED (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 % resolving gel</td>
<td>3.305</td>
<td>2.25</td>
<td>3.375</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>12 % resolving gel</td>
<td>3.98</td>
<td>2.25</td>
<td>2.7</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>17 % resolving gel</td>
<td>3.7</td>
<td>2.25</td>
<td>2.35</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>4 % Stacking gel</td>
<td>2.59</td>
<td>1</td>
<td>0.35</td>
<td>50</td>
<td>10</td>
</tr>
</tbody>
</table>

**Table 2.2: Quantities of each component used to prepare gels depending on the percentage acrylamide required.**
Electrophoresis was carried out at 140 volts for approximately 1.5 h. Staining of the gels was carried out using a solution of 0.2 % (w/v) Coomassie brilliant blue, 10 % (v/v) acetic acid and 10 % (v/v) isopropanol for ~ 1 h. Destaining was carried out in 10 % (v/v) acetic acid and 10 % (v/v) isopropanol overnight. SimplyBlue™ SafeStain (Invitrogen) was used when a more sensitive staining method was required. The freshly run gels were repeatedly washed with water 3 times and then left for 3 h in ~20 ml SafeStain. Destaining was carried out in water. All gels were then stored in water until dried between cellophane sheets.

**Isoelectric focusing**

Isoelectric focusing (IEF) is an electrophoresis technique that separates proteins on the basis of their isoelectric point (pI). The pI of a protein is the pH at which the protein has no net charge and does not move in an electric field. IEF gels effectively create a pH gradient so proteins separate according to their pI. A protein sample is loaded onto the gel and an electric charge applied, the anode is at the acidic end of the gradient and the cathode is at the basic end of the gradient. Proteins with a net positive charge on the surface will migrate to the cathode and negatively charged proteins will move towards the anode. When the protein reaches an environment where the net surface charge is zero, it will no longer move. At this point the protein becomes "focused" and a band is formed in the gel. For this application the Novex® pH 3-10 IEF Buffer Kit (Invitrogen) was used.

### 2.2: Ion exchange

**Theory**

Ion exchange depends on the forces of attraction between a charged matrix and an oppositely charged protein. There are two different types, cationic (negative) exchange and anionic (positive) exchange. The binding of the protein can firstly be achieved by applying the protein to a buffered environment of a specific pH causing the protein to gain a net charge opposite to the matrix. Secondly the protein solution should be applied to the column under the same conditions. The binding of the protein is reversible and can be eluted by an increase in salt concentration, usually NaCl. The weakly bound molecules elute first and with
increasing the gradient of NaCl the more strongly bound molecules elute resulting in their separation.

**Purification of the IFN-α2b inclusion bodies**

The pI of IFN-α2b had been calculated to 6 and so anionic exchange was chosen initially. The column used was a 1 ml HiTrap Q Sepharose™ FF column and for the cationic exchange a 1 ml SP Sepharose™ FF column was used (G.E. Healthcare). The flow rate was 1 ml/min and after each run the column was cleaned with 1 M sodium hydroxide (NaOH) followed by water. All ion exchange was carried out by hand using a 20 ml syringe to apply the buffer. Initially the ion exchange experiments were carried out at a reduced urea concentration of 4 M however this was eventually increased to 8 M urea to ensure the IFN-α2b was kept denatured. A PD-10 column (G.E. Healthcare) was used initially for buffer exchange of the guanidine-HCl solubilised samples but due to the results this was substituted by dialysis to ensure complete buffer exchange. The dialysis was carried out for 16 h at either 4 °C or room temperature. The elution was performed by an increasing range of NaCl concentrations. The results and all the conditions tried can be seen in table 4.1, chapter 4.

**Purification post refolding**

The ion exchange post successful refolding was carried out on the same 1 ml HiTrap Q FF column used for the initial purification of the inclusion bodies. Instead of by hand an ÄKTAprime™ system (G.E. Healthcare) was used. The flow rate was 1 ml/min and the load volume was 10 ml. The ion exchange was carried out at pH 8.0, 8.4 and 8.6 from a guanidine-HCl solubilised and refolded inclusion bodies. The ion exchange was also carried out on the refolded samples from urea solubilised inclusion bodies but only at pH 8.0. The eluate was collected in 1 ml fractions.
2.3: Gel filtration

Theory

Gel filtration (also known as size exclusion) is a method of separating molecules on the basis of their size. The matrix is made up of spherical particles all porous. The technique relies on the smaller molecules passing into the matrix and therefore eluting later than larger molecules which are unable to pass into the beads therefore eluting early. Gel filtration has advantages over other chromatographic methods as it does not depend on binding of the protein to the matrix and the conditions used can be varied to suit the protein of interest. Superdex™ 75 media was chosen for this application. Superdex™ is based on cross-linked agarose particles covalently bonded to dextran. This gives the media both chemical and physical stability, important here due to the use of high concentrations of urea and guanidine-HCl. Superdex™ 75 has a fractionation range of 3000 to 70000 Da.

Column packing and efficiency

The Superdex™ media was supplied as free resin stored in 20 % ethanol. A XK16 column was packed with the Superdex™ 75 gel filtration media. The tallest bed height was aimed for with the available media. The packing of the column was carried out using water at a flowrate of 0.7 ml/min increasing to 1 ml/min to finish. The bed height was 11.5 cm giving a total column volume of 23 ml. To confirm the columns performance the number of theoretical plates and peak asymmetry were determined. The typical values for Superdex™ provided by G.E. Healthcare are an efficiency of \( N > 10000 \) and a peak asymmetry between 0.7-1.3.

To measure both the number of plates and the asymmetry the column was equilibrated with 20 mM Tris-HCl pH 8.0 until a stable baseline was reached. The column was then spiked with 0.5 ml (approx 2 % column volume) 1 M NaCl and the conductivity recorded until the NaCl had eluted and a stable baseline achieved.
A selection of molecular weight markers were also applied to the gel filtration column to ensure the efficient running of the column. These were cytochrome C (12.5 kDa), carbonic anhydrase (29.5 kDa), β-amylase (200 kDa) and blue dextran (2000 kDa). Both the blue dextran and β-amylase should illustrate the void volume of the column. All samples were loaded at a volume of 0.5 ml at an approximate concentration of 1 mg/ml; carbonic anhydrase was loaded at a concentration of approx 0.1 mg/ml due to availability. The running buffer was 20 mM Tris-HCl, 0.15 M NaCl pH 8.0.

Purification of the IFN-α2b inclusion bodies
The flowrate for the purification of the inclusion bodies was 0.5 ml/min and the load volume was 10 ml. The column was pre-equilibrated with the specified running buffer and the sample not applied until a steady baseline had been reached. The fractions were collected as 1 ml aliquots and pooled on the basis of the absorbance spectrum at 280 nm (A_{280}). After each run the column was washed with water, followed by 1 column volume of 0.5 M sodium hydroxide and washed with water a second time. The column was stored in 20 % (v/v) ethanol. The running buffers were the solubilisation buffers; 6 M guanidine-HCl, 20 mM Tris-HCl pH 8.0 or 8 M urea, 20 mM Tris-HCl pH 8.0, with or without 5 mM DTT.

2.4: Protein Concentration Determination

Measurements at 280 nm
The total protein concentration can be calculated by how much light passes through the protein sample.

\[ A = \log \frac{I_o}{I} \]  

(2.1)

Where A = Absorbance, I_o = Incident light intensity and I = Transmitted light intensity.

The Beer-Lambert law shows that the concentration of sample and the samples absorbance have a linear relationship.
Beer-Lambert law: \[ A = \varepsilon \cdot c \cdot l \] (2.2)

Where \( \varepsilon \) = molar extinction coefficient, \( c \) = molar concentration of the absorbing substance and \( l \) = pathlength of the light (cm).

All protein concentrations were determined by absorbance at 280 nm using the provided (by Cobra) molar extinction coefficient 19200 \( \text{cm}^{-1} \text{ mol}^{-1} \). A Cary 4E UV-Visible absorbance spectrophotometer was used along with a pair of 1 cm path length Hellma quartz cuvette.

Bicinochoninic Acid Protein Assay (BCA)
The BCA assay is similar to the Lowry procedure (Lowry et al., 1951), as they both rely on the formation of a \( \text{Cu}^{2+} \) and protein complex under alkaline conditions followed by the reduction to \( \text{Cu}^{1+} \) from \( \text{Cu}^{2+} \). The amount of reduction is proportional to the amount of protein present. The change in colour of the BCA with the \( \text{Cu}^{1+} \) to a purple-blue allows the change to be monitored at 560 nm. The BCA working reagent is made up of reagent A and reagent B. Reagent A is bicinchoninic acid solution pH 11.25 and Reagent B is 4 % (w/v) copper (II) sulphate pentahydrate. The protein standard Bovine Serum Albumin (BSA) is provided at 1 mg/ml. The working BCA reagent is prepared by mixing reagent A to reagent B at a ratio of 50:1. This solution appears green in colour and is added to the protein solution at a ratio of 20:1. The samples are incubated for 30 minutes at 37 °C followed by absorbance measurement at 560 nm.

2.4: \( N-(1\text{-pyrene})\text{maleimide} \) labelling of IFN-\( \alpha2b \) solubilised inclusion bodies

The solubilised inclusion bodies were labelled with \( N-(1\text{-pyrene})\text{maleimide} \). \( N-(1\text{-pyrene})\text{maleimide} \) is non-fluorescent probe exhibiting monomer emission peaks at 375 nm and 395 nm when excited at \( \sim345 \) nm. When the pyrene molecules are brought into close proximity and are excited they form excited dimers (excimers) that emit at longer wavelengths usually around 470 nm (Sahoo et al., 2002).
The samples were incubated for 2 h at room temperature with a 10 X molar excess of Tris-HCl(2-carboxyethyl)phosphine hydrochloride (TCEP) to reduce the four cysteine residues in IFN-α2b. A 5 M molar excess of N-(1-pyrene) maleimide solution was added to the protein samples and incubated overnight at room temperature with agitation. Following incubation excess N-(1-pyrene) maleimide was removed by use of a PD-10 column. The eluate was collected and analysed by fluorescence and absorbance. The fluorescence was measured between 350 -600 nm after excitation at 338 nm. The absorbance was carried out between 250-370 nm using an extinction coefficient of 40000 M$^{-1}$ cm$^{-1}$ at 344 nm (Drury and Narayanaswami, 2005).

2.5: Circular dichroism spectroscopy

CD was used to investigate the secondary and tertiary structure of IFN-α2b using wavelengths in the far UV region (250-190 nm) and the near UV region (320-250 nm). All CD spectroscopy was carried out on a Jasco J-810 spectropolarimeter and all cuvettes were from Hellma. All spectra were calculated by subtraction of a buffer baseline followed by conversion to Δε (Mol$^{-1}$ cm$^{-1}$l$^{-1}$). For far UV CD the mean residue weight (MRW) used for IFN-α2b was 116. The spectra were measured at a HT (V) value <600. The conditions for all CD analysis are shown in table 2.3.

<table>
<thead>
<tr>
<th></th>
<th>Far UV</th>
<th>Near UV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Band Width (nm)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Response (sec)</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Measurement wavelength (nm)</td>
<td>250-190</td>
<td>320-250</td>
</tr>
<tr>
<td>Data Pitch (nm)</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Scanning speed (nm/min)</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Accumulations</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Size of cuvette (cm)</td>
<td>0.02</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2.3: Conditions of all far and near UV CD analysis.
Initial characterisation of the IFN-α2b Cobra standard

Initial characterisation studies were carried out on purified and folded IFN-α2b. IFN-α2b for these studies was provided by Cobra Biomanufacturing PLC as EP interferon (CTL2003#1010M) in an unknown buffer at a concentration of 8.47 mg/ml, 10 aliquots were provided.

Circular dichroism spectroscopy of folded IFN-α2b

IFN-α2b was diluted and dialysed into 0.2 M sodium phosphate, 100 mM sodium chloride pH 7.0. IFN-α2b was diluted to 0.5 mg/ml and left to dialyse for 16 h at 4 °C. Both the far UV and near UV CD were analysed. DTT was added to the folded IFN-α2b at a final concentration of 5 mM and then incubated overnight with agitation before the far and near UV CD was measured also.

Circular dichroism spectroscopy of denatured IFN-α2b

The Cobra supplied IFN-α2b was diluted to 0.5 mg/ml. Two stock solutions of 8 M urea and 6 M guanidine-HCl were used to both dilute and dialyse the two IFN-α2b samples. The samples were incubated for 16 h at room temperature giving a final denaturant concentration of 5 M guanidine-HCl and 7 M urea. Both far-UV and near-UV analysis was performed on these samples.

2.6: Thermal stability of IFN-α2b

Thermal melt determination by loss in secondary structure

Thermal stability of IFN-α2b was investigated at different pH values to determine the transition temperatures. Melting temperatures (T_m) were determined at pH 7.0, 5.0 and 9.0, using; 0.2 M phosphate, 100 mM sodium chloride pH 7.0, 100 mM citric acid pH 5.0 and 25 mM sodium borate pH 9.0. An initial far UV CD scan was taken of the samples at a concentration of 0.1 mg/ml in these three buffers to ensure the secondary structure was still intact. The thermal unfolding was also to be carried out at the same concentration. Thermal unfolding was carried out between 20-95 °C at a rate of 1 °C/min in a 0.1 cm stoppered cuvette. The CD signal was followed at 223 nm. The samples also had a far-UV scan taken every 5 °C. This experiment was also repeated with the addition of DTT to
a final concentration of 5 mM. Samples containing DTT were incubated at 20 °C overnight prior to thermal denaturation.

**Differential scanning microcalorimetry (DSC)**

Differential scanning microcalorimetry (DSC) involves the heating of a protein solution at a constant rate alongside a reference cell containing the same buffer as the protein solution. The difference in the heat energy uptake between the sample and the reference required to maintain an equal temperature corresponds to a heat capacity which can be used to determine a melting temperature (Tm). The DSC was performed on a Micro-Cal VP-DSC (MicroCal Europe, Milton Keynes, UK). The initial scan was carried out on an IFN-α2b sample of 0.5 mg/ml followed by 0.1 mg/ml due to the precipitation encountered. Both samples were in the buffer 0.2 M phosphate 0.1 M NaCl pH 7.0 and were filtered and degassed prior to use. The scan rate was 1 °C/min.

**2.7: Intrinsic fluorescence**

Intrinsic fluorescence was used to analyse IFN-α2b due to the presence of 5 tyrosine and 2 tryptophan residues. The emission was measured between 296-380 nm. The excitation wavelength was 280 nm, a 5 mm cuvette (Hellma) was used and all measurements were carried out at 20 °C. The protein concentration used for fluorescence analysis was 0.1 mg/ml unless otherwise stated. All measurements were carried out on a Varian Cary Eclipse fluorescence spectrophotometer.

Alongside the maximum intensity being monitored the barycentric mean was also used to compare samples (Lakey et al., 1989). The λ_max is the wavelength at which the emission reaches its highest point. However this is not the preferred method of analysing fluorescence data, as λ_max is sensitive to noise in the sample. The λ_bary measures the 'centre of mass' of the data giving a wavelength which takes into account all emissions over a chosen wavelength range. The λ_bary is calculated by equation 1.3 where λ is wavelength and i is intensity.
Fluorescence analysis of the folded IFN-α2b
The fluorescence of the folded IFN-α2b was analysed at a total protein concentration of 0.1 mg/ml. To this sample 5 mM DTT was added to measure the effect, if any, as with the CD analysis.

Fluorescence analysis of IFN-α2b in urea and guanidine-HCl
The IFN-α2b was diluted to 0.1 mg/ml and dialysed into the denaturants giving final concentrations of 5 M guanidine and 7 M urea. Samples were left to dialyse for 16 h at room temperature. Following fluorescence analysis of the denatured sample DTT was then added at a final concentration of 5 mM. The fluorescence of this sample was measured immediately (T = 0), at T = 60 minutes, at T = 120 minutes and after incubation overnight.

Determination of unfolding curves for IFN-α2b using both urea and guanidine-HCl.
The unfolding of IFN-α2b was investigated using intrinsic fluorescence and in the far UV region measured at 223 nm. The sample of IFN-α2b at 0.05 mg/ml was prepared with guanidine-HCl and urea added to give a range of concentrations from 0 M to 7 M (urea) and 6.5 M (guanidine-HCl). The samples were left to incubate with agitation overnight. The exact concentration of the stock solution of guanidine-HCl was determined using a Bellingham and Stanley refractometer. This measures the difference in refractive index between the guanidine-HCl buffer and water. Equation 1.4 was used to calculate the exact concentration of guanidine-HCl and equation 1.5 was used to determine the urea concentration.
Guanidine-HCl

\[57.147(\Delta N) + 38.68(\Delta N)^2 - 91.60(\Delta N)^3\]  \hspace{1cm} (2.4)

Urea

\[117.66(\Delta N) + 29.753(\Delta N)^2 + 185.56(\Delta N)^3\]  \hspace{1cm} (2.5)

\(\Delta N\) is the difference in the refractive index of the denaturant solution and water at the sodium D line (Pace, 1986).

The range of concentrations for the urea samples were every 0.5 M from 0 M to 7 M. For the guanidine-HCl experiments this was used up to 6.5 M however around the mid point a more narrow range was measured at increments of every 0.15 M. The samples were analysed by both far UV CD and intrinsic fluorescence. DTT was added at 5 mM to a second set of samples at the same denaturant concentration. Once the data had been collected the guanidine-HCl results from both the fluorescence and the CD experiments were used for the calculation of the conformational stability of IFN-\(\alpha\)2b in the absence of guanidine-HCl, \(\Delta G(H_2O)\) and \(m\), the dependence of \(\Delta G\) on denaturant concentration. For this calculation a two state folding mechanism is assumed,

\[f_f + f_u = 1\]  \hspace{1cm} (2.6)

\(f_f\) = fraction folded

\(f_u\) = fraction unfolded

Using the data collected

\[K = (y_f - y)/(y_f - y_u)\]  \hspace{1cm} (2.7)

Where \(y_f\) is the value of the folded protein and \(y_u\) is the value of the unfolded protein, \(y\) is the data at that specific guanidine-HCl concentration.

\[\Delta G = -RT \ln K\]  \hspace{1cm} (2.8)

\(R\) is the gas constant 1.98 cal/K/mol, \(T\) is the temperature in Kelvin 298.
\[ \Delta G = \Delta G(\text{H}_2\text{O}) - m[D] \]  

(2.9)

\( \Delta G(\text{H}_2\text{O}) \) = the y intercept

\( m \) = the gradient of the line

\([D]\) = Denaturant concentration

In some cases the detergent 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) which is a nondenaturing zwitterionic detergent was added at 1% (w/v) to the urea samples with DTT. This would possibly break the possible hydrophobic core of the IFN-α2b which may have formed due to the inefficient nature denaturation with urea (Pasquali et al, 1997). The far UV CD was measured at 1, 3, 5 and 7 M urea.

**8-Anilino-1-naphthalenesulphonate binding**

8-Anilino-1-naphthalenesulphonate (ANS) is a fluorescent probe which has an affinity for the hydrophobic regions within a polypeptide chain (Stryer, 1965). This affinity is at its most extensive when a molten globule structure is present. ANS weakly binds the denatured and native structure (Semisotnov et al., 1987; 1991). ANS is negatively charged and a non-covalent fluorescence probe which emits fluorescence when exposed to highly hydrophobic environments. ANS shifts as with tryptophan fluorescence to shorter wavelengths when in a more hydrophobic environment.

**Figure 2.2: Chemical structure of ANS.** ANS is used to investigate the hydrophobic nature of protein molecules. Drawn using Chemwindow® 6.
ANS was added to the IFN-α2b standard, the refolded IFN-α2b samples and the solubilised inclusion bodies. ANS was prepared by making a concentrated solution, allowing it to dissolve followed by filtration using a 0.2 µm filter. The concentration was measured using an extinction coefficient of 5000 M⁻¹cm⁻¹ at 360 nm (Matulis and Lovrien, 1998). The ANS was added to the sample at a final concentration of 0.1 mM, the samples were well mixed and the fluorescence emission measured between 400-600 nm after excitation at 360 nm.

2.8: Growth media and bacterial strains

All expression of IFN-α2b was carried out in autoclaved Luria Bertani media (LB), 10g/l tryptone, 5g/l yeast extract, 10g/l sodium chloride. For the initial isolation of the bacterial colonies bacterial agar 2 g of agar was added to 100 ml of LB and set in Petri dishes. Both the agar and LB were supplemented with the antibiotic kanamycin at a final concentration of 30 µg/ml. The LB plates were kept at 4 °C for short term storage.

The strains used for the initial expression of IFN-α2b were BL21 (DE3), JM105 and DH5α.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>BL21 (DE3)</td>
<td>E.coli B F⁻ dcm ompT hsdS(rB mB-) gal λ(DE3)</td>
<td>(Studier et al., 1990)</td>
</tr>
<tr>
<td>JM105</td>
<td>thi rpsL endA sscB15 hspR4 Δ(lac-proAB) [F' tarD36 proAB lacZΔM15]</td>
<td>(Yanischperron et al., 1985)</td>
</tr>
<tr>
<td>DH5α</td>
<td>F'80dlacZΔM15 Δ(lacZYA-argF) U169 recA endA1 hsdR17(rk, mK+) phoA supE44 thi-1 gyrA96 relA1</td>
<td>(Woodcock et al., 1989)</td>
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</table>

Table 2.4: The genotype of the bacterial strains used for the initial expression of IFN-α2b.
The plasmid shown in figure 2.3 was provided by Cobra Biomanufacturing PLC and used throughout for all IFN-α2b expressions. The plasmid provided consisted of the DNA sequence for expression IFN-α2b. The expression was under the temperature control of the lambda cl857 repressor.

Transformation of competent BL21 (DE3), DH5α and JM105 cells with pSL-A2b(Kan)+

The vector pSL-A2bkan(+) containing the IFN-α2b insert (2 μl) was added to 200 μl of thawed competent cells of all three strains; BL21 (DE3), DH5α and JM105. Samples were incubated on ice for 30 minutes before a heat shock at 42 °C for 2 minutes allowing cell permeabilisation. All three strains were immediately incubated on ice for a further 5 minutes. Following this was the addition of 500 μl of sterile LB to the cells and incubation at 37 °C for 1 h. The transformants were
then plated out onto LB agar supplemented with kanamycin and incubated overnight at 37 °C.

2.9: Expression
All expression was carried out in E. coli strains with induction by increased temperature from 30 °C to 43 °C.

Small scale expression of IFN-α2b in BL21, DH5α and JM105
LB media tubes (5 ml) containing kanamycin at a final concentration of 30 μg/ml were inoculated with a single colony for the agar plate. The culture was incubated overnight at 30 °C repressing the lambda promoter. 250 ml flasks containing 50 ml LB (containing kanamycin) were inoculated by the addition of 1 ml of the 5 ml overnight culture. The 50 ml cultures were left to grow at 30 °C until the optical density at 600 nm (OD$_{600}$) of the culture reached 0.6-0.7. Once the culture reached the appropriate density the flasks containing each strain were transferred to a pre-heated incubator at 43 °C. Flasks of each strain were left at 30 °C to act as controls. The flasks at 43 °C and 30 °C were sampled every h and the OD$_{600}$ measured, these samples were also set aside for subsequent SDS polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Thermal induction was allowed to proceed at 43 °C for 5 h. The expression was carried out in these three strains to find a suitable system for future expressions. BL21 (DE3) was chosen.

Large scale expression of IFN-α2b using BL21 (DE3).
The strain chosen for future IFN-α2b expression was BL21 (DE3). The expression was scaled up to 500 ml cultures to begin treatment of the inclusion bodies. The transformation was carried out as above. The overnight 30 °C culture was scaled up from 5 ml to 50 ml. From this overnight 50 ml culture 5 ml was added to 500 ml of sterile LB. This larger culture was grown at 30 °C until and OD$_{600}$ of 0.6 and then the temperature increased to 43 °C. After a steady OD$_{600}$ reading of 2 had been reached the culture was centrifuged at 7025 x g (average speed) for 30 minutes at 4 °C using a Beckman Avanti J-25 centrifuge (rotor JA10). The pellets were kept frozen at −20 °C.
10 l fermentation of IFN-α2b

Due to the success of the IFN-α2b expression a large scale preparation was set up to increase yields. The fermenter vessel (Bioflow 3000 Batch/continuous Bioreactor, New Brunswick Scientific Edison, N.J. USA) was appropriately washed and sterilised by autoclaving. 10 l of LB was added to the fermenter vessel and autoclaved for a second time. Sigma antifoam 287 was added to the media to prevent excessive foaming of the culture whilst growing. To inoculate the culture 250 ml of overnight culture grown at 30 °C was added to the fermenter. Samples were taken every 30 minutes and the absorbance measured until an OD$_{600}$ of 0.6 was reached, the temperature of the fermenter was then increased to 43 °C and the culture grown until a steady OD$_{600}$ was reached. Samples were still taken every 30 minutes. The culture was harvested as detailed in section 2.10.

2.10: Harvesting of the IFN-α2b inclusion bodies

The cells from the expression were resuspended in 20 mM Tris-HCl pH 8.0 followed by sonication on ice. The inclusion bodies were subjected to repeated washing and centrifugation with 20 mM Tris-HCl and 1 % Triton X-100. Figure 2.4 illustrates the individual steps in the treatment of the inclusion bodies.
Resuspend Cell Pellets in 20 mM Tris-HCl pH 8.0

Sonication

Centrifuge Supernatant 16556 x g for 1 h at 4 °C

Resuspend Pellet in 20 mM Tris-HCl 1% Triton pH 8.0
Homogenise Pellet for 30 minutes at 37 °C

Centrifuge at 4709 x g for 1 h at 4 °C

Resuspend Pellet 20 mM Tris-HCl 1% Triton pH 8.0
Homogenise Pellet for 30 minutes at 37 °C

Centrifuge at 4709 x g for 45 minutes at 4 °C

Resuspend Pellet in 20 mM Tris-HCl 1% Triton pH 8.0
Homogenise Pellet for 30 minutes at 37 °C

Centrifuge at 4709 x g for 30 minutes at 4 °C

Solubilisation of the pellet in either 6 M guanidine-HCl,
20 mM Tris-HCl
pH 8.0 or 8 M urea, 20 mM Tris-HCl pH 8.0
Leave to solubilise overnight at room temperature

Centrifuge at 106255 x g for 1.5 h at 4 °C

Store solubilised IFN-α2b at -20 °C

Figure 2.4: Flowchart illustrating the treatment of the inclusion bodies before solubilisation. Centrifugation speeds are an average value.
The cell pellet was suspended in 20 mM Tris-HCl pH 8.0. To this suspension protease inhibitors were added; Benzamidine (100 μg/ml), AEBSF (100 μg/ml), Aprotonin (0.5 μg/ml), Leupeptin (1 μg/ml) and Pepstatin A (1 μg/ml). DNase 1 (10 μg/ml) and RNase (10 μg/ml) were also added. The cells were broken using Sonication (MISONIX sonicator 3000) to release the IFN-α2b inclusion bodies. The solution was centrifuged at 16556 x g (average speed) for 1 h in Beckman L780 ultracentrifuge (rotor Ti70) to pellet the inclusion bodies. The supernatant was removed and the pellet retained.

The inclusion body pellet was resuspended in 20 mM Tris-HCl/1 % Triton X-100 pH 8.0. This was added to a homogeniser, stationed in a 37 °C water bath. The pellet was homogenised, solubilising the outer membrane proteins in the Triton X-100 detergent. The homogenised pellet was centrifuged for 1 h at 4709 x g (average speed) in the ultracentrifuge (rotor Ti70). The pellet was resuspended and centrifuged as above, the centrifugation however was reduced to 45 minutes and then 30 minutes respectively. The supernatants were retained after each centrifugation for analysis. The washed inclusion bodies were stored at -20 °C.

**Solubilisation of the IFN-α2b inclusion bodies with 8 M urea and 6 M guanidine-HCl**

1 g (wet weight) of inclusion bodies was solubilised in 10 ml of denaturant. DTT was added wherever stated to a final concentration of 5 mM. The solutions were left overnight with agitation at room temperature. Centrifugation of the solubilised inclusion bodies was carried out at 106255 x g (average speed) for 1.5 h. The supernatants were diluted 1 in 10 and 0.01 g of the four pellets was resuspended in 100 μl of buffer for SDS-PAGE analysis. The supernatants were stored frozen at -20 °C. The inclusion bodies solubilised in 6 M guanidine-HCl required dialysis into 8 M urea in order to be analysed by SDS-PAGE, this was carried out at 1 ml in 1 l.

**Addition of 2 M and 4 M NaCl to 8 M urea for solubilisation**

Solubilisation buffers were prepared of 2 M and 4 M NaCl with 8 M urea, 20 mM Tris-HCl pH 8.0 solubilisation buffer. DTT was added to where stated. The
solubilisation of 1 g of inclusion bodies was carried out overnight at room temperature with agitation; all following steps were as above.

**SDS-PAGE analysis of reduced and oxidised forms of IFN-α2b**

Analysis by SDS-PAGE of the samples obtained after solubilisation indicated the presence of a protein between the molecular weights of 37-25 kDa. As IFN-α2b has been found to run at a molecular weight of 15 kDa this band could signify dimer formation. To confirm this and discover if any further purifications steps were required, DTT was added at a final concentration of 50 mM. Iodoacetamide (\(\text{ICH}_2\text{CO}_2\text{NH}_2\)), an alkylating agent was then added to a final concentration of 5 mM (Creighton, 1978) in order to block the cysteine residues.

The oxidation of IFN-α2b was carried out by the addition of copper sulphate to a final concentration of 0.1 mM and phenanthroline at 0.5 mM to the original DTT reduced sample. This sample was left to incubate overnight and then analysed by SDS-PAGE. For this sample a non-reducing sample buffer was used at a ratio of 1:1 with the sample.

2.11: Refolding

2.11.1: Refolding with no purification

The refolding was to be started simply based on a dilution and dialysis method. The formation of the disulphide bonds was to be encouraged by the addition of oxidised glutathione at a final concentration of 0.1 mM.

Refolding at 4 °C and 24 °C

The initial refolding experiments were carried out at both 4 °C and 24 °C with inclusion body samples solubilised in both 8 M urea and 6 M guanidine-HCl. The refold was as follows:

- 1 in 10 dilution into 20 mM Tris, 1 mM DTT pH 8.0
- 3 h incubation
- Addition of 0.1 mM oxidised glutathione
- 4 h incubation
- Dialysis into 20 mM Tris-HCl pH 8.0

The final samples from these four refolding experiments were analysed by far UV CD, fluorescence and SDS-PAGE.

Refolding with longer oxidised glutathione incubation time from both 8 M urea or 6 M guanidine-HCl solubilised inclusion bodies
These refolding experiments were carried out on four different samples in four different solubilisation buffers. The four conditions were 6 M guanidine-HCl, 20 mM Tris-HCl pH 8.0, 6 M guanidine-HCl, 20 mM Tris-HCl, 1 mM DTT pH 8.0, 8 M urea, 20 mM Tris-HCl pH 8.0 and 6 M urea, 20 mM Tris-HCl, 1 mM DTT pH 8.0. The refold was carried out as stated above but with a 16 h incubation time with oxidised glutathione.

Standard denaturation

Freeze thaw
The standard was diluted to 0.1 mg/ml and the fluorescence measured on excitation at 280 nm. This same sample was frozen overnight and the fluorescence under the same conditions measured. This was repeated with this same sample 5 times to indicated any change in fluorescence of the standard on freeze thawing.

Denaturation of the standard with DTT and heat
The standard was diluted to a concentration of 0.1 mg/ml in 6 M guanidine-HCl, 20 mM Tris-HCl pH 8.0. The sample was mixed thoroughly using a vortex and left at room temperature for 1 h. DTT was then added to the sample at a final concentration of 50 mM. This sample mixed thoroughly and left at room temperature for 2 h. The fluorescence was measured. Finally the sample was heated to 95 °C for 15 minutes. The fluorescence was measured.
2.11.2: Refolding after gel filtration

Initial refolding straight in 20 mM Tris-HCl

A sample of the guanidine-HCl solubilised inclusion bodies was dialysed straight into 20 mM Tris-HCl pH 8.0 at 4 °C. This was not successful previously but with the removal of contamination it may give a more positive result.

Refolding after gel filtration in three steps.
The refolding is based on a 1 in 10 dilution under reducing conditions followed by two dialysis steps, the first under reducing conditions and the second to remove the DTT.

Figure 2.5: Flowchart to illustrate the refolding of the samples after gel filtration.
Following the refolds the far and near UV CD were measured along with the intrinsic fluorescence, ANS binding and SDS-PAGE of the final samples.

2.12: Functional assays

Both the antiviral and the reporter gene assays were carried out at the National Institute for Biological Standards and Control (NIBSC, Potters Bar, Herts, UK). The IFN-α2b reference standard 95/566 and all reagents were all provided by NIBSC. An aliquot of the Cobra Biomanufacturing reference standard was taken frozen on dry ice, along with the refolded samples. Each refolded sample was divided into two; one was kept in 20 mM Tris-HCl pH 8.0 and the second was dialysed into phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.47 mM NaH₂PO₄ pH 7.4).

Antiviral assay
The cell lines tested were 2D9 and A549 (PBS samples only). Both the Tris-HCl and PBS samples were tested on the 2D9 cells. The cell lines were trypsinised and resuspended as single cell suspensions at 5 x 10⁵ cells per ml of growth medium Roswell Park Memorial Institute (RPMI) 1640 (Moore and Woods, 1976) supplemented with penicillin and streptomycin to prevent contamination, L-glutamine at 300 mg/l, 6% fetal calf serum, trypsin versene, sodium pyruvate. The cells were seeded onto a 96 well plate and incubated for 24 h at 37 °C. Serial dilutions of the samples were carried out in the growth medium and then transferred to the plates in duplicate. The plates were incubated for 24 h at 37 °C. Following the incubation the medium was replaced by medium containing 2% fetal calf serum containing encephalomyocarditis virus (EMCV). The plates were incubated for 24 h at 37 °C.

Staining was carried out by washing the plates with PBS with added Ca²⁺ and Mg²⁺ and then addition of 0.05 % naphthol blue. The stain was left for 25 minutes and then removed. A fixative was added to the wells consisting of 10 % formalin, 0.1 M sodium acetate and 9 % acetic acid solution. This was left for 2-5 minutes and removed by washing with water. The plates were left to dry for 3 h at 37 °C. The stain was eluted using 0.1 M sodium hydroxide and the plates read at 630 nm.
Plate setup of the antiviral assays

### Plate 1

|   | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  |
|---|
| A | CC  | Std | Std | CStd| CStd| G1a | G1a | G1b | G1b | G2a | G2a | VC  |
| B |     |     |     |     |     |     |     |     |     |     |     |     |
| C |     |     |     |     |     |     |     |     |     |     |     |     |
| D |     |     |     |     |     |     |     |     |     |     |     |     |
| E | VC  |     |     |     |     |     |     |     |     |     |     |     |
| F |     |     |     |     |     |     |     |     |     |     |     |     |
| G |     |     |     |     |     |     |     |     |     |     |     |     |
| H |     |     |     |     |     |     |     |     |     |     |     |     |

### Plate 2

|   | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  |
|---|
| A | CC  | G2b | G2b | Std | Std | U1  | U1  | U2  | U2  | UF  | UF  | VC  |
| B |     |     |     |     |     |     |     |     |     |     |     |     |
| C |     |     |     |     |     |     |     |     |     |     |     |     |
| D |     |     |     |     |     |     |     |     |     |     |     |     |
| E | VC  |     |     |     |     |     |     |     |     |     |     |     |
| F |     |     |     |     |     |     |     |     |     |     |     |     |
| G |     |     |     |     |     |     |     |     |     |     |     |     |
| H |     |     |     |     |     |     |     |     |     |     |     |     |

**Figure 2.6:** Plate set up of the antiviral assay carried out on refolded if samples.  
CC = Cell control, cells only no IFN-α2b or virus. CV = Virus control, cells and virus only.  
The sample layout can be seen along the top row. The samples were diluted 2 fold towards the bottom of the plate. For abbreviations see table 2.5.
The international standard for human IFN-α2b, 95/566, with an assigned potency of 70,000 international units (IU) per ampoule.

Cobra Biomanufacturing provided standard

Sample solubilised in guanidine-HCl and refolded

Sample solubilised in guanidine-HCl and dialysed into urea and refolded

Sample solubilised in guanidine-HCl and dialysed into urea and refolded

Sample solubilised in urea and refolded

Sample solubilised in urea 1 mM DTT and refolded

Sample solubilised in urea and attempted to be refolded (unfolded sample)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Starting Concentration</th>
<th>Abbreviation</th>
</tr>
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<tbody>
<tr>
<td>The international standard for human IFN-α2b, 95/566, with an assigned potency of 70,000 international units (IU) per ampoule.</td>
<td>31.25 pg/ml</td>
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<td>Sample solubilised in urea and attempted to be refolded (unfolded sample)</td>
<td>0.39 μg/ml</td>
<td>UF</td>
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Table 2.5: Concentrations of the samples used for the antiviral assay.

Reporter gene assay

Investigation of the protein inducing activity of the IFN-α2b. This experiment was only carried out on the PBS samples. The cell line used was a transfected (Human embryonic kidney cell line) HEK 293 cell line harbouring a secreted alkaline phosphatase cDNA linked to the IRES promoter. Cells were seeded in a 96 well microtitre plate at 2 x 10⁴ cells/ml in 0.1 ml colourless Dulbecco’s modified Eagle’s medium (DMEM) growth medium, and incubated for 24 h at 37 °C. After incubation, serial dilutions of the IFN-α2b samples were made on a separate plate and then transferred onto the assay plate. These plates were then incubated for 48 h at 37 °C. The 0.05 ml p-nitophenyl phosphate (pNPP) substrate (1 mg/ml in 10% ethanolamine buffer pH 9.8) was added to the plates.
and they were centrifuged to remove any bubbles. The plates were left for 20 minutes and then read at 405 nm using an ELISA reader.

**Plate setup of the reporter gene assays**

**Plate 1**

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*Figure 2.7: Plate set up of the reporter gene assay carried out on refolded if samples. The sample layout can be seen along the top row. The samples were diluted 2 fold towards the bottom of the plate.*
<table>
<thead>
<tr>
<th>Sample</th>
<th>Starting Concentration</th>
<th>Abbreviation</th>
</tr>
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<tbody>
<tr>
<td>The international standard for human IFN-α2b, 95/566, with an assigned potency of 70,000 international units (IU) per ampoule.</td>
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<td>Cobra Biomanufacturing provided standard</td>
<td>4.8 ng/ml</td>
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<td>Sample solubilised in guanidine-HCl and refolded</td>
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<td>Sample solubilised in guanidine-HCl and dialysed into urea and refolded</td>
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<tr>
<td>solubilised in guanidine-HCl and dialysed into urea and refolded</td>
<td>3.3 ng/ml</td>
<td>G2b</td>
</tr>
<tr>
<td>Sample solubilised in urea and refolded</td>
<td>530 ng/ml</td>
<td>U1</td>
</tr>
<tr>
<td>Sample solubilised in urea 1 mM DTT and refolded</td>
<td>890 ng/ml</td>
<td>U2</td>
</tr>
<tr>
<td>Sample solubilised in urea and attempted to be refolded (unfolded sample)</td>
<td>3.5 μg/ml</td>
<td>UF</td>
</tr>
</tbody>
</table>

Table 2.6: Concentrations of the samples used for the reporter gene assay.
Chapter 3
Chapter 3

Biophysical characterisation of the Cobra IFN-α2b reference standard

3.1: Introduction

In order to analyse the refolded material expressed and refolded during this project it was advantageous to investigate the IFN-α2b standard that Cobra Biomanufacturing use for their analysis. This IFN-α2b was provided by Cobra at a total protein concentration of 8.47 mg/ml as EP interferon (CTL2003#1010M). As Cobra do not have access to circular dichroism (CD) and fluorescence equipment it seemed appropriate that in-depth unfolding characterisation of the IFN-α2b standard should be carried out. This provided an understanding of how this molecule behaved in solution natively and non-natively but also provided experience using the techniques which will became useful tools during the further work. The far and near UV CD provided a fingerprint for the IFN-α2b molecule along with the intrinsic fluorescence, to which future refolded IFN-α2b was compared.

Unfolding of IFN-α2b was investigated using both guanidine-HCl and urea, both well utilised denaturants capable of non-covalently disrupting protein structure (Greene and Pace, 1974). Following the methods by Pace and Scholtz, (1997) the free energy (ΔG) of unfolding was calculated providing information on the stability of IFN-α2b. Along with guanidine-HCl and urea denaturation by DTT was investigated. IFN-α2b contains two disulphide bonds and they may be important in the molecule’s stability and crucial to unfolding or more importantly refolding. The unfolding using the two denaturants was monitored by far and near UV CD analysis but also by intrinsic fluorescence and 8-Anilino-1-naphthalenesulphonate (ANS) binding (Semisotnov et al., 1987). These are all good sensitive biophysical indicators of the presence of both secondary and tertiary structure.
The far UV CD spectra provided information on the secondary structure present in IFN-α2b. As IFN-α2b is made up of five alpha helices then strong signals at 208 and 220 nm were expected and consequently observed. The intrinsic fluorescence can provide information on the environments of the aromatic residues present in IFN-α2b particularly focusing on tryptophan and tyrosine, these results supported by the near UV CD analysis. As discussed in chapter 2, section 2.7, ANS binds to exposed hydrophobic regions of proteins (Stryer, 1965). This fluorescent probe confirmed any molten globule structure present in IFN-α2b during the unfolding.

3.2: Results

3.2.1: Far and near UV CD analysis of the IFN-α2b standard

The IFN-α2b standard was analysed in the far (190–250 nm) and near (250-320 nm) UV region.

Far and near UV CD of the natively folded IFN-α2b standard

To investigate the IFN-α2b standard the protein was diluted to 0.5 mg/ml and dialysed overnight into 0.2 M phosphate, 0.1 M sodium chloride pH 7.0. The far UV measurements were carried out using a 0.02 cm pathlength demountable cuvette and the near UV analysis carried out in a 1 cm pathlength low volume (150 µl) cuvette. Measurements were carried out at 20 °C and were an average of 10 accumulations.
Figure 3.1: The far UV CD spectrum of natively folded IFN-α2b. The far UV CD of IFN-α2b shows an alpha helical structure with negative peak intensities at 222 nm and 208 nm. Sample concentration was 0.5 mg/ml in 0.2M phosphate, 0.1 M sodium chloride pH 7.0 at 20 °C, the result is an average of 10 accumulations.

The far UV CD of the IFN-α2b standard shown in figure 3.1 is a characteristic alpha helical spectrum. The negative intensities seen at 208 nm and 222 nm are exactly what would be expected from a protein consisting of mainly alpha helical structure, confirming structural data found by Radhakrishnan et al., (1996). This spectrum shall act as a good reference for all future work. It is assumed that the IFN-α2b standard received from Cobra is natively folded. Units of Δε are M⁻¹cm⁻¹l⁻¹.
The near UV CD spectrum of natively folded IFN-α2b. The near UV CD of IFN-α2b shows two negative signals between 280 nm and 300 nm probably resulting from the two tryptophan residues present. The sample concentration was 0.5 mg/ml in 0.2M phosphate, 0.1 M sodium chloride pH 7.0 at 20 °C, the result is an average of 10 accumulations.

The near UV CD analysis in figure 3.2 displays a strong signal in the near UV region indicative of tertiary structure. The strong signals between 280 nm and 300 nm are characteristic of tryptophan residues being in a fixed environment (Kelly et al., 2005). Between 275 and 282 nm the signals are due to the five tyrosine residues and between 255 and 270 nm the signals are from the phenylalanine residues (Kelly et al., 2005).

Guanidine-HCl and urea treated IFN-α2b analysed by far and near UV CD.

This analysis involved incubating the IFN-α2b standard in high concentrations of both guanidine-HCl and urea. From this the unfolded state of IFN-α2b should be obtained. The samples were incubated in the buffers; 5 M guanidine-HCl, 0.2 M phosphate pH 7.0 and 7 M urea, 0.2 M phosphate pH
7.0 for 16 hours at room temperature with constant agitation. Both the far and near UV CD were measured.

![Graph](image)

**Figure 3.3:** Far UV CD spectrum of the IFN-α2b standard in the presence of 7 M urea and 5 M guanidine-HCl. The guanidine-HCl shows complete loss of secondary structure whereas structure still remains in the urea sample. The intensity of the urea sample has decreased which may be due to a concentration difference. Sample concentrations were 0.5 mg/ml and the measurements were taken at 20 °C. Due to the absorptive properties of guanidine-HCl and urea the data collected ends at around 208 nm.
Far and near UV analysis of the IFN-α2b standard with DTT

IFN-α2b has two disulphide bonds so the addition of DTT to the folded IFN-α2b will establish, if any, whether reduced cysteine residues have a
destabilising effect on the IFN-α2b molecule. DTT was added at a final concentration of 5 mM and the sample incubated overnight with constant agitation. The far and near UV CD was measured.

![Graph of Far UV CD of IFN-α2b after addition of 5 mM DTT](image)

**Figure 3.5:** Far UV CD of IFN-α2b after addition of 5 mM DTT. The spectrum shows a decrease in the intensities at 208 nm and 222 nm. The helical secondary structure is reduced but is not entirely lost.

![Graph of Near UV analysis of the folded IFN-α2b standard with 5 mM DTT](image)

**Figure 3.6:** Near UV analysis of the folded IFN-α2b standard with 5 mM DTT. The tertiary structure signal is reduced when the disulphides are broken.
The addition of DTT to the folded IFN-α2b standard does have an effect on the protein's secondary (figure 3.5) and tertiary structure (figure 3.6). The results suggest that the protein remains largely folded even when the disulphides are broken. This implies that the disulphides are not required for the folded conformation of IFN-α2b, but may stabilise the native state.

3.2.3: Intrinsic fluorescence of the IFN-α2b standard
As well as CD analysis intrinsic fluorescence was used to analyse these treated IFN-α2b samples. The results from the intrinsic fluorescence will hopefully support the near UV results as they are both based on aromatic residues and their environment. Fluorescence is a very sensitive technique which does not require the high protein concentrations needed for CD analysis.

Intrinsic fluorescence of the IFN-α2b standard on addition of DTT
The fluorescence emission of IFN-α2b was measured after excitation at 280 nm, thereby selecting for both tryptophan and tyrosine emission of which there are two and five residues respectively. The sample concentration for fluorescence analysis was 0.1 mg/ml measured in a 5 mm cuvette at 20 °C and the DTT was added to a final concentration of 5 mM. Incubation times were recorded from immediate addition, after 1 h and after overnight incubation (ON).
Figure 3.7: **Intrinsic fluorescence of the folded IFN-α2b standard in the presence of 5 mM DTT.** The fluorescence shows a small shift to the longer wavelengths when the DTT has been added and left to incubate overnight (ON). DTT added straight away (sa), DTT added and incubation for 1 hour (1h).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Barycentric mean (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-α2b standard</td>
<td>340.02</td>
</tr>
<tr>
<td>IFN-α2b standard + DTT sa</td>
<td>340.58</td>
</tr>
<tr>
<td>IFN-α2b standard + DTT 1 h</td>
<td>340.82</td>
</tr>
<tr>
<td>IFN-α2b standard + DTT ON</td>
<td>340.96</td>
</tr>
</tbody>
</table>

**Table 3.1:** *Table of values of the barycentric mean of the IFN-α2b standard on addition of 5 mM DTT for increasing incubation time.* There is no difference to the fluorescence emission on addition of DTT. Measured between 306 and 380 nm.

The fluorescence analysis shown in figure 3.7 shows tryptophan emission. Tryptophan in water emits at 348 nm (Lakowicz, 1983). When subjected to hydrophobic environments this value shifts to the shorter wavelengths. The IFN-α2b folded standard, without any additives emits at 340.02 nm (calculated
between 306 and 380 nm, see table 3.1). This illustrates the two tryptophan residues in IFN-α2b are within a hydrophobic environment. Once DTT is added the results support the near UV analysis in figure 3.6. Both the near UV CD and the intrinsic fluorescence show that the tertiary structure or more specifically, the environment of the aromatic residues, mainly tryptophan, remains unchanged when the disulphides are broken. The barycentric mean changes by ~ 1nm which does not indicate unfolding of the IFN-α2b molecule and exposure of the two tryptophan residues to a more polar environment. Intrinsic fluorescence of the IFN-α2b in high concentrations of urea and guanidine-HCl should be carried out to help support these findings. This will also provide a barycentric mean for the unfolded IFN-α2b.

**Intrinsic fluorescence analysis of the IFN-α2b standard after incubation with guanidine-HCl and urea.**

These experiments were carried out under the same conditions as in section 3.2. The measurements were taken on immediate addition of the denaturant (sa), after 1 hour and after overnight incubation (ON) with agitation. The final concentrations of denaturants were 5 M guanidine-HCl and 7 M urea.
Figure 3.8: Fluorescence analysis of the unfolding of the IFN-α2b, in the presence of guanidine-HCl. The guanidine-HCl was added to the IFN-α2b standard and was measured sa = straight away (black dashed line), after 1 hour (red line) and ON = overnight (green line). The fluorescence intensity decreases on immediate addition of the guanidine-HCl and further reduction in intensity is seen after overnight incubation.

On addition of 5 M guanidine-HCl to the IFN-α2b standard there is a decrease in the fluorescence intensity see figure 3.8. Once the sample has been incubated overnight the intensity decreases again. There is a very small shift to the longer wavelengths indicating that some unfolding is taking place within the molecule but much less than expected from the far UV and near UV CD data.
Figure 3.9: Unfolding of IFN-α2b on addition of 5 M guanidine-HCl followed by 5 mM DTT analysed by intrinsic fluorescence. The fluorescence results demonstrate a decrease in intensity on addition of guanidine-HCl with increasing incubation time with the 5 mM DTT indicating unfolding (sa = straight away, ON = overnight incubation).

When DTT is added to the IFN-α2b alongside the guanidine-HCl a more definitive unfolding pattern is seen (figure 3.9). The fluorescence intensity decreases and a red shift in the emission is observed with increasing incubation time.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Barycentric mean (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folded IFN-α2b</td>
<td>341.39</td>
</tr>
<tr>
<td>Folded IFN-α2b + 5 M guanidine-HCl straight away</td>
<td>341.15</td>
</tr>
<tr>
<td>Folded IFN-α2b + 5 M guanidine-HCl 1 h</td>
<td>341.25</td>
</tr>
<tr>
<td>Folded IFN-α2b + 5 M guanidine-HCl overnight</td>
<td>341.58</td>
</tr>
<tr>
<td>Folded IFN-α2b + 5 M guanidine-HCl + 5 mM DTT straight away</td>
<td>341.58</td>
</tr>
<tr>
<td>Folded IFN-α2b + 5 M guanidine-HCl + 5 mM DTT 1 h</td>
<td>343.93</td>
</tr>
<tr>
<td>Folded IFN-α2b + 5 M guanidine-HCl + 5 mM DTT 2 h</td>
<td>343.78</td>
</tr>
<tr>
<td>Folded IFN-α2b + 5 M guanidine-HCl + 5 mM DTT overnight</td>
<td>343.54</td>
</tr>
</tbody>
</table>

Table 3.2: Barycentric means from the unfolding of IFN-α2b with guanidine-HCl and DTT. Increase in the Barycentric mean on addition of DTT alongside guanidine-HCl suggests unfolding. Measured between 306 and 380 nm.
The barycentric means listed in table 3.2 show very little difference in the fluorescence emission when guanidine-HCl is added to the IFN-α2b. This is surprising as the far UV shows complete loss of secondary structure on addition of guanidine-HCl which should lead to exposure of the aromatic residues. When DTT is added to the molecule a change in the fluorescence spectra is observed. The intensity is reduced and the barycentric mean, after overnight incubation shifts from 341.58 nm to 343.54 nm. The same experiment was carried out with 7 M urea.

Figure 3.10: Fluorescence analysis of the unfolding of the IFN-α2b, in the presence of urea. The urea was added to the IFN-α2b standard and was measured immediately (sa), after 1 hour and left overnight (ON). The fluorescence intensity decreases on addition of the urea however does not further reduce with increasing incubation time.

Figure 3.10 shows the fluorescence intensity of the IFN-α2b incubated with urea. There is a small decrease in intensity on immediate addition of urea. The intensity does not change after incubation for 1 hour or overnight. This
supports the far and near UV data of urea’s inability to successfully denature the IFN-α2b.

Figure 3.11: Unfolding of IFN-α2b on addition of 7 M urea followed by 5 mM DTT analysed by intrinsic fluorescence. The fluorescence results demonstrates the small decrease in intensity on addition of urea, but with increasing incubation time with the 5 mM more unfolding takes place DTT (sa = straight away, ON = overnight incubation).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Barycentric mean (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folded IFN-α2b</td>
<td>341.39</td>
</tr>
<tr>
<td>Folded IFN-α2b + urea straight away</td>
<td>341.70</td>
</tr>
<tr>
<td>Folded IFN-α2b + urea 1 h</td>
<td>341.56</td>
</tr>
<tr>
<td>Folded IFN-α2b + urea overnight</td>
<td>341.62</td>
</tr>
<tr>
<td>Folded IFN-α2b + urea + DTT straight away</td>
<td>342.97</td>
</tr>
<tr>
<td>Folded IFN-α2b + urea + DTT 1 h</td>
<td>344.15</td>
</tr>
<tr>
<td>Folded IFN-α2b + urea + DTT 2 h</td>
<td>344.07</td>
</tr>
<tr>
<td>Folded IFN-α2b + urea + DTT overnight</td>
<td>344.70</td>
</tr>
</tbody>
</table>

Table 3.3: Barycentric means of the unfolding of the IFN-α2b with urea and DTT. With the addition of DTT alongside the urea some unfolding is achieved due to the increase in barycentric mean. Measured between 306 and 380 nm.
The fluorescence results on addition of urea show little difference in the emission spectrum when urea solely is added to the IFN-α2b. On addition of DTT a similar relationship to that of the guanidine-HCl treated IFN-α2b is observed where with increasing incubation time the more unfolding takes place.

The final barycentric mean (table 3.3) of the 7 M urea treated IFN left overnight with DTT was 344.70 nm which is longer than the guanidine-HCl overnight incubated IFN-α2b with DTT which gives 343.54 nm. This may indicate that 5 M guanidine-HCl is not sufficient to completely denature the IFN or that the incubation time may not be long enough for the guanidine-HCl to disrupt the IFN-α2b structure.

3.2.4: Urea and guanidine-HCl unfolding curves for IFN-α2b

The IFN-α2b standard was subjected to unfolding by guanidine-HCl and urea to gain curves of concentration dependence that can be used to calculate ΔG(H2O) and m of IFN-α2b. Unfolding curves can be determined using many techniques. Here only intrinsic fluorescence and far UV CD were investigated. The method relies on calculation of equilibrium constants at a range of denaturant concentrations for the reaction below

\[
\text{Folded (F) } \leftrightarrow \text{ Unfolded (U)} \quad (3.1)
\]

Calculation of the equilibrium constant enables the free energy (ΔG) of unfolding at each denaturant concentration to be obtained. By extrapolation not only can the free energy change for unfolding in the absence of denaturant termed ΔG(H2O) can be achieved but also m, the dependence of ΔG(H2O) on denaturant concentration (Pace and Scholtz, 1997). Co-operative unfolding curves consist of three regions; the pre transition region, the transition region and the post transition region. All curves were fitted in Origin™ using the Analysis: Fit Sigmoidal menu, which uses the Boltzmann equation for fitting:
\[ y = \frac{A_1 - A_2}{1 + e^{(x-x_0)/d_x}} + A_2 \quad (3.2) \]

Where

\( x_0 \) = centre, \( dx \) = width, \( A_1 \) = initial Y value: \( y(-\infty) \) \( A_2 \) = final Y value: \( y(+\infty) \)

The Y value at \( x_0 \) is half way between the two limiting values, therefore providing a mid point of unfolding. The fitting using a least squares procedure based on the Levenberg-Marquardt (LM) algorithm. The mid point shows the centre of the transitional region allowing data surrounding the mid point to be used to calculate the free energy of unfolding.

Unfolding of IFN-\( \alpha \)2b with guanidine-HCl analysed by intrinsic fluorescence

![Graph showing unfolding curve of IFN-\( \alpha \)2b with guanidine-HCl monitored by intrinsic fluorescence. The curve shows cooperative unfolding of the IFN-\( \alpha \)2b molecule with the addition of guanidine-HCl. The transition region is used to calculate the free energy of unfolding and also the mid point. The mid-point was found to be 4.5 M. Curve fitted by Origin\textsuperscript{TM} using a Boltzmann function.](image)

Figure 3.12: Unfolding curve of IFN-\( \alpha \)2b with guanidine-HCl monitored by intrinsic fluorescence. The curve shows cooperative unfolding of the IFN-\( \alpha \)2b molecule with the addition of guanidine-HCl. The transition region is used to calculate the free energy of unfolding and also the mid point. The mid-point was found to be 4.5 M. Curve fitted by Origin\textsuperscript{TM} using a Boltzmann function.
The unfolding curve of the IFN-α2b standard can be seen in figure 3.12 where intrinsic fluorescence was used for the analysis. The mid point of unfolding was found to be 4.5 M. A refractometer was used for determination of the stock guanidine-HCl and urea solutions to ensure accuracy throughout these experiments (Pace and Scholtz, 1997). The three different regions of the curve are labelled illustrating a two state transition of IFN-α2b from the folded to the unfolded.

To calculate \( \Delta G(H_2O) \) and \( m \) the unfolding has to be reversible. Samples from the experiment carried out in figure 3.12 were diluted down to specific guanidine-HCl concentrations and left for 16 hours and measured a second time. The dilution should enable the samples to begin refolding showing reversibility.

<table>
<thead>
<tr>
<th>Original samples</th>
<th>Diluted samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guanidine-HCl concentration (M)</td>
<td>Barycentric mean (nm)</td>
</tr>
<tr>
<td>2</td>
<td>341</td>
</tr>
<tr>
<td>2.5</td>
<td>341</td>
</tr>
<tr>
<td>3</td>
<td>341</td>
</tr>
<tr>
<td>4.1</td>
<td>342</td>
</tr>
<tr>
<td>4.9</td>
<td>348</td>
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<tr>
<td>5.5</td>
<td>348</td>
</tr>
<tr>
<td>6.5</td>
<td>348</td>
</tr>
</tbody>
</table>

Table 3.4: Samples in 6 M guanidine-HCl diluted to ensure reversibility. The reduction in the barycentric mean of samples where the guanidine-HCl has been diluted down show reformation of structure and therefore confirmation that the unfolding reaction with guanidine-HCl is reversible.

The results from the diluted samples shown in table 3.4 confirm that the unfolding process of IFN is reversible. The calculation of \( \Delta G(H_2O) \) and \( m \) can be calculated by firstly determining the equilibrium constant for a range of denaturant concentrations in the transition region;

\[
K = \frac{(y_F - y)}{(y - y_U)}
\]  

(3.3)
Where $y_F$ is the value of the folded protein and $y_U$ is the value of the unfolded protein, $y$ is the data at that specific guanidine-HCl concentration. Once $K$ has been calculated this can then be incorporated into the calculation for the free energy:

$$\Delta G = -RT \ln K$$  \hfill (3.4)$$

$R$ is the gas constant (1.98 cal/K/mol), $T$ is the temperature in Kelvin, 298. $K$ is the equilibrium constant.

![Graph showing the relationship between $\Delta G$ and guanidine-HCl concentration.](image)

**Figure 3.13:** Relationship between $\Delta G$ and guanidine-HCl concentration using intrinsic fluorescence data. This data was used to calculate $\Delta G(H_2O)$ (y intercept) and $m$ (gradient of the line). $\Delta G(H_2O)$ was found to be 13.50 kcal/mol ± 0.97 and $m$ was 3.01 kcal/mol/M.

The data from the unfolding experiment was used to calculate $\Delta G$ which could then be used to calculate $\Delta G(H_2O)$ and $m$ for the folded state, see figure 3.13. $\Delta G(H_2O)$ was found to be 13.50 kcal/mol ± 0.97 and $m$ was 3.01 kcal/mol/M.
Unfolding of IFN-α2b with guanidine-HCl analysed by far UV CD

The previous experiment investigated the unfolding of IFN-α2b by intrinsic fluorescence, in this experiment the unfolding was monitored by a decrease in secondary structure at 223 nm.

![Graph](image)

**Figure 3.14:** Unfolding curve of the IFN-α2b standard using an increasing range of guanidine-HCl concentrations followed at 223 nm. The mid point of unfolding was found to be 4 M. Curve fitted by Origin™ using a Boltzmann function.

The mid point using far UV CD (figure 3.14) was found to be 4 M lower than the midpoint of 4.6 M found using intrinsic fluorescence. This data was used to calculate $\Delta G(H_2O)$ and $m$ of unfolding as with the intrinsic fluorescence results.

$\Delta G(H_2O)$ was found to be 5.90 kcal/mol ± 3.62 and $m$ was 1.49 kcal/mol/M. The value for $\Delta G(H_2O)$ was found to be lower that the value for the intrinsic fluorescence however the data from the intrinsic fluorescence experiment are less noisy than that of the CD data.
Unfolding curves using guanidine-HCl and 5 mM DTT

**Figure 3.15:** Unfolding curve of the IFN-α2b standard using guanidine-HCl with 1 mM DTT monitored by far UV CD. The decrease in secondary structure was followed at 223 nm. The mid point at 3.7 M was found to be lower than with guanidine-HCl alone. Curve fitted by Origin™ using a Boltzmann function.

**Figure 3.16:** Unfolding curve of the IFN-α2b standard using guanidine-HCl with 1 mM DTT monitored by intrinsic fluorescence. The mid point at 2.6 M was found to be lower than with guanidine-HCl alone and guanidine with 1 mM DTT followed at 223 nm. Curve fitted by Origin™ using a Boltzmann function.
The unfolding curves in figures 3.15 and 3.16 show the unfolding of IFN-α2b in the presence of guanidine-HCl and 5 mM DTT. The curves do not give as well defined co-operative unfolding as the guanidine-HCl only. This is probably due to the destabilising effect the DTT has on the IFN-α2b structure. The intrinsic fluorescence gives a mid-point of 2.6 M and the CD gives a mid-point of 3.7 M.

**Unfolding of IFN-α2b with urea**

The initial unfolding of IFN-α2b using urea does not appear to yield unfolded IFN-α2b. The unfolding curves were carried out in case any co-operative unfolding takes place.

![Unfolding curve of the IFN-α2b standard using urea followed at 223 nm.](image)

*Figure 3.17: Unfolding curve of the IFN-α2b standard using urea followed at 223 nm. The linear trend indicates little co-operative unfolding. It does show that the amount of secondary structure does however decrease in the presence of increasing urea concentrations.*
Figure 3.18: Unfolding curve of the IFN-α2b standard using urea followed by fluorescence. As with the CD results some unfolding of the IFN-α2b takes place however it is not co-operative due to the linear relationship observed.

The unfolding of the IFN-α2b using only urea shown in both figures 3.17 and 3.18 show no indication of cooperative unfolding. The initial unfolding experiments also showed that urea alone was not capable of denaturing the IFN-α2b.

Unfolding of IFN-α2b with urea and 5 mM DTT

As the initial unfolding experiments with urea indicated no unfolding, an unfolding curve was obtained in the presence of DTT as that did allow some unfolding (figure 3.11). This experiment will show whether this unfolding is cooperative as with the guanidine experiments.

Figure 3.19: Unfolding curve of the IFN-α2b standard using urea with 1 mM DTT followed by far UV CD at 223 nm. The linear relationship indicates no cooperative unfolding.
Figure 3.20: Unfolding curve of the IFN-α2b standard using urea with 1 mM DTT measured using intrinsic fluorescence. The use of both urea and DTT has little effect on the IFN-α2b again showing no co-operative unfolding.

The unfolding of IFN with urea and DTT shown in figures 3.19 and 3.20 do not show any co-operative unfolding. To the urea with 5 mM DTT samples 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS); a detergent was added. This would hopefully penetrate the hydrophobic core of the IFN-α2b inducing more co-operative unfolding.

Figure 3.21: Unfolding of the IFN-α2b standard with urea and CHAPS. The CHAPS was added to disrupt the hydrophobic core of the protein to enable unfolding to take place by the urea. The result was the same as the previous results for urea in that the IFN-α2b still does not unfold co-operatively.
The CHAPS was added to the IFN-α2b standard to disrupt the hydrophobic core of the protein in order to initiate the cooperative unfolding of the IFN-α2b molecule. From the results shown in figure 3.21 this has not worked. The unfolding of the IFN-α2b at high concentrations of urea still provides a linear relationship. The secondary structure in the presence of CHAPS does however appear to be reduced.

3.2.5: Thermal unfolding of the IFN-α2b standard

Thermal unfolding of the IFN-α2b standard using far UV CD
The Cobra IFN-α2b was also unfolded using temperature to gain a mid point of unfolding using a different method of denaturation. The IFN-α2b was heated up to 90 °C using the peltier in the CD machine at 1 °C/min and the reduction in secondary structure monitored at 223 nm. This unfolding was carried out a second time in the presence of 5 mM DTT. The fluorescence had shown that the IFN-α2b structure is still intact when the disulphides are broken; this experiment will illustrate how stable the molecule remains under reducing conditions. The thermal unfolding was initially carried out at pH 7.0 however to investigate the stability of the IFN-α2b further pH 9.0 and 5.0 was analysed also.
Figure 3.22: Thermal unfolding curves of IFN-α2b standard monitored at 223 nm at 1 °C/min at pH 7.0. The mid point of unfolding of the IFN-α2b standard is 70 °C (black line). With the addition of DTT (red line) the mid point is 40 °C. This decrease in the mid point of unfolding indicates the breaking of the disulphides weakens the IFN-α2b structure. Curve fitted by Origin™ using a Boltzmann function.

The unfolding of the IFN-α2b at pH 7.0 can be seen in figure 3.22. The mid point without DTT was found to be 70 °C and on addition of 5 mM DTT 40 °C. The decrease in temperature when DTT is added confirms that the reduced IFN-α2b is less stable. However; the shape of the curve is still sigmoidal suggesting that the molecule is still folded and unfolds cooperatively even when the disulphides are broken. The pH values of 9.0 and 5.0 were investigated with and without DTT and the results can be seen in figure 3.24. The far UV CD of the IFN at the three different pH values can be seen in figure 3.23, this was to ensure the secondary structure of the IFN-α2b was still intact.
Figure 3.23: Far UV CD of IFN at pH 7.0, 9.0 and 5.0. The analysis shows that at pH 9.0 and 5.0 the secondary structure is still intact.

Figure 3.24: Thermal unfolding of the IFN-α2b standard at pH 5.0 and pH 9.0 with and without DTT. The $T_m$ for both pH values decreases on addition of DTT as with pH 7.0 and co-operative unfolding can still be seen. Curve fitted by Origin™ using a Boltzmann function.
The temperature at which 50 % of the molecules are unfolded can be calculated by two methods the first by the Jasco software and the second by importing the data into the statistical software Origin™ which uses a Boltzmann function to fit a sigmoidal curve.

<table>
<thead>
<tr>
<th>Method</th>
<th>pH</th>
<th>Origin™</th>
<th>Jasco</th>
</tr>
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<tbody>
<tr>
<td>7.0</td>
<td>70</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>7.0 + DTT</td>
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<tr>
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<td></td>
</tr>
<tr>
<td>9.0</td>
<td>68</td>
<td>67</td>
<td></td>
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<tr>
<td>9.0 + DTT</td>
<td>44</td>
<td>49</td>
<td></td>
</tr>
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</table>

Table 3.5: Table of mid-points of unfolding from thermal unfolding of the IFN-α2b with and without DTT at three pH values. The mid-points at pH 7.0, 9.0 and 5.0 are between 67-70 °C. On addition of DTT the three pH values give mid points of unfolding are between 38-49 °C. Measured by both the Jasco CD software and Origin™.

The mid-points of unfolding at the three pH values measured by two different methods can be seen in table 3.5. The samples without DTT all have mid-points around 70 °C. On addition of DTT the mid-points decrease to around 38-49 °C. This demonstrates the stability of IFN-α2b. All show cooperative unfolding by the sigmoidal shaped curves. On addition of the DTT the mid-points decrease but again at all three pH values cooperative unfolding is seen. This indicates that when the disulphides are broken the alpha helical bundle does not unfold. This supports the CD and fluorescence data and may prove useful when devising a refolding protocol for IFN-α2b.
Figure 3.25: Illustration of the decrease in alpha helical structure of IFN-α2b on heating at pH 7.0.

Figure 3.25 illustrates the unfolding of IFN-α2b during the thermal melt monitored at 223 nm. Every 5 °C a far UV spectrum was measured. The unfolding shows the decrease in the alpha helical structure and an increase in the β structure also reported by Beldarrain et al. (2001) at pH 7.0 with heating and Luykx, et al. (2005) who observed it by the addition of increasing concentrations of acetonitrile.

Thermal unfolding measured by intrinsic fluorescence

Figure 3.26: Thermal unfolding of IFN-α2b monitored by intrinsic fluorescence. The data shows cooperative unfolding and gives a $T_m$ of 68 °C. Curve fitted by Origin™ using a Boltzmann function.
The thermal unfolding followed by intrinsic fluorescence supports the previous thermal unfolding with a mid point being reached of 68 °C.

Figure 3.27: DSC results of IFN-α2b heated to 90 °C at 0.5 mg/ml and 0.1 mg/ml. The DSC result for 0.5 mg/ml shows precipitation of the IFN-α2b. The reduced concentration of 0.1 mg/ml does not allow determination of a Tm.

The DSC results were reproducibly unsuccessful and therefore not used for any analysis on the stability of IFN-α2b. The results shown in figure 3.27 show the precipitation of the IFN-α2b at temperatures above 80 °C which led to the decrease in total protein concentration from 0.5 mg/ml (red line) to 0.1 mg/ml (black line), the spectra collected however did not provide good enough results to be used. The DSC technique was not used any further for any analysis of the refolded material.

3.2.6: ANS binding of the IFN-α2b standard

1-anilinonaphthalene-8-sulfonic acid (ANS) is a fluorescent probe capable of non-covalently binding to exposed hydrophobic regions on protein molecules. ANS is barely fluorescent when free in solution, once bound to a protein the fluorescence increases. In these experiments ANS was used to investigate the folded and unfolded forms of IFN-α2b.
ANS binding of native IFN-α2b with DTT

As with the previous analysis the natively folded IFN-α2b was subjected to ANS binding followed by addition of DTT. The results can be seen in figure 3.28.

![Graph showing ANS fluorescence of folded IFN-α2b standard with and without DTT](image)

**Figure 3.28: ANS fluorescence of the folded IFN-α2b standard with and without DTT.** Increase in the intensity and a shift to the shorter wavelengths.

The data obtained from the IFN-α2b with and without DTT shows a large increase in ANS binding on addition of 5 mM DTT. This shows that the ANS has gained access to the hydrophobic core of the protein. The barycentric mean of the IFN-α2b standard was 511 nm and on addition of DTT 503 nm displaying a blue shift. This blue shift is indicative of ANS moving into a more hydrophobic environment. As with folded proteins their hydrophobic areas and not readily accessible to the ANS and so an emission at a longer wavelength is expected along with a decrease in intensity.
ANS binding on addition of guanidine-HCl to the IFN-α2b standard

In this experiment guanidine-HCl to a final concentration of 5 M was added to the IFN-α2b standard. ANS was added to this sample, the fluorescence measured and DTT added to a final concentration of 5 mM and the fluorescence measured again.

![Graph showing ANS binding of the IFN-α2b standard on addition of 5 M guanidine-HCl followed by 5 mM DTT. The intensity decreases on addition of the guanidine-HCl illustrating the denaturing effects of the guanidine-HCl on the IFN-α2b standard.]

Table 3.6: Barycentric mean of the ANS binding of the folded IFN-α2b on addition of guanidine-HCl and DTT. Measured between 420 and 600 nm.
On addition of guanidine-HCl to the IFN-α2b standard the ANS fluorescence intensity drops and shifts to a longer wavelength (table 3.6), this illustrates shows the disruption of the hydrophobic core as the protein unfolds. This results in little ANS binding therefore a reduction in emission intensity, see figure 3.29. The blue shift of the guanidine-HCl sample when DTT is added is odd however with such a reduction in intensity unfolding has taken place and the barycentric mean does not shift to the same wavelength as the IFN-α2b standard.

**ANS binding on addition of urea to the IFN-α2b standard**

![Fluorescence vs Wavelength Graph](image_url)

**Figure 3.30: ANS binding of the IFN-α2b standard on addition of urea.** When Urea is added to the IFN-α2b a red shift is seen (red line) and a decrease in intensity. On addition of DTT (green line) the fluorescence shifts back to a shorter wavelength of 502 nm, the fluorescence intensity increases higher than the folded IFN-α2b (black dashed line). This indicates that the urea does not unfold the protein and on addition of DTT the hydrophobic areas of the protein are exposed and are able to bind ANS.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Barycentric mean (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folded IFN-α2b</td>
<td>511</td>
</tr>
<tr>
<td>Folded IFN-α2b + 7 M urea</td>
<td>519</td>
</tr>
<tr>
<td>Folded IFN-α2b + 7 M urea + 5 mM DTT</td>
<td>502</td>
</tr>
</tbody>
</table>

Table 3.7: Barycentric mean of the ANS binding of the folded IFN-α2b on addition of 7 M urea followed by DTT. Measured between 420 and 600 nm.

The ANS results for the IFN-α2b on addition of urea are in some respects consistent with the guanidine-HCl results. When Urea is added to the IFN-α2b a red shift is seen and a decrease in intensity (figure 3.30). When DTT is added the fluorescence shifts back to a shorter wavelength of 502 nm (table 3.7) which is shorter than the wavelength for the folded IFN-α2b, the fluorescence intensity increases higher than the folded IFN-α2b. This indicates that the urea does not unfold the protein and on addition of DTT the hydrophobic areas of the protein are exposed and can bind ANS.

3.3: Conclusion

The biophysical characterisation of the IFN-α2b standard provided by Cobra Biomanufacturing has highlighted some potentially useful properties and characteristics of the IFN-α2b molecule. These findings should improve the refolding of IFN-α2b. The strong CD features of the IFN-α2b in both the far and near UV regions shall be used as references during the refolding work. In the far UV region the IFN-α2b gives two strong signals at 208 and 220 nm indicating an alpha helical structure (Alder et al., 1973). In the near UV region there are strong tryptophan signals between 280 and 300 nm. The far and near UV CD results support structural data already published (Radhakrishnan et al., 1996). Leukocyte IFN A was investigated by CD in 1982 by Bewley et al., indicating both the alpha helical nature of IFN but also the near UV fingerprint of the IFN A molecule.

It was clear that IFN-α2b reacted differently to the two denaturants guanidine-HCl and urea. Guanidine-HCl has proved to be the more efficient denaturant
whereas urea results in inconsistent results and unsuccessful denaturation. The far UV CD and intrinsic fluorescence analysis of unfolding with guanidine-HCl enabled the $\Delta G(H_2O)$ and $m$-value of unfolding to be calculated. The $\Delta G(H_2O)$ is the stability of the native state of the protein in the absence of denaturant and the $m$-value is the dependence of $\Delta G$ on the denaturant concentration.

The values calculated were $\Delta G(H_2O) = 13.50 \text{ kcal/mol} \pm 0.97$ and $m = 3.01 \text{ kcal/mol/M}$ from the intrinsic fluorescence measurements and $\Delta G(H_2O) = 5.9 \text{ kcal/mol} \pm 3.6$ and $m = 1.49 \text{ kcal/mol/M}$ from the far UV CD measurements. The values for $m$ are a measurement of the steepness of the transition between the native folded form and the unfolded. It would have been interesting to compare the values for $m$ from the unfolding of IFN-α2b with and without DTT due to the lower $T_m$ calculated. However, the results with the addition of DTT does not result in a two state transition required for his type of analysis.

The addition of urea did not result in any denaturation according to the CD results. On addition of guanidine-HCl the fluorescence intensity decreased and the spectrum shifted to the longer wavelengths both indicators of unfolding. Urea showed this also but only when DTT was added alongside; the urea as alone was not enough. This suggested that the two disulphides had a stabilising effect on the IFN-α2b molecule. When DTT was added to both the guanidine-HCl and the urea treated samples the fluorescence did decrease and a red shift was observed.

Thermal unfolding of IFN-α2b was carried out to investigate how stable the IFN-α2b was when subjected to increasing temperatures with and without DTT. The guanidine-HCl unfolding had shown a two state co-operative relationship when unfolding, would temperature show the same relationship? The results supported the guanidine-HCl unfolding. Thermal unfolding was measured over a range of 20 to 90 °C at 1 °C/min. The $T_m$ at pH 7.0 was calculated to be 70 °C, three other pH values were investigated; pH 5.0 and 9.0. The far UV CD suggested little effect of the pH extremes on the IFN-α2b
molecule. Beldarrain et al., (2001) also showed that IFN-α2b at pH 3.5 showed little difference in structure in both the far and near UV region compared to pH 7.0. The Tₘ values at these two different pH values were 70 °C for pH 5.0 and 68 °C for pH 9.0, calculated using Origin™. The thermal unfolding was carried out at all three pH values with the addition of DTT, this showed co-operative unfolding but a lower Tₘ. The Tₘ values were 40 °C for pH 7.0 + DTT, 41 °C for pH 5.0 and 44 °C or pH 9.0 calculated using the software Origin™. These results implied that IFN-α2b did not collapse on reduction of the two disulphide bonds, but was less stable. In addition to the Tₘ values the thermal unfolding at pH 7.0 monitored by the loss in structure in the far UV region was subject to far UV scans taken at specific temperatures to produce a figure of the decreasing structure with increasing temperature (figure 3.26). This figure illustrated the reduction in the alpha helical signals but also the formation of β-sheets again observed by Beldarrain et al., (2001) at pH 7.0 with increasing temperature but also by Luykx et al., (2005) when concentrations equal and above 30 % acetonitrile are added to IFN-α2b. The thermal unfolding using the DSC was consistently unsuccessful and not used for any further analysis throughout this project.

The next part of the project was to focus on the refolding of IFN-α2b from inclusion bodies. The first step in refolding from inclusion bodies is solubilisation. With the unfolding of IFN-α2b producing different results depending on whether guanidine-HCl or urea is used may have implications for this solubilisation stage. Due to the apparent stabilising effect of the disulphides and the ability of IFN-α2b to remain alpha helical without them may result in a refolding protocol where the IFN-α2b is refolded under reducing conditions and only during the final stages the reducing agent is removed forming the disulphides.

Disulphide bonds are able to be present in the native folded form of protein molecules but also the unfolded state and so are not necessarily required for stability of the folded structure (Creighton, 1992). According to Creighton (1992) proteins with reduced disulphides tend to be unfolded because of the stabilising effect of the disulphide bond however it also depends on the
stability of the proteins structure as a whole. The stabilisation provided by disulphide bonds has been debated by two conflicting views and due to limited strong evidence supporting either view conclusions can only be reached through the experimental evidence itself. The first view from Flory (1956) is that the disulphide bond provides an increase in free energy of the denatured state by decreasing the conformational entropy. The second view is of Doig and Williams (1991) which suggests that it is both the entropy and enthalpy involved of the protein molecule and the surrounding system which are involved.

Experimental evidence supporting both views has been provided from the investigation of the stabilising effects of disulphide bonds (Betz, 1993). The mutation of the cysteine residues of human lysozyme to alanine was investigated by Kuroki et al., (1992). The native protein containing the disulphide had a higher free energy than the non-crosslinked and a higher Tm in keeping with the IFN-α2b results found here. The introduction of a disulphide at different positions within the enzyme Bacillus circulans xylanase enabled its thermal stability to be increased (Davoodi et al., 2007). Any changes in the structure of the enzyme were analysed by both far and near UV CD and all but one mutant were identical to the wild type in the near UV region and all identical in the far UV region. The removal of disulphides not by mutation but by reduction as is the case in the IFN-α2b work may have negative steric effects due to large groups being bound to the cysteines and that this may well have the destabilising effect.

The work carried out by Pace et al., (1988) on RNase T1, showed that the protein remains folded and can be folded without the need for the disulphides to be intact. Reduction of the four cysteines present does reduce the thermal and urea stability of the protein but a two step unfolding curve can be generated. For RNase T1, only one disulphide is buried within the proteins core and the other is solvent accessible. With IFN-α2b the disulphides are in regions of high mobility suggesting they are solvent accessible especially as the first disulphide connects the N terminus of helix E to the AB1 loop (Radhakrishnan et al., (1996). The second disulphide is between Cys29 on
the $3_{10}$A helix and Cys138 within helix E. The residues forming the $3_{10}$A helix are solvent accessible and so it is quite possible that the disulphides present within IFN-α2b are not buried as with RNase T₁ allowing the proteins to remain folded under reducing conditions. These proteins behave in contrast to proteins investigated for their disulphides properties such as Bovine pancreatic trypsin inhibitor (BPTI) which unfolds when all three of the disulphides are broken. As an intact protein BPTI is deemed a very stable protein with a $T_m$ of 95 °C (Creighton and Goldenberg, 1984). This suggests, as discussed in chapter 1, section 1.2, page 16, that protein stability cannot simply be due to one force alone and that all interactions whether covalent or not play a part in protein stability.
Chapter 4
Expression, purification and initial refolding studies

4.1: Introduction

This chapter details the expression and consequent harvesting of the IFN-α2b. Included also are initial refolding trials. The expression of the IFN-α2b was under the control of the temperature sensitive lambda cl857 repressor (Sussman and Jacob, 1962). This technique was new to this laboratory and so various _E. coli_ strains were investigated to provide successful expression, this ultimately lead to one strain being chosen to be taken through further experiments. The IFN-α2b expressed resulted in inclusion bodies, this was in keeping with Cobra’s previous data. Once the cells had been lysed by sonication, the inclusion bodies underwent a series of washing stages to remove contamination such as cell debris and nucleic acids. The solubilisation initially carried out using 8 M urea was investigated to compare to the solubilisation using 6 M guanidine-HCl. Valente et al., (2006) found there to be a difference in yield of IFN-α2b between the two denaturants.

Purification of the solubilised inclusion bodies was attempted by both anion exchange chromatography after dialysis of the guanidine-HCl solubilised inclusion bodies into urea and gel filtration. Ion exchange has been used in the past to purify IFN-α2b however usually this is carried out after the refolding step (Beldarrain et al., 2001; Srivastava et al., 2005). The gel filtration involved the packing of a specific column and subsequent analysis ensuring column efficiency. The initial refolding studies provided early information of the effects of certain parameters on the IFN-α2b molecule such as temperature. This was carried out as a mixture of ‘trial and error’ techniques revealing how the protein reacts to differing environmental temperatures and the effect of the different solubilisation denaturants. The methodology was based on work done by Srivastava et al. (2005) which involved a 1 in 10 dilution followed by dialysis.
4.2: Results

4.2.1: Expression of IFN-α2b

A diagnostic digest using EcoR I and Sal I was carried out, this confirmed that the plasmid and IFN-α2b band were the correct sizes. The plasmid used for the expression of the IFN-α2 is presented in chapter 2, figure 2.3. Initial expression experiments were carried out to find a compatible strain and conditions to express the IFN-α2b protein. The expression of IFN-α2b was under the control of the temperature sensitive lambda c1857 repressor. The cells were grown to an optical density at 600 nm (OD_{600}) of 0.6 at 30 °C, the temperature of the incubator was then increased to 43 °C to induce expression. As temperature induction had not been widely used in this laboratory it was decided that three E. coli strains should be tested initially to find out how well this type of induction method worked. The three E. coli strains available at the time were BL21 (DE3), DH5α and JM105, all three were tested.

Small scale expression of IFN-α2b in strains BL21 (DE3), DH5α and JM105

Expression in three different strains was carried out to find a strain capable of successfully expressing IFN-α2b to be used in all future experiments. This was initially carried out as a 50 ml culture.

![Figure 4.1: 12 % SDS-PAGE analysis of the 50 ml expression of IFN-α2b by the three E. coli strains; BL21 (DE3), DH5α and JM105. All three strains expressed IFN-α2b (lanes 5, 9 and 13). BL21 (DE3) was chosen for future use. Lanes 3, 4, 7, 8, 11 and 12 show the flasks which were not induced, SN = supernatant. IFN-α2b standard loaded to 3 μg.](image-url)
Figure 4.1 shows the expression of IFN-α2b by the strains BL21 (DE3), DH5α and JM105. Lanes 3, 7 and 11 represent the pellets from the flasks kept at 30 °C. They do not show expression of IFN-α2b. Lanes 5, 9 and 13 are the induced flasks, all three strains show expression due to the large band present at the same molecular weight as the IFN-α2b standard in lane 2. The strain BL21 (DE3) was chosen for future use. Expression using the JM105 strain resulted in an apparently higher level of contamination (lanes 11 and 13). BL21 (DE3) grew the fastest out of all three strains, an OD₆₀₀nm of 0.6 being reached in 3 hours compared to 4 and 5 hours using DH5α and JM105 respectively. The death of one of the DH5α cultures (lane 7) made their reliability suspect.

Expression of IFN-α2b in BL21 (DE3) in 0.5 l cultures

BL21 (DE3) had been chosen as the strain to express IFN-α2b. Figure 4.2 shows the expression of a larger culture (500 ml). Two flasks are represented to show the increase in IFN-α2b levels after induction over a 3 hour period, the first flask was induced at 43 °C and the second was not.

Figure 4.2: 12 % SDS-PAGE of the 0.5 l expression in the E. coli strain BL21 (DE3) over three hours. Lanes 3, 5 and 7 show the increase in expression over the three hours. Lanes 9, 11 and 13 are the samples taken from the flasks left at 30 °C, these show no expression of IFN-α2b. IFN-α2b standard loaded to 3 μg.
Figure 4.2 shows the increasing expression of IFN-α2b in BL21 (DE3) over 3 hours. This confirmed BL21 (DE3) as a reliable strain for the IFN-α2b expression at larger scales.

10 l fermentation of IFN-α2b

A 10 l fermentation of IFN-α2b was set up as the expression worked well on the smaller scales. Figure 4.3 shows the OD_{600} readings over 5 h; the total time it took for the fermentation of IFN-α2b.

![Growth curve of the BL21 (DE3) strain used to express IFN-α2b in the 10 l fermentation.](image)

The fermentation of IFN-α2b was successful and resulted in 52.46 g of wet cell pellet, which led to 17.95 g of washed inclusion bodies.

4.2.3: Harvesting and solubilisation of the IFN-α2b inclusion bodies

**Harvesting of IFN-α2b**

Following fermentation the cells were broken using sonication and the inclusion bodies subjected to many washing steps, detailed in chapter 2, section 2.10.
Once the inclusion bodies had been isolated they were solubilised and so refolding studies could begin. The initial solubilisation of the inclusion bodies with 8 M urea proved to be useful if subsequent purification was needed, however guanidine-HCl proved to be the more efficient denaturant. As a consequence both were investigated along with their effects, if any, on the success of the refolding.

**Summary of inclusion body treatment**

- Resuspension of the cell pellet in 20 mM Tris-HCl pH 8.0
- Sonication.
- Centrifugation at 16556 x g (average speed) for 1 h at 4 °C.
- Resuspension of pellet in 20 mM Tris-HCl 1 % Triton X-100 with homogenisation at 37 °C for 30 minutes.
- Centrifugation at 4709 x g (average speed) for 1.5 h at 4 °C.
- Repeated resuspension and centrifugation twice reducing the centrifugation time by 15 minutes each time.
- Solubilisation of the inclusion bodies.
- Centrifugation at 106255 x g (average speed) for 1.5 h at 4 °C.
SDS-PAGE analysis of the inclusion body harvesting

Figure 4.4: 17 % SDS-PAGE of samples taken throughout the harvesting and washing of the IFN-α2b inclusion bodies. A: The solubilisation of the cells and their sonication followed by the washing steps. B: The last two washing steps alongside the centrifugation. The IFN-α2b standard in lane 2 on both gels shows splitting of the band, this is assumed to be degradation due to the repeated freeze thawing of the IFN-α2b standard gel sample. All centrifugation speeds are averages. IFN-α2b standard loaded to 3 μg.

The gel in figure 4.4 shows the gradual removal of higher molecular weight contamination and limited IFN-α2b in the soluble fractions indicating successful isolation of the IFN-α2b inclusion bodies. The inclusion bodies were initially solubilised in 8 M urea, 20 mM Tris-HCl pH 8.0 due to the potential need for further purification.
Mass spectrometry

Matrix-assisted laser desorption/ionization (MALDI) protein identification mass spectrometry was carried out to confirm the product from the expression was IFN-α2b. The results are shown in figure 4.5.

![Chemical structure of lodoacetamide](image)

Reduced forms of IFN-α2b

This experiment aimed to determine whether there was dimer present in the IFN-α2b solubilised inclusion bodies or higher molecular weight contamination. It involved two methods of reduction. Reduction was achieved firstly using DTT and secondly using both 50 mM DTT and 5 mM iodoacetamide. Iodoacetamide (chemical structure shown in figure 4.6) blocks the cysteine residues preventing the two disulphide bonds forming therefore highlighting any contamination.

**Figure 4.5:** The amino acid sequence of IFN-α2b. The matched peptides found by mass spectrometry are shown in red. Mass spectrometry was carried out by Pinnacle, University of Newcastle upon Tyne, Nov 2005. The machine used was an ABI Voyager-DE™ STR Biospectrometry™ Workstation MALDI-TOF Mass Spectrometer. The Mowse score of 83 confirmed the protein expressed to be IFN-α2b.

The mass spectrometry carried out on the solubilised inclusion body samples resulted in a Mowse score of 83 and above for IFN-α. This confirmed it was the correct protein as the cut off for significance is 67.
Figure 4.7: 12 % SDS-PAGE analysis of reduced IFN-α2b to illustrate the presence of any contamination. The dimer band has been reduced on addition of the reducing agents however there are still many high molecular weight contaminating bands present confirming the need for further purification. IFN-α2b standard loaded to 3 µg.

Figure 4.7 shows IFN-α2b in two reduced forms one reduced by DTT, lane 7 and the second reduced by both DTT and iodoacetamide, lane 9. Lane 5 contains IFN-α2b as run previously illustrating the presence of possible dimer. This gel confirms the presence of both dimer and other high molecular weight multimeric species. The use of the iodoacetamide confirms this as all disulphide bonds are broken and as such IFN-α2b can only be present as monomer preventing the formation of any multimeric species, the monomer is represented by the two large bands in lanes 7 and 9 at approx 15 kDa. The presence of the other bands in lanes 7 and 9 suggest contamination and the requirement for further purification.
Reduced and oxidised forms of IFN-α2b

As well as reduction of the IFN-α2b a sample was also oxidised in the presence of copper sulphate and phenanthroline to illustrate the formation of multimeric species.

![Image of SDS-PAGE analysis](image)

Figure 4.8: 12% SDS-PAGE analysis of IFN-α2b in its reduced and oxidised form. Lane 7 shows the shift of the IFN-α2b band to a higher molecular weight than the samples in lanes 3 and 5. Lane 9 illustrates the sample oxidised with copper sulphate and phenanthroline, the IFN-α2b band is faint as the sample has remained in the stacking gel due to the formation of oligomeric species. IFN-α2b standard loaded to 3 μg.

This gel in figure 4.8 shows the reduced IFN-α2b in lane 7 and the oxidised IFN-α2b in lane 9. The oxidised IFN-α2b has formed multimeric species which has resulted in a large proportion remaining in the stacking gel. This result would indicate the advantage of keeping IFN-α2b reduced during the purification stages to prevent the formation of oligomers and increase yields. A further observation from both figure 4.7 and 4.8 is that the reduced form of IFN-α2b runs at a higher molecular weight than the non-reduced form; something that could be used to decide whether the two disulphides have formed correctly.
Differences in solubilisation with 6 M guanidine-HCl and 8 M urea with and without DTT

This experiment was carried out to ensure that the 8 M urea was effectively solubilising the IFN-α2b inclusion bodies. A comparison with 6 M guanidine-HCl was incorporated. To solubilise the inclusion bodies 1 g (wet weight) of inclusion bodies was solubilised in 10 ml of denaturant, the solubilisation was carried out overnight with agitation at room temperature. DTT was added to a final concentration of 5 mM. After centrifugation at 106255 x g (average speed) for 1.5 h the supernatants were diluted 1 in 10 with SDS-PAGE sample buffer and 0.01 g (wet weight) of the four pellets post centrifugation were resuspended in 100 µl of SDS-PAGE loading buffer. 15 µl of the samples were loaded onto the gel.

Figure 4.9: A 17 % SDS-PAGE of the solubilised inclusion bodies after centrifugation using the four conditions; 6 M guanidine-HCl, 6 M guanidine-HCl 1 mM DTT, 8 M urea and 8 M urea 1 mM DTT pH 8.0. Pellets are shown in lanes 2, 4, 6 and 8. Supernatants are shown in lanes 3, 5, 7 and 9. The small amount of IFN-α2b present in lanes 7 and 9 versus the large amount in lanes 6 and 8 illustrates the ineffectiveness of urea as the solubilisation agent. IFN-α2b standard loaded to 3 µg.

Figure 4.9 shows that solubilisation with 6 M guanidine-HCl is more effective than with 8 M urea. This is shown by the large amount of IFN-α2b left in the pellet when 8 M urea is used (lanes 6 and 8). Lanes 7 and 9 show very little soluble IFN-α2b. The presence of DTT does not seem to make a difference in the efficiency of the solubilisation in both cases. Both guanidine-HCl (Beldarrain et
al., 2001) and urea (Srivastava et al., 2005) have been used for the successful solubilisation of IFN-α2b inclusion bodies however it was Valente et al., (2006) who showed that guanidine-HCl had a much greater effect on the inclusion bodies solubilisation. Valente et al., (2006) found a difference in yield of IFN-α2b of 88 % when guanidine-HCl was used compared to 51 % using urea alongside DTT.

4.2.4: Purification of the IFN-α2b solubilised inclusion bodies

The SDS-PAGE analysis of the solubilised inclusion bodies highlighted the need for a purification step. This will ensure that only IFN-α2b is being refolded and not being affected by any contamination.

Ion exchange

In order to purify the inclusion bodies further ion exchange chromatography was chosen. The pI of IFN-α2b has been calculated to be 5.9 (Gasteiger E et al., 2003). Anionic ion exchange media was chosen and run initially at pH 8.0, this was not successful. Further conditions tried can be seen in table 4.1. The elution was performed by an increasing concentration of sodium chloride concentrations up to 1 M.
<table>
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<th>Ion Exchange Run</th>
<th>Buffer A</th>
<th>pH</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4M Urea, 50 mM Tris-HCl, 1 mM DTT</td>
<td>8.0</td>
<td>Dialysis into buffer A at 4°C, Linear Gradient, Cloudy after dialysis</td>
</tr>
<tr>
<td>2</td>
<td>4 M Urea, 50 mM Tris-HCl, 1 mM DTT</td>
<td>8.0</td>
<td>PD10 column used for buffer exchange, Step elution 250 mM, 500 mM, 750 mM and 1 M NaCl.</td>
</tr>
<tr>
<td>3</td>
<td>4 M Urea, 50 mM Tris-HCl, 1 mM DTT</td>
<td>8.0</td>
<td>New PD10 column used for buffer exchange, Step elution</td>
</tr>
<tr>
<td>4</td>
<td>4 M Urea, 30 mM Ethanolamine, 1 mM DTT</td>
<td>9.5</td>
<td>PD10 column used for buffer exchange, Step elution</td>
</tr>
<tr>
<td>5</td>
<td>8 M Urea, 50 mM Tris-HCl, 1 mM DTT</td>
<td>8.0</td>
<td>Dialysis into buffer A at 4°C, Step elution</td>
</tr>
<tr>
<td>6</td>
<td>8 M Urea, 30 mM Ethanolamine, 1 mM DTT</td>
<td>9.5</td>
<td>Dialysis into buffer A at 4°C, Urea came out of solution left for 3 hours at room temperature, Step elution</td>
</tr>
<tr>
<td>7</td>
<td>8 M Urea, 50 mM Tris-HCl, 1 mM DTT</td>
<td>9.5</td>
<td>Dialysis into buffer A at room temperature, Step elution</td>
</tr>
<tr>
<td>8</td>
<td>8 M Urea, 50 mM Tris-HCl, 1 mM DTT</td>
<td>9.5</td>
<td>Dialysis into buffer A at room temperature, pH adjustment carried out with acetic acid, Step elution</td>
</tr>
<tr>
<td>9</td>
<td>8 M Urea, 0.1 M Citric acid, 1 mM DTT</td>
<td>4.0</td>
<td>Dialysis into buffer A at room temperature, Step elution</td>
</tr>
<tr>
<td>10</td>
<td>8 M Urea, 20 mM Tris-HCl, 1 mM DTT</td>
<td>8.0</td>
<td>After thoroughly cleaning the column, Dialysis into buffer A at room temperature, Step elution</td>
</tr>
<tr>
<td>11</td>
<td>8 M Urea, 1 mM DTT, 0.2 M Phosphate</td>
<td>7.0</td>
<td>Dialysis into buffer A at room temperature, Step elution</td>
</tr>
<tr>
<td>12</td>
<td>8 M Urea, 1 mM DTT, 10 mM Bis Tris-HCl.</td>
<td>7.0</td>
<td>Dialysis into buffer A at room temperature, Elution of 500 mM and 1 M.</td>
</tr>
</tbody>
</table>

**Table 4.1:** All the different ion exchange conditions that were tried in order to purify the denatured IFN-α2b. None of these conditions were successful.

From table 4.1 it can be seen the range of conditions tried and tested. None of the conditions proved successful as a purification technique. Examples of SDS-PAGE analysis of ion exchange runs 5 and 6 can be seen in figure 4.10.
Figure 4.10: Examples of the SDS-PAGE analysis carried out after the ion exchange runs shown in table 4.1. A: Ion exchange run 4, B: ion exchange run 5, the two most successful ion exchange runs. It can be seen from these two gels the extent of protein loss during the ion exchange experiments, see lane 5. IFN-α2b standard loaded to 3 μg.

The example gels from the ion exchange attempts shown in figure 4.10 highlight a great loss of protein in the flowthrough which is not pure IFN-α2b itself. Figure 4.10B does show a small amount of IFN-α2b eluting in the 250 mM NaCl fraction however the yield is too low to consider this as an efficient purification technique at this stage. The results of the ion exchange confirm it to be an unproductive stage in purifying the inclusion bodies. This maybe because of the high concentrations of urea present interfering with the proteins access to the resin or the urea is producing cyanate which is causing carbamylation of the IFN-α2b.

**Ion exchange using Amberlite® treated buffers**

Urea has a tendency to break down producing cyanate which is able to carbamylate proteins. To eliminate the possibility of this happening to the IFN-
α2b, the urea buffers were treated with Amberlite®, a mixed-bed ion exchange media before the ion exchange run at pH 8.0. 10 g of Amberlite® was added to 100 ml of the running buffers and left stirring for 16 hours at room temperature (in accordance with manufacturer's instructions). The buffers were then 0.2 μm filtered and degassed under vacuum.

![Figure 4.11: SDS-PAGE analysis of the samples taken during the ion exchange at pH 8.0 using Amberlite® treated buffers. The gel shows that even when the buffers are treated with Amberlite® the protein still does not bind to the Q ion exchange resin.](image)

The ion exchange run using Amberlite® treated buffers does not result in improved purification, see figure 4.11. There is no indication of the sample binding to the column suggesting that the unsuccessful ion exchange is not due to breakdown products of the urea interfering with the binding of the IFN-α2b to the column.

**Isoelectric focusing analysis**

Isoelectric focusing (IEF) gels can be used to separate proteins according to their charge. The pH at which the protein has a net charge of zero is termed the pl and can be determined by IEF analysis. Once the pl is known the conditions of the ion exchange can be assessed and optimised. The running of an IEF gel should confirm the pl of IFN-α2b.
Figure 4.12: IEF gel of the IFN-α2b standard and solubilised inclusion bodies. The calculated pI of IFN-α2b is 6 the IFN-α2b standard runs just above 6 but less than 6.9. The solubilised IFN-α2b inclusion bodies cannot be seen on the gel which was assumed to be due to the incompatibility of the solubilisation buffer with the running conditions.

The solubilised inclusion body sample did not show up on the IEF gel shown in figure 4.12 which may be due to the high concentration of urea interfering with the efficient running of the gel. The IFN-α2b standard did confirm the pI as being just above 6, the calculated pI. This suggests that the pH at which the ion exchange is being carried out (above 6) is not the cause of the unsuccessful purification. These results conclude that maybe the high concentration of urea is reducing the IFN-α2b access to the resin preventing it from binding.

Pure Inclusion bodies

Due to the unsuccessful nature of the ion exchange purification the solubilised inclusion bodies were accepted as pure enough for the refolding to be carried out without the need for purification. The samples were treated with iodoacetamide (final concentration 5 mM) and analysed by SDS-PAGE.
Figure 4.13: 12 % SDS-PAGE analysis of the reduced solubilised inclusion bodies with DTT and iodoacetamide. This illustrates the relatively pure inclusion bodies due to the reduced level of contamination. IFN-α2b standard loaded to 1 μg.

The gel shown in figure 4.13 shows the SDS-PAGE analysis carried out on the samples gained from the inclusion body solubilisation. The gel shows suitably pure inclusion bodies to have refolding studies started and will therefore not undergo further purification.

4.2.5: Refolding without purification
The following results are examples of the initial refolding experiments carried out on the solubilised IFN-α2b inclusion bodies without any purification after inclusion body preparation.

Refolding at 4 °C and 24 °C
The initial refolding experiments focused on finding an optimum temperature at which to carry out the refolding. The two temperatures chosen were 4 °C and 24 °C (room temperature). Whether the protein refolded differently when solubilised in urea or guanidine-HCl was also investigated. This was thought to be important due to the differences in behaviour observed during the characterisation work and the differences in the extent of the solubilisation using the two denaturants. For details see chapter 2, section 2.11.1.
Refolding of IFN-α2b at 4 °C and 24 °C

The initial refolding experiments were carried out at both 4 °C and 24 °C with inclusion body samples solubilised in both 8 M urea and 6 M guanidine-HCl, 20 mM Tris-HCl, 1 mM DTT pH 8.0. The refold was as follows;

- 1 in 10 dilution of the protein sample into 20 mM Tris-HCl, 1 mM DTT pH 8.0, 3 hour incubation.
- Addition of 0.1 mM oxidised glutathione, 4 hour incubation.
- Dialysis into 20 mM Tris-HCl pH 8.0.

The final samples under these four refold conditions;

1. Refolded from urea at room temperature
2. Refolded from urea at 4 °C
3. Refolded from guanidine-HCl at room temperature
4. Refolded from guanidine-HCl at 4 °C

All were analysed by far UV CD, intrinsic fluorescence and SDS-PAGE.
Far UV CD analysis

![Graph showing Far UV CD analysis](image)

**Figure 4.14:** Far UV CD of the final samples from the refold carried out at both 24 °C and 4 °C solubilised in both 6 M guanidine-HCl and 8 M urea. The only two with alpha helical structure are the two samples solubilised in guanidine-HCl (red and green lines). The samples showing alpha-helical structure are not as intense as the IFN-α2b standard (black dotted line) confirming that they are not yet natively refolded.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Initial Concentration (mg)</th>
<th>Final concentration (mg)</th>
<th>Yield(%)</th>
<th>Concentration for CD analysis (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guanidine-HCl refold at 4 °C</td>
<td>19.8</td>
<td>1.14</td>
<td>5.75</td>
<td>0.38</td>
</tr>
<tr>
<td>Guanidine-HCl refold at 24 °C</td>
<td>19.8</td>
<td>0.75</td>
<td>3.78</td>
<td>0.22</td>
</tr>
<tr>
<td>Urea refold at 4 °C</td>
<td>9.8</td>
<td>2.5</td>
<td>26</td>
<td>0.25</td>
</tr>
<tr>
<td>Urea refold from 24 °C</td>
<td>10.5</td>
<td>3</td>
<td>28</td>
<td>0.3</td>
</tr>
</tbody>
</table>

**Table 4.2:** Concentrations determined by $A_{280}$ of the samples before and after the first refold attempt. The urea samples appear to give the greatest yields. The final samples were concentrated for far UV CD analysis.
Fluorescence analysis

Figure 4.15: The fluorescence results of the final samples from the refold carried out at 4 °C and room temperature. Only the urea solubilised sample refolded at room temperature (red line) shows tryptophan fluorescence similar to that of the IFN-α2b standard. The other samples appear to be giving a mixture of tryptophan and tyrosine fluorescence not previously seen from the folded IFN-α2b standard. Sample concentrations determined by A$_{280}$ were 0.1 mg/ml.

The far UV CD of the final samples can be seen in figure 4.14. The only samples to give the correct alpha helical signal are the two samples solubilised in 6 M guanidine-HCl especially when DTT is added. This may indicate that the protein is fully unfolded when DTT is used alongside guanidine-HCl for the solubilisation of the inclusion bodies but the refolding does not provide the correct conditions for fully folded IFN-α2b of the same intensity as the standard. Without the protein being fully denatured it is unable to refold correctly to its native conformation. The fluorescence results shown in figure 4.15 show the correct tryptophan emission from the inclusion bodies solubilised in 8 M urea. The samples which had been solubilised in 6 M guanidine-HCl gave a mixture of tyrosine and tryptophan fluorescence. This is the first indication of the different behaviour in refolding by
IFN-α2b starting from solubilisation in either urea or guanidine-HCl. A possible explanation for this is that the starting material in urea is not completely denatured and unfolded whereas it is with the guanidine-HCl. There are 5 tyrosine residues present in IFN-α2b and 2 tryptophans and so tryptophan emission is expected. The tyrosine fluorescence has not been observed from the IFN-α2b standard during the characterisation work and so could be a difference in the proteins structure due to the mixture of tyrosine and tryptophan emission seen or a possible contaminant not removed during the washing of the inclusion bodies.

SDS-PAGE analysis of the refolded samples

A. 24 °C Refold Gel

<table>
<thead>
<tr>
<th>Lanes</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1. MWMM</td>
<td></td>
</tr>
<tr>
<td>2. IFN Std heated to 95 °C</td>
<td></td>
</tr>
<tr>
<td>3. IFN Std not heated</td>
<td></td>
</tr>
<tr>
<td>4. IFN Std heated and reduced</td>
<td></td>
</tr>
<tr>
<td>5. Sample heated</td>
<td></td>
</tr>
<tr>
<td>6. Sample not heated</td>
<td></td>
</tr>
<tr>
<td>7. Sample heated and reduced</td>
<td></td>
</tr>
<tr>
<td>8. Pellet after refold</td>
<td></td>
</tr>
</tbody>
</table>

B. 4 °C Refold Gel

<table>
<thead>
<tr>
<th>Lanes</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1. MWMM</td>
<td></td>
</tr>
<tr>
<td>2. IFN Std heated to 95 °C</td>
<td></td>
</tr>
<tr>
<td>3. IFN Std not heated</td>
<td></td>
</tr>
<tr>
<td>4. IFN Std heated and reduced</td>
<td></td>
</tr>
<tr>
<td>5. Sample heated</td>
<td></td>
</tr>
<tr>
<td>6. Sample not heated</td>
<td></td>
</tr>
<tr>
<td>7. Sample heated and reduced</td>
<td></td>
</tr>
<tr>
<td>8. Pellet after refold</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.16: 12 % SDS-PAGE analysis of the final samples form the 4 °C and 24 °C refold from guanidine-HCl solubilised inclusion bodies. Lane 8 on both gels illustrates the loss of protein through precipitation during the 24 °C refold. The smearing effect is due to incomplete disulphide bond formation. IFN-α2b standard loaded to 3 μg.

The SDS-PAGE analysis shown in figure 4.16 shows the shift of the refolded IFN-α2b to a higher molecular weight, seen previously when the IFN-α2b standard samples have been reduced. There is no difference between the band position whether the samples are heated or not. The gels also illustrate the great loss of protein by precipitation during the refold at 24 °C. This is shown in lane 8 in both figure A and B. The refold at 24 °C shall not be used in future experiments due to this great loss. This loss in protein is however not reflected in the yields of the
Refold shown in table 4.2. A much smaller yield would be expected from the guanidine-HCl refold carried out at 24 °C. There appears to be splitting of the IFN-α2b band, potentially indicating different combinations of disulphide formation. These bands run to the same molecular weight as the heated and reduced samples (lanes 5 and 6). In this experiment the results of the refolding are not successful but it does highlight the usefulness of SDS-PAGE in confirming the disulphides have formed and also that 24 °C is not a satisfactory temperature for the refold.

Refold with longer oxidised glutathione incubation time

The previous refold was carried out with an incubation time with oxidised glutathione of 3 hours, in this experiment the incubation time was increased to 16 hours. Results were analysed by far UV CD and fluorescence.

Far UV CD results

![Far UV CD results](image)

**Figure 4.17: The far UV CD results of the final samples of the refold involving a 16 hour incubation time with oxidised glutathione.** The only sample showing any alpha helical structure is the sample solubilised in 6 M guanidine-HCl 1 mM DTT (red line). The remaining samples show signals of unfolded structure.
In figure 4.17 the only sample to give an alpha helical signal is the sample solubilised in 6 M guanidine-HCl, 1 mM DTT.

**Intrinsic fluorescence analysis**

![Graph showing fluorescence results](image)

**Figure 4.18:** Fluorescence results of the refold at 4 °C from both urea and guanidine-HCl solubilised inclusion bodies with a 16 hour incubation time with oxidised glutathione. Tryptophan emission can be seen from the guanidine-HCl solubilised inclusion bodies (black and red lines) and tyrosine emission from the urea solubilised inclusion bodies (green and blue lines). Tyrosine emission is not expected from the refolded IFN-α2b.

The fluorescence results in figure 4.18 show the correct tryptophan emission from the inclusion bodies solubilised in guanidine-HCl with or without DTT. The samples which had been solubilised in urea now give tyrosine fluorescence. It is however only the sample solubilised in 6 M guanidine-HCl, 1 mM DTT which appears folded by far UV CD. The intensities of the fluorescence emissions are low and noisy even though their total protein concentrations are 0.1 mg/ml. This indicates that there is a contamination which is interfering with the absorbance measurements.
Conclusions from the refolding without purification

The refolding without purification was carried out due to relatively pure inclusion bodies samples being obtained and the unsuccessful nature of the ion exchange as a purification method. The initial aim was to investigate a temperature at which the IFN-α2b could be refolded minimising the possibility of aggregation. The IFN-α2b seemed to undergo large precipitation when refolded at room temperature giving rise to small yields. The yields at 4 °C did not appear much better which was odd as the same level of precipitation had not been observed. It was the inclusion bodies solubilised in guanidine-HCl which resulted in alpha helical structure regardless of the temperature (figure 4.14). The urea samples showed signs of unfolded species due to the large negative intensities below 210 nm. The intrinsic fluorescence from this study gave unexpected results. Although the guanidine-HCl solubilised samples were showing alpha helical structure they were displaying a mixture of tyrosine and tryptophan fluorescence something not seen from the IFN-α2b standard, see figure 4.15. The case was different for the urea solubilised inclusion bodies. The refold carried out at 24 °C gave tryptophan emission and the 4 °C refold gave tyrosine emission, again unexpected. There are 5 tyrosines and 2 tryptophans in IFN-α2b and usually only the tryptophan fluorescence is seen in a protein containing both when excited at 280 nm (Schmid, 1997). From this refold the conclusions were that the refold should be carried out at 4 °C to reduce the level of aggregation. The SDS-PAGE highlighted the extent of the aggregation when the refold is carried out at 25 °C. A second function of the SDS-PAGE analysis is that they illustrate the formation of the disulphide bonds. In both gels the IFN-α2b band is more of a smear compared to the IFN-α2b standard and the reduced sample, see figure 4.16. This suggests that the disulphides have not formed completely or maybe incorrectly. The next stage was to investigate the disulphide formation still using oxidised glutathione.

The second refold was based on increasing the incubation time with oxidised glutathione from 3 to 16 hours, to improve disulphide bond formation. The inclusion bodies had now been solubilised in both urea and guanidine-HCl with and without DTT and so all four were refolded. The results from this second refold gave interesting results again. The only sample showing alpha helical
structure was from the inclusion bodies solubilised in guanidine-HCl with DTT, the remaining three showed strong characteristic unfolded signals below 210 nm (figure 4.17). The fluorescence results show strong tyrosine emission from the urea solubilised inclusion bodies and tryptophan emission from the guanidine-HCl solubilised inclusion bodies (figure 4.18). However inconsistent the results, both experiments highlight the differences in refolding of inclusion bodies solubilised in guanidine-HCl and urea; the guanidine-HCl solubilised samples can lead to an alpha helical structure whereas the urea solubilised samples do not. The fluorescence is odd as tyrosine emission is unexpected as not previously seen during the characterisation work in chapter 3, section 3.2.3. The tyrosine fluorescence must be seen in the IFN-α2b standard or the purification needs to be re-introduced.

4.2.6: Standard Denaturation

Repeated freeze thaw

An aliquot of IFN-α2b standard at 0.01 mg/ml was freeze thawed for 5 days at -20°C and the fluorescence emission measured after excitation at 280 nm.

![Graph showing fluorescence emission over 5 days](image)

Figure 4.19: Fluorescence emission of continuous freeze thawing the IFN-α2b standard over a period of five days. There is no indication of the appearance of any tyrosine fluorescence due to freeze thawing.
The freeze thawing of the IFN-α2b standard shown in figure 4.19 did not result in tyrosine fluorescence. It did however show the decrease in intensity of the fluorescence emission over the five days which could indicate possible denaturation and in future freeze thawing of the IFN-α2b should be kept to a minimum.

**Denaturation with Guanidine-HCl, DTT and heat**

This second experiment was also designed to encourage tyrosine fluorescence from the IFN-α2b standard. A solution of the folded IFN-α2b standard was subjected to treatment with 6 M guanidine-HCl, 50 mM DTT and heating to 95 °C.

![Graph](image)

**Figure 4.20:** The fluorescence emission of the IFN-α2b standard denatured using guanidine-HCl and heat. The fluorescence emission from treatment with guanidine-HCl, DTT and heat does not result in tyrosine emission, tryptophan emission remains.

The fluorescence results from the standard denaturation work did not present tyrosine fluorescence, see figure 4.20. Repeated freeze thawing indicated possible denaturation of the protein with the decrease in intensity. The deliberate
denaturation using guanidine-HCl and heat also illustrates denaturation but no tyrosine emission.

The results indicate that a purification step is essential to gain more reliable and successful refold results especially with the urea solubilised samples.

4.2.7: Purification using gel filtration

As ion exchange had proved unsuccessful as a purification stage for IFN-α2b gel filtration was investigated as a possible alternative. Gel filtration would remove the need for dialysis of the guanidine-HCl solubilised inclusion bodies into urea in order to be purified allowing the two conditions to be investigated separately. A suitable gel filtration column would firstly have to be prepared as analytical columns available would be too small for this preparative work.

Preparation of the gel filtration column

A XK16 column (G.E. Healthcare, Amersham, UK) was packed with Superdex™ 75 media (G.E. Healthcare). The bed height was 11.5 cm giving a total column volume of 29 ml. To check the packing efficiency of the column NaCl was used. The column was equilibrated with 20 mM Tris-HCl pH 8.0, and when a stable baseline had been reached the column was spiked with 0.5 ml (approx 2 % column volume) 1 M NaCl. From the results the number of theoretical plates and the asymmetry can be calculated. The methods and equations used were taken from the Amersham Biosciences handbook on gel filtration 18-1022-18 edition Al (2002).
Figure 4.21: Chromatography spectrum of the XK16 packed to 29 ml with Superdex™ 75 resin spiked with 1 M NaCl. The peak was used to calculate the asymmetry and the number of theoretical plates. $V_e$ is the peak elution volume, $W_{1/2}$ is the peak width at half the peak height and $L$ is the bed height (mm).

The results of the NaCl spike in figure 4.21 enabled the number of theoretical plates to be calculated by the following equation

$$N = 5.54 \left(\frac{V_e}{W_{1/2}}\right)^2 \times 1000/L \quad (4.1)$$

Where $V_e$ is the peak elution volume, $W_{1/2}$ is the peak width at half the peak height and $L$ is the bed height (mm).

The result gave a theoretical number of plates of 31452. The pre-packed Superdex™ gel filtration columns from G.E. Healthcare are usually packed to 30000 plates (Technical services at G.E. Healthcare) or as the Amersham gel filtration handbook (2002) recommends $> 10000$, indicating the packed column is sufficiently packed for this application.
The asymmetry was calculated by the following equation

\[ A_s = \frac{B}{A} \]  

(4.2)

\( A = \) Leading half peak width at 10 % peak height
\( B = \) Trailing half peak width at 10 % peak height

The result gave an asymmetry of 0.92 which indicates good asymmetry, for Superdex™ media the asymmetry should lie between 0.7–1.3.

As the column was well packed molecular weight markers were applied to the column to ensure the column was as effective with protein samples. The samples used were cytochrome C (12.5 kDa), carbonic anhydrase (29.5 kDa) and β-amylase (200 kDa). The samples were applied to the column pre-equilibrated with 20 mM Tris-HCl, 0.15 M NaCl pH 8.0. The load volume was 0.5 ml and the protein concentration was approx 1 mg/ml. Blue dextran (2000 kDa) was also used to elucidate the void volume.

![Absorbance vs. Volume graph](image)

**Figure 4.22: Elution profiles of the molecular weight markers used to calibrate the Superdex™ 75 column to be used for the purification of the IFN-α2b inclusion bodies.** Carbonic anhydrase was added at a lower concentration due to availability. The peak elution volumes can be used to elucidate when the IFN-α2b should elute.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Molecular weight (kDa)</th>
<th>Elution volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome C</td>
<td>12.5</td>
<td>17.45</td>
</tr>
<tr>
<td>Carbonic anhydrase</td>
<td>29</td>
<td>15.29</td>
</tr>
<tr>
<td>Beta amylase</td>
<td>200</td>
<td>10</td>
</tr>
<tr>
<td>Blue dextran</td>
<td>2000</td>
<td>11.37</td>
</tr>
<tr>
<td>IFN-α2b</td>
<td>19.5</td>
<td>13.4</td>
</tr>
</tbody>
</table>

**Table 4.3: Table of the molecular weight calibration markers along with their elution volumes.** The Blue dextran and the beta amylase elute at similar volumes even though their molecular weights are different, this is probably due to them both eluting in the void volume as they are too large for separation by the Superdex™75. Unusually the IFN-α2b elutes before the carbonic anhydrase which is larger, this may be due to the loading concentration being different, the IFN-α2b was loaded to a much higher concentration.

Table 4.3 and figure 4.22 shows the molecular weights and the elution volumes of the protein standards used to calibrate the gel filtration column. The calibration shows that the smaller proteins elute late and the large proteins elute early. Blue dextran and β-amylase elute early in the void volume due to their large size. The IFN-α2b elutes around 13 ml, it would be expected that IFN-α2b would elute between cytochrome C and carbonic anhydrase however due to total protein concentration and load volume differences the elution may differ.

**Figure 4.23: 17% SDS-PAGE analysis of the solubilised inclusion bodies before and after purification by gel filtration.** The gel filtration appears to be purifying the guanidine-HC1 solubilised inclusion bodies however there remains little difference between the urea solubilised inclusion bodies purity before and after gel filtration.
The SDS-PAGE analysis of the urea solubilised inclusion bodies before and after gel filtration shown in figure 4.23 confirms a similar amount of contamination present in each. The guanidine-HCl solubilised sample gives a better result after the gel filtration. There appears to be less high molecular weight contamination and reduced smearing of the sample. The apparent contamination may be aggregation during running of the gel.

Gel filtration of solubilised inclusion bodies with and without the use of Triton X-100

The following figure illustrates the difference in gel filtration profile with and without Triton X-100.

![Gel filtration profile](image)

**Figure 4.24:** Example of the differences in gel filtration of guanidine-HCl solubilised inclusion bodies with and without Triton X-100. The red line shows a large second peak after 20 ml indicating the removal of the Triton X-100. The black line shows the separation of a sample having never come into contact with Triton X-100.

The gel filtration profile of the guanidine-HCl solubilised inclusion bodies when Triton X-100 is used displays a large peak above 20 ml (figure 4.24). This peak is present in the samples where no Triton X-100 was used but at a much lower
concentration. The elution volume of the contamination suggests it is small in size and so may not be visible by SDS-PAGE; the fluorescence of this peak shall be investigated.

Intrinsic fluorescence of the gel filtration of Triton X-100 washed inclusion bodies

The two fractions were chosen from the centre of the two peaks and excited at 280 nm.

![Fluorescence spectra](image)

**Figure 4.25:** Fluorescence spectra of two fractions taken during the gel filtration of guanidine-HCl solubilised inclusion bodies with Triton X-100 present in the wash buffer. The fraction taken at 14 ml shows tryptophan emission and the fraction taken at 25 ml shows tyrosine emission suggesting that the later fractions are not IFN-α2b.

The fluorescence obtained from the two fractions revealed tyrosine fluorescence from the peak above 20 ml, see figure 4.25. It is possible that the tyrosine emission seen in the earlier refolds may have been caused by the Triton X-100. To confirm this, the fluorescence emission of pure Triton X-100 was compared to the emission of N-acetyl-L-tyrosinamide, a compound used to mimic tyrosine in a protein-like environment. Both the N-acetyl-L-tyrosinamide and the Triton X-100 were diluted to approx 0.1 mM and the absorbance and the fluorescence emission measured after excitation at 280 nm.
Absorbance of N-acetyl-L-tyrosinamide and Triton X-100

![Absorbance spectrum of N-acetyl-L-tyrosinamide and Triton X-100](image)

**Figure 4.26:** Absorbance spectrum of N-acetyl-L-tyrosinamide and Triton X-100. Both scans show the peak at 275 nm followed by the shoulder at 280 nm characteristic of tyrosine absorption. The figure suggests the tyrosine fluorescence is a result of the Triton X-100. The compounds were diluted to approx 0.1 mM for fluorescence analysis. Chemical structures drawn using Chemwindow® 6.

Both absorbance spectra in figure 4.26 show similar results with the shoulder at 280 nm and a peak at 275 nm, a characteristic of tyrosine absorption. These samples were then subjected to excitation at 280 nm and their fluorescence emission measured.
Intrinsic fluorescence analysis of N-acetyl-L-tyrosinamide and Triton X-100.

![Fluorescence emission of N-acetyl-L-tyrosinamide and Triton X-100](image)

**Figure 4.27: Fluorescence emission of N-acetyl-L-tyrosinamide and Triton X-100.**

The barycentric mean of both samples show that they are very similar confirming Triton X-100 gives a strong tyrosine emission implicating it in the contamination of the IFN-α2b inclusion bodies.

The fluorescence emission of N-acetyl-L-tyrosinamide and Triton X-100 give the expected tyrosine emission spectrum (figure 4.27). The barycentric mean, calculated between 286 and 337 nm for the N-acetyl-L-tyrosinamide is 308.4 nm and for the Triton X-100 is 307.8 nm indicating the cause of the “tyrosine” emission was the Triton X-100.

The A$_{280}$ spectrum shown in figure 4.26 illustrates the strong protein-like signal given by the Triton X-100. This suggests the total protein concentrations of the inclusion bodies and possibly the refolded samples displaying tyrosine fluorescence had been overestimated therefore explaining the unusual results obtained during the initial refolding. Removal of the Triton X-100 from the washing of the inclusion bodies will make total protein calculations more reliable. The gel filtration shall be kept even when Triton X-100 had not been used during the wash steps. This was due to there still being some small molecular weight
contamination between 20-30 ml but also due to the first peak not solely being of the correct IFN-α2b A_{280} spectrum. The fractions collected from the first peak were pooled on the basis of their A_{280} spectrum. The fractions pooled usually eluted between 14 and 18 ml (inclusive). Examples of the A_{280} spectra from a guanidine-HCl separation can be seen in figure 4.28.

**A_{280} profiles of the gel filtration fractions**

![A_{280} Profiles](image)

*Figure 4.28: Examples of the absorbance scans obtained of the guanidine-HCl fractions collected during the gel filtration. The fractions were pooled on the basis of their A_{280} profile. The red line indicates the possible contamination which absorbs around 260-280 nm and the black line indicates the IFN-α2b fractions pooled and taken through the refolding.*

The absorbance spectra shown in figure 4.28 shows the difference in absorbance of two fractions taken throughout the gel filtration where no Triton X-100 has been used. The spectra show the presence of either contamination that also absorbs around 260 nm, possibly DNA or mis-folded IFN-α2b. Due to these differences in the A_{280} spectra the BCA assay was introduced as a comparison to the A_{280} values. The BCA assay was used on the solubilised inclusion bodies.
where Triton X-100 had been used to discover the correct total protein concentrations. The standard curves can be seen in figure 4.29.

![Figure 4.29: Standard curves for BCA assay. The assay was carried out with the buffers 8M urea, 6 M guanidine-HCl and 20 mM Tris-HCl and used to work out the total protein concentrations of subsequent samples (R values were 0.999, 0.9992 and 0.9966 respectively).]
3.1 Expression Yields

| Cell Pellet | 16.75 g |
| Inclusion bodies | 4.2 g |

<table>
<thead>
<tr>
<th>Triton X-100</th>
<th>Weight of Inclusion bodies solubilised (g)</th>
<th>$A_{280}$ (mg/ml)</th>
<th>BCA (mg/ml)</th>
<th>Volume (ml)</th>
<th>BCA Total mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>0.5</td>
<td>0.78</td>
<td>0.58</td>
<td>20</td>
<td>11.6</td>
</tr>
<tr>
<td>Urea DTT</td>
<td>0.5</td>
<td>0.53</td>
<td>0.29</td>
<td>20</td>
<td>5.8</td>
</tr>
<tr>
<td>Guanidine-HCl</td>
<td>0.5</td>
<td>1.83</td>
<td>0.82</td>
<td>20</td>
<td>16.4</td>
</tr>
<tr>
<td>Guanidine-HCl DTT</td>
<td>0.5</td>
<td>1.76</td>
<td>0.71</td>
<td>20</td>
<td>14.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Without Triton X-100</th>
<th>$A_{280}$ (mg/ml)</th>
<th>BCA (mg/ml)</th>
<th>Volume (ml)</th>
<th>BCA Total mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>0.5</td>
<td>0.72</td>
<td>0.44</td>
<td>20</td>
</tr>
<tr>
<td>Urea DTT</td>
<td>0.5</td>
<td>0.99</td>
<td>0.25</td>
<td>20</td>
</tr>
<tr>
<td>Guanidine-HCl</td>
<td>0.5</td>
<td>1.23</td>
<td>1.22</td>
<td>20</td>
</tr>
<tr>
<td>Guanidine-HCl DTT</td>
<td>0.5</td>
<td>1.26</td>
<td>1.3</td>
<td>20</td>
</tr>
</tbody>
</table>

Table 4.4: Concentrations of the washed IFN-α2b inclusion bodies with and without Triton X-100. The BCA assay tends to give lower protein concentrations when Triton X-100 has been added to the inclusion bodies indicating Triton X-100 interference with $A_{280}$ readings. There is still a discrepancy between the urea solubilised inclusion bodies when Triton X-100 has not been used however the guanidine-HCl solubilised inclusion bodies give similar values. The guanidine-HCl solubilised inclusion bodies always give higher total protein concentrations indicating more solubilisation.

The concentrations from the 3.1 expression of IFN-α2b can be seen in table 4.4. The inclusion bodies were split and half were treated with washing steps containing Triton X-100. There is a large amount of variability in the values for the total protein between the two methods used; $A_{280}$ and the BCA assay when Triton X-100 is used during the washing. This highlights the problem with Triton X-100 contributing to the calculation of total protein concentration using $A_{280}$. The
$A_{280}$ is almost double the BCA value. The washing of the inclusion bodies without Triton X-100 provides much more consistent results between methods especially for the guanidine-HCl solubilised inclusion bodies, the urea solubilised inclusion bodies still have a degree of variability. The BCA assay was used to calculate the total amount of IFN-α2b in milligrams (mg); this showed in the highest case that from the guanidine-HCl solubilisation the amount of protein was almost three times the level from the urea solubilisation.

<table>
<thead>
<tr>
<th>Triton X-100</th>
<th>Solubilised inclusion bodies (mg)</th>
<th>Pools after gel filtration (mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>$A_{280}$ 7.8  BCA 5.8</td>
<td>$A_{280}$ 0.19  BCA 0.38</td>
<td>2.4</td>
</tr>
<tr>
<td>Urea DTT</td>
<td>$A_{280}$ 5.3  BCA 2.9</td>
<td>$A_{280}$ 0.26  BCA 0.24</td>
<td>4.9</td>
</tr>
<tr>
<td>Guanidine-HCl</td>
<td>$A_{280}$ 18.3 BCA 8.2</td>
<td>$A_{280}$ 0.6  BCA 0.6</td>
<td>3.3</td>
</tr>
<tr>
<td>Guanidine-HCl DTT</td>
<td>$A_{280}$ 17.6 BCA 7.1</td>
<td>$A_{280}$ 4.65  BCA 3.85</td>
<td>26.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Without Triton X-100</th>
<th>Solubilised inclusion bodies (mg)</th>
<th>Pools after gel filtration (mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>$A_{280}$ 0.72 BCA 0.44</td>
<td>$A_{280}$ 0.11 BCA 0.095</td>
<td>15.3</td>
</tr>
<tr>
<td>Urea DTT</td>
<td>$A_{280}$ 0.99 BCA 2.5</td>
<td>$A_{280}$ 0.225 BCA 0.75</td>
<td>22.7</td>
</tr>
<tr>
<td>Guanidine-HCl</td>
<td>$A_{280}$ 2.3 BCA 2.2</td>
<td>$A_{280}$ 0.5  BCA 0.52</td>
<td>21.7</td>
</tr>
<tr>
<td>Guanidine-HCl DTT</td>
<td>$A_{280}$ 2.6 BCA 3</td>
<td>$A_{280}$ 0.425 BCA 0.41</td>
<td>16.3</td>
</tr>
</tbody>
</table>

**Table 4.5: Concentrations from the 3 l fermentation before and after purification by gel filtration.** Both the inclusion bodies treated with and with out Triton X-100 are shown. After the gel filtration the concentrations of the inclusion bodies treated with Triton X-100 give more consistent values between methods confirming the removal of the Triton X-100.
10 l Fermentation Yields

<table>
<thead>
<tr>
<th>Weight (g)</th>
<th>$A_{280}$ (mg/ml)</th>
<th>BCA (mg/ml)</th>
<th>Volume (ml)</th>
<th>Total using BCA result (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>1</td>
<td>2.3</td>
<td>2.2</td>
<td>20</td>
</tr>
<tr>
<td>Urea DTT</td>
<td>1</td>
<td>2.9</td>
<td>2.2</td>
<td>20</td>
</tr>
<tr>
<td>Guanidine-HCl</td>
<td>1</td>
<td>3.3</td>
<td>3.3</td>
<td>20</td>
</tr>
<tr>
<td>Guanidine-HCl DTT</td>
<td>1</td>
<td>3.6</td>
<td>3.4</td>
<td>20</td>
</tr>
</tbody>
</table>

Table 4.6: Concentrations of washed IFN-$\alpha$2b inclusion bodies from the 10 l fermentation. No Triton X-100 was used during the wash steps. The total protein concentrations between the guanidine-HCl solubilised inclusion bodies and urea do not vary to the same extent as the 3 l expression, see table 4.3. The solubilisation with guanidine-HCl still provides a higher total protein concentration.

As with the 3 l expression the concentrations of the solubilised inclusion bodies from the 10 l fermentation were measured and investigated. No Triton X-100 was used during the washing of the 10 l fermentation inclusion bodies and so only four conditions were analysed, see table 4.6. The concentration of the solubilised inclusion bodies did not differ to the same extent as the 3 l expression. Gel filtration was also carried out on the 10 l fermentation solubilised inclusion bodies and the total protein concentrations can be seen in tables 4.5 and 4.7.
10 l fermentation yields from gel filtration

<table>
<thead>
<tr>
<th>Sample</th>
<th>Solubilised inclusion bodies (mg)</th>
<th>Pools after gel filtration (mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$A_{280}$</td>
<td>BCA</td>
<td>$A_{280}$</td>
</tr>
<tr>
<td>Urea</td>
<td>23</td>
<td>22</td>
<td>6.4</td>
</tr>
<tr>
<td>Urea DTT</td>
<td>29</td>
<td>22</td>
<td>6.85</td>
</tr>
<tr>
<td>Guanidine-HCl</td>
<td>33</td>
<td>33</td>
<td>12.1</td>
</tr>
<tr>
<td>Guanidine-HCl DTT</td>
<td>36</td>
<td>34</td>
<td>13.4</td>
</tr>
</tbody>
</table>

Table 4.7: Yields from the gel filtration run carried out on the inclusion bodies from the 10 l fermentation. No Triton X-100 was used during the washing steps. As expected all the yields from the guanidine-HCl solubilisation are higher than the values for the urea solubilisation.

The yields calculated in from both the 3 l and 10 l expressions show that the yields of IFN-α2b after gel filtration are lower when Triton X-100 has been used for the washing of the inclusion bodies washing. This demonstrates that the ion exchange has been successfully replaced with gel filtration and that the removal of Triton X-100 from the washing steps actually improves the yields of IFN-α2b. Refolding of the IFN-α2b solubilised inclusion bodies can now go ahead confident that contamination should not interfere.

4.3: Conclusions
This chapter has revealed some important characteristics of IFN-α2b. The differences in solubility of the IFN-α2b inclusion bodies is clear with guanidine-
HCl being the favoured denaturant as shown in figure 4.9 and previously by Valente et al., (2006). Urea however is the preferred denaturant for Biomanufacturing and thus was fully explored as a denaturant. The differences in the results gained from the refolding, depending on which denaturant has been used for the solubilisation was clear. Only the inclusion bodies solubilised in guanidine-HCl resulted in alpha helical secondary structure (figures 4.14 and 4.17). However the intrinsic fluorescence results revealed “tyrosine” emission which had not been previously observed during the characterisation work in chapter 3, section 3.2.3, it is also known that in proteins containing a mixture of tryptophan and tyrosine residues it is the tryptophan residues which dominate (Lackowicz, 1983). Beldarrain et al., (2001) carried out detailed biophysical characterisation of folded IFN-α2b and there was no mention of tyrosine fluorescence. Therefore to ensure this tyrosine emission had been caused by the IFN-α2b, denaturation trials were carried out involving repeated freeze thawing (figure 4.19) along with denaturation with guanidine-HCl, DTT and heat (figure 4.20). None however resulted in the IFN-α2b standard producing a mixture of both tyrosine and tryptophan fluorescence.

The next stage was to investigate everything which had been added to the protein or used throughout the expression and treatment of the inclusion bodies. As Triton X-100 had been used during the wash steps and is known to absorb at around 280 nm it was investigated. Triton X-100 is a non-ionic detergent used extensively in the treatment of membrane proteins but also for its ability to breakdown lipids, a useful characteristic for washing away any contaminating lipids from inclusion bodies. Triton X-100 has been known to interfere with protein absorption spectra (Tiller, et al., 1984). The ability of detergents such as Triton X-100 to interact with membrane proteins has been investigated and is due to the binding of the detergent molecule to the protein’s hydrophobic regions allowing it to remain soluble in aqueous solution (Moller and le Maire, 1993). This binding can take place whether the Triton X-100 is in the form of a monomer or as a micelle above its critical micelle concentration (CMC) (Clarke, 1975). The CMC is the concentration at which detergent molecules convert from monomers to micelles. The CMC of Triton X-100 is 0.2 mM as stated in the product literature. The solution used here was a 1 % (v/v) solution providing a final Triton
X-100 concentration of 15.5 mM. This suggests that the Triton X-100 was either binding to any exposed hydrophobic regions of the denatured IFN-α2b molecules or the concentration remaining after washing was high enough, due to such a high concentration being used initially, that the formed micelles were too large to pass through the dialysis tubing and be removed.

The introduction of the gel filtration resulted in the Triton X-100 being removed from the solubilised inclusion body samples. The conclusion however that removal of Triton X-100 altogether did not have a negative effect on the yield of IFN-α2b allowed Triton X-100 to be removed completely. The gel filtration was kept even though no Triton X-100 was involved as a small peak late in the elution profile highlighted to continued need for a purification step, see figure 4.24. The next stage is to use this gel filtration step to purify the solubilised inclusion bodies and begin refolding this purified IFN-α2b using the same method of a 1 in 10 dilution followed by dialysis at 4 °C.
Chapter 5
Chapter 5

Refolding of IFN-α2b purified by gel filtration and investigation of the solubilised inclusion bodies

5.1: Introduction

The gel filtration proved successful in removing contamination from the IFN-α2b inclusion bodies. Refolding was therefore started. The refold method consisted of a dilution step followed by dialysis. The IFN-α2b was kept under reducing conditions by use of DTT until the final stage when it was removed using a second dialysis step. The addition of oxidised glutathione was investigated as the only additive. The refolding work detailed in this section concentrated on samples made up from pooled fractions from the gel filtration where no Triton X-100 has been used to wash the inclusion bodies. Once the refolding had been carried out the refolded material underwent functional analysis through an antiviral assay and a reporter gene assay. Alongside these experiments the refolded material was further purified to remove any mis-folded species and elucidate if ion exchange on IFN-α2b could be successful without the influence of urea.

Due to the differences observed in chapter 3, section 3.2.4, in the unfolding of IFN-α2b along with the solubilisation of the inclusion bodies with urea and guanidine-HCl the solubilised inclusion bodies were investigated using the available techniques. Any differences in the starting material may have been the reason for such differences in the success of the refolding and so was investigated. The occurrence of secondary structure in unfolded proteins has been investigated by nuclear magnetic resonance (NMR) (Shortle and Ackerman, 2001), small angle x-ray scattering (SAXS) (Lattman, 1994) and Fourier transform infrared spectroscopy (FTIR) (Ami et al., 2006). Here far and near UV CD, intrinsic fluorescence, ANS fluorescence, N-(1-pyrene) maleimide binding were investigated. These techniques are more widely available and may provide an insight into the difference observed during refolding.
5.2: Refolding

5.2.1: Refold of a one step dialysis into 20 mM Tris pH 8.0

The first refold experiment carried out was a short dialysis step of 1 ml sample of guanidine-HCl solubilised inclusion bodies dialysed into 1 l of 20 mM Tris pH 8.0 to investigate if the quick approach suits this protein. The refold was carried out at 24 °C as well as 4 °C and at this sample to buffer ratio all denaturant should be removed.

![Fluorescence results](image)

**Figure 5.1: Fluorescence results of the final supernatants from the refold consisting of one dialysis step.** Both samples (black and red lines) show tryptophan fluorescence but appear shifted to the longer wavelengths in comparison to the IFN-α2b standard (green line).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Barycentric Mean (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folded IFN-α2b standard</td>
<td>341.6</td>
</tr>
<tr>
<td>4 °C</td>
<td>343.2</td>
</tr>
<tr>
<td>Room temperature</td>
<td>343.5</td>
</tr>
</tbody>
</table>

**Table 5.1: Barycentric mean of the samples refolded by one dialysis step at 4 °C and 24 °C.** The results shows results similar to the IFN-α2b standard suggesting some of the IFN-α2b has refolded.
Table 5.2: Concentrations and yields of the refolded samples after one dialysis step. The results show a large loss of protein indicating it is not an efficient method of refolding IFN-α2b.

The refold of one dialysis step shown in figure 5.1 illustrates that IFN-α2b does refold to look similar to the IFN-α2b standard (table 5.1) however the yields listed in table 5.2 of 8.4 % and 6.78 % for 4 °C and 24 °C respectively suggest that it is not a suitable way of refolding IFN-α2b.

5.2.2: Refolding under reducing conditions

In this refold the protein underwent the following:

- 1 in 10 dilution into 20 mM Tris, 1 mM DTT pH 8.0.
- Dialysis into the same buffer to remove any denaturant.
- Dialysis to remove the DTT.

All steps were carried out at 4 °C. The end samples were concentrated for CD analysis using a Vivaspin 5000 MWCO concentrator. An aliquot of each of the guanidine-HCl pools was dialysed into 8 M urea, 20 mM Tris pH 8.0 with and without DTT. This was to investigate, again whether the starting denaturant has an effect on the refolding now with the Triton X-100 removed. The concentrations were measured throughout the refold and can be seen in table 5.3.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Starting concentration (mg/ml)</th>
<th>Final concentration (mg/ml)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 °C</td>
<td>1</td>
<td>0.084</td>
<td>8.4</td>
</tr>
<tr>
<td>24 °C</td>
<td>1</td>
<td>0.0678</td>
<td>6.78</td>
</tr>
<tr>
<td>Sample</td>
<td>Starting Concentration (mg/ml)</td>
<td>Dilution</td>
<td>Dialysis 1</td>
</tr>
<tr>
<td>-------------------------------------</td>
<td>-------------------------------</td>
<td>----------</td>
<td>------------</td>
</tr>
<tr>
<td></td>
<td>BCA</td>
<td>A$_{280}$</td>
<td>BCA</td>
</tr>
<tr>
<td>Urea</td>
<td>0.27</td>
<td>0.3</td>
<td>0.11</td>
</tr>
<tr>
<td>Urea DTT</td>
<td>0.25</td>
<td>0.29</td>
<td>0.12</td>
</tr>
<tr>
<td>Guanidine-HCl</td>
<td>1.3</td>
<td>2.4</td>
<td>0.19</td>
</tr>
<tr>
<td>Guanidine-HCl DTT</td>
<td>1.26</td>
<td>2.6</td>
<td>0.26</td>
</tr>
<tr>
<td>Guanidine-HCl dialysed into urea</td>
<td>0.88</td>
<td>2</td>
<td>0.23</td>
</tr>
<tr>
<td>Guanidine-HCl DTT dialysed into urea DTT</td>
<td>0.98</td>
<td>1.9</td>
<td>0.21</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>Yield (%)</th>
<th>BCA</th>
<th>A$_{280}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>35</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>Urea DTT</td>
<td>33</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>Guanidine-HCl</td>
<td>116</td>
<td>117</td>
<td></td>
</tr>
<tr>
<td>Guanidine-HCl DTT</td>
<td>69</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>Guanidine-HCl dialysed into urea</td>
<td>91</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>Guanidine-HCl DTT dialysed into urea DTT</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.3: Concentrations of the samples taken throughout the refold under reducing conditions. The concentrations have been measured by both the BCA method and A$_{280}$. The variation in sample concentration between methods highlights the difficulties experienced in measuring the concentrations throughout these experiments especially when the refold is unsuccessful. Throughout the refold a broad A$_{280}$ spectrum does indicate the incorrect folding of the IFN-α2.
The concentrations in table 5.3 highlight the variability between the two methods of protein concentration determination. Yields were calculated using the values from both assays and appear to give similar results. The greatest yields came from the samples where the inclusion bodies had been solubilised in guanidine-HCl. The samples solubilised in guanidine-HCl and then dialysed into urea give a yield of 100%. A 100% and above in refolding of proteins is unrealistic especially as there is loss of protein to precipitation throughout the refold. The values do however show the differences between refolding from urea and guanidine-HCl.

Intrinsic fluorescence

![Intrinsic fluorescence graph](image)

**Figure 5.2: Intrinsic fluorescence results of the final samples of the refold.** The results all show tryptophan fluorescence similar to that of the folded IFN-α2b standard (black dashed line). If not already at 0.1 mg/ml the samples were diluted to 0.1 mg/ml for fluorescence analysis using the A$_{280}$ value of the concentrated samples. From these curves the barycentric mean was calculated (table 5.4).

The fluorescence results in figure 5.2 show tryptophan emission in all six samples. There is no indication of tyrosine fluorescence confirming the possible
cause of this was the Triton X-100. The Cobra reference standard can be seen as the purple line with the highest intensity. The other six samples can be seen to be lower in fluorescence intensity and more red shifted than the standard indicating that the tertiary structures are not quite as well folded as the standard. The barycentric means of the curves can be seen in table 5.4 measured between 306 and 380 nm.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Barycentric Mean (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guanidine start</td>
<td>349</td>
</tr>
<tr>
<td>Guanidine <strong>end</strong></td>
<td><strong>344.2</strong></td>
</tr>
<tr>
<td>Guanidine DTT start</td>
<td>349</td>
</tr>
<tr>
<td>Guanidine DTT <strong>end</strong></td>
<td><strong>343.7</strong></td>
</tr>
<tr>
<td>Urea Start</td>
<td>348</td>
</tr>
<tr>
<td>Urea <strong>end</strong></td>
<td><strong>344.4</strong></td>
</tr>
<tr>
<td>Urea DT start</td>
<td>348</td>
</tr>
<tr>
<td>Urea DTT <strong>end</strong></td>
<td><strong>346.7</strong></td>
</tr>
<tr>
<td>Guanidine dialysed into urea start</td>
<td>346</td>
</tr>
<tr>
<td>Guanidine dialysed into urea <strong>end</strong></td>
<td><strong>344.5</strong></td>
</tr>
<tr>
<td>Guanidine DTT dialysed into urea DTT start</td>
<td>346</td>
</tr>
<tr>
<td>Guanidine DTT dialysed into urea DTT <strong>end</strong></td>
<td><strong>343.1</strong></td>
</tr>
<tr>
<td>IFN-α2b standard</td>
<td><strong>341.6</strong></td>
</tr>
</tbody>
</table>

**Table 5.4:** Barycentric means of the final samples of all six refolded samples. The closest samples to the folded IFN-α2b standard were the samples dialysed into urea DTT after being solubilised in guanidine-HCl and DTT. The start values represent the solubilised inclusion bodies and the end represents the values calculated from figure 5.2. The Barycentric mean was calculated between 306-380 nm.
The barycentric means in table 5.4 for all six samples support the results seen in figure 5.2. The samples are red shifted to the longer wavelengths in comparison the IFN-α2b standard indicating they are not quite as well folded as the IFN-α2b standard.

ANS binding of the final samples from the refold

The samples used for the intrinsic fluorescence analysis were also subjected to ANS binding. ANS was added to the sample to a final concentration of 0.1 mM, the samples were excited at 360 nm.

![Graph showing ANS binding of final samples](image)

**Figure 5.3: ANS binding of the final samples from the refold.** The ANS binding of the final samples initially solubilised in guanidine-HCl (pink and royal blue lines) and the dialysed samples (green and red lines) show a larger emission than the IFN-α2b standard. The urea solubilised samples (brown and turquoise lines) show similar intensity of fluorescence but are blue shifted compared to the standard.
Table 5.5: Table of the barycentric means of the final samples binding of ANS.

Out of all six samples the closest to the IFN-α2b standard is the sample solubilised in urea. The samples appear to be blue shifted to the IFN-α2b standard. Barycentric mean measured between 420 -600 nm.

The ANS binding of the final refolded samples shows that the samples are again not exactly the same as the IFN-α2b standard. The intensity of the guanidine-HCl solubilised samples and the samples dialysed into urea show a large increase in fluorescence intensity and a shift to the shorter wavelengths; see figure 5.3 and table 5.5. The large increase in intensity would suggest a more molten globule state than the fully native state. This supports the intrinsic fluorescence results as they also show indications of partially folded IFN-α2b.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Barycentric mean (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-α2b standard</td>
<td>511.1</td>
</tr>
<tr>
<td>Guanidine-HCl</td>
<td>502.7</td>
</tr>
<tr>
<td>Guanidine-HCl DTT</td>
<td>497.8</td>
</tr>
<tr>
<td>Urea</td>
<td>506.0</td>
</tr>
<tr>
<td>Urea DTT</td>
<td>505.2</td>
</tr>
<tr>
<td>Guanidine-HCl dialysed into urea</td>
<td>499.8</td>
</tr>
<tr>
<td>Guanidine-HCl DTT dialysed into urea DTT</td>
<td>499.7</td>
</tr>
</tbody>
</table>
Far and near UV analysis

The samples were concentrated and far and near UV analysis carried out to gain information about secondary and tertiary structure.

Figure 5.4: Far UV CD to show the final samples of the refold. The far UV shows alpha helical structure in all six samples. The guanidine-HCl solubilised samples (brown and green lines) show alpha helical structure most similar to the IFN-α2b standard (black dashed line).

Figure 5.5: Near UV CD to show the final samples of the refold. The near UV shows tertiary structure similar to that of the standard in both the guanidine-HCl solubilised samples (red and green lines). The remaining samples do not give a near UV signal.
The far UV analysis in figure 5.4 shows alpha helical structure in all six samples. The closest secondary structure to the IFN-α2b standard comes from the two samples solubilised in 6 M guanidine with or without DTT. The samples dialysed into urea then have the second best alpha helical structure and finally it is the urea samples with the least amount of secondary structure. The near UV (figure 5.5) shows the same characteristics as the standard from both guanidine-HCl solubilised samples, indicating that the tertiary structure of the refolded samples is very similar to that of the IFN-α2b standard. The concentrations used for CD analysis can be seen in table 5.6.

<table>
<thead>
<tr>
<th>Sample</th>
<th>A_{280} Concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>0.11</td>
</tr>
<tr>
<td>Urea DTT</td>
<td>0.11</td>
</tr>
<tr>
<td>Guanidine-HCl</td>
<td>0.5</td>
</tr>
<tr>
<td>Guanidine-HCl DTT</td>
<td>0.52</td>
</tr>
<tr>
<td>Guanidine-HCl dialysed into urea</td>
<td>0.17</td>
</tr>
<tr>
<td>Guanidine-HCl DTT dialysed into urea DTT</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Table 5.6: Table of concentrations used for far and near UV analysis.

SDS-PAGE analysis of the refolded samples

Figure 5.6: SDS-PAGE analysis of the final samples of the refold. The IFN-α2b shows a shift up the gel under reducing conditions (carried out by addition of DTT and iodoacetamide). The refolded samples also show a shift. The urea samples cannot be seen and may require a more sensitive staining procedure than the Coomassie blue used here. IFN-α2b standard loaded to 3 μg.
Figure 5.7: Thermal melt trace of the sample refolded from guanidine-HCl with DTT. This sample was the only sample which gave a trace to which a curve could be fitted to calculate a $T_m$. The $T_m$ was found to be 59 °C. The IFN-α2b standard has a $T_m$ of 70 °C. Curve fitted by Origin™ using a Boltzmann function.

Conclusions from the refolding experiments

Samples solubilised in guanidine-HCl
The results show successful refolding from the two samples solubilised in 6 M guanidine-HCl. The far UV CD for these two samples shows similar signals and intensities at 208 nm and 220 nm to that of the IFN-α2b standard. The barycentric means for both guanidine-HCl solubilised samples are not the same as the folded IFN-α2b standard indicating some incorrect folding therefore the sample may be a mixture of folded and mis-folded species and thus require further purification. The near UV shows tertiary structure in the solubilised guanidine-HCl sample supporting the intrinsic fluorescence as the barycentric mean is almost the same as the IFN-α2b standard. The SDS-PAGE analysis in figure 5.6 shows the shift of the band from the reduced sample to a higher molecular weight but it also
highlights the presence of some aggregation. After each step of the refold the samples are centrifuged to remove any precipitation which may occur so this may be soluble oligomers which have formed. Figure 5.7 showing the thermal melt is poorly cooperative and gives a melting temperature of 59 °C whereas the IFN-α2b standard has a T_m of 70 °C. This result indicates the guanidine-HCl refolded samples are not as stable or as well folded as the IFN-α2b standard.

**Samples solubilised in guanidine-HCl and dialysed into urea**

The samples dialysed into urea after being solubilised into guanidine-HCl show far UV CD spectrum that does indicate alpha helical structure but is not as intense does as the signals from the folded IFN-α2b standard or the guanidine-HCl solubilised samples (figure 5.4). The intrinsic fluorescence does not shift to 341.6 nm; the barycentric means for the samples are 346 nm (without DTT) and 343 nm (with DTT) however the near UV does not show any native tertiary structure.

**Urea solubilised samples**

The inclusion bodies solubilised in urea were the least successful of all six samples. The starting concentrations are lower due to the poor solubilisation. Fluorescence analysis was carried out on both samples and tryptophan emission was observed however the barycentric means did not match the value from the IFN-α2b standard, table 5.5. The far UV CD also did not match the IFN-α2b standard, alpha helical signals were obtained but were not intense enough to confirm a structure equivalent to that of the IFN-α2b standards.

**5.2.3: Refold with 0.1mM oxidised glutathione**

In order to ensure formation of the two disulphide bonds 0.1 mM oxidised glutathione was added after the first dialysis step in the hope the correct two disulphides would form, which may had been the reason the structure seen previously was not identical to that of the IFN-α2b standard. The oxidised glutathione was added at 0.1 mM and the incubation time was 1 h at 4 °C.
### Table 5.7: Table of concentrations from the refold with 0.1 mM oxidised glutathione.

The refolded samples from guanidine-HCl show the highest yield measure by the BCA assay however the urea solubilised and refolded inclusion bodies show the highest yield measured by \( A_{280} \), this confirms interference of contaminants which no longer include the Triton X-100.

Table 5.7 details the protein concentrations measure throughout the refold for each condition. The highest yields can be seen to be gained from the refolded...
guanidine-HCl solubilised inclusion bodies and the urea refolded are the least successful.

**Fluorescence analysis of the refold with 0.1 mM oxidised glutathione**

![Fluorescence graph](image)

**Figure 5.8:** *Intrinsic fluorescence results of the refold using 0.1 mM oxidised glutathione.* The results all show tryptophan fluorescence similar to that of the folded IFN-α2b standard however the fluorescence from the urea solubilised (green and blue lines) and refolded material have a lower emission intensity.

The fluorescence results in figure 5.8 show tryptophan emission from all six samples. The urea solubilised samples give the lowest intensities suggesting less tertiary structure present compared to the other samples. The barycentric means of all six samples before the refold and after the refold can be seen in table 5.8.
Table 5.8: Table to show the barycentric mean of the final samples of all six refolds before and after 0.1 mM oxidised glutathione was used. Both the refolded samples that were solubilised in guanidine-HCl with or without DTT have barycentric means the same as the folded IFN-α2b.

The table shows that the inclusion bodies solubilised in guanidine-HCl with or without DTT give a barycentric mean the same as the IFN-α2b standard indicating the successful refolding of these samples has taken place.
ANS binding of the refolded samples

![Fluorescence graph showing ANS binding of the refolded samples](image)

**Figure 5.9:** ANS binding of the final samples from the refold using 0.1 mM oxidised glutathione. The samples solubilised in guanidine-HCl ± DTT (maroon and red lines) and urea (green line) show similar fluorescent intensities as the IFN-α2b standard.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Barycentric mean (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-α2b standard</td>
<td>511.13</td>
</tr>
<tr>
<td>Guanidine-HCl</td>
<td>505.43</td>
</tr>
<tr>
<td>Guanidine-HCl DTT</td>
<td>505.02</td>
</tr>
<tr>
<td>Urea</td>
<td>508.57</td>
</tr>
<tr>
<td>Urea DTT</td>
<td>517.35</td>
</tr>
<tr>
<td>Guanidine-HCl dialysed into urea</td>
<td>497.17</td>
</tr>
<tr>
<td>Guanidine-HCl DTT dialysed into urea DTT</td>
<td>500.60</td>
</tr>
</tbody>
</table>

**Table 5.9:** Barycentric means of the ANS binding of the final samples of the refold with 0.1 mM oxidised glutathione. The closest to the IFN-α2b standard is the samples solubilised in urea.
The ANS results in figure 5.9 show the inclusion bodies solubilised in guanidine-HCl and then dialysed into urea give higher intensities than the IFN-α2b standard indicating molten globule structure present in these samples suggesting a less well folded structure. The barycentric means for the fluorescence analysis can be seen in table 5.9.

**Far and near UV CD analysis**

![Graph showing CD analysis](image)

**Figure 5.10:** Far UV CD to show the final samples of the refold using 0.1 mM oxidised glutathione. The far UV CD spectra show some alpha helical structure in all six samples. The samples which show alpha helical structure closest to the folded IFN-α2b standard are the samples solubilised in guanidine-HCl (yellow) and guanidine-HCl with 1 mM DTT (red).
Figure 5.11: Near UV CD to show the final samples of the refold using 0.1 mM oxidised glutathione. The near UV CD spectra shows tertiary structure similar to that of the standard in both the guanidine-HCl solubilised samples (pink and red lines), there is also a little tertiary structure in the dialysed samples (blue and green lines). The urea samples did not give a signal indicating any structure (data not shown).

<table>
<thead>
<tr>
<th>Sample</th>
<th>$A_{280\text{nm}}$ Concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>0.12</td>
</tr>
<tr>
<td>Urea DTT</td>
<td>0.12</td>
</tr>
<tr>
<td>Guanidine-HCl</td>
<td>0.31</td>
</tr>
<tr>
<td>Guanidine-HCl DTT</td>
<td>0.32</td>
</tr>
<tr>
<td>Guanidine-HCl dialysed into urea</td>
<td>0.17</td>
</tr>
<tr>
<td>Guanidine-HCl DTT dialysed into urea DTT</td>
<td>0.18</td>
</tr>
</tbody>
</table>

Table 5.10: Sample concentrations for CD analysis. The concentrations shown here were used in the analysis of the far UV and near UV CD data shown in figures 5.10 and 5.11.
Figure 5.12: 17 % SDS-PAGE analysis of the final samples of the refold with 0.1 mM oxidised glutathione. The IFN-α2b shows a splitting of the in band in lane 2 as both the reduced and non-reduced samples were applied to the same lane. The samples also show a shift. The urea samples cannot be seen once again, the gel was stained using the more sensitive SimplyBlue™ Safe stain. IFN-α2b standard loaded to 1.5 μg.

Thermal denaturation

Figure 5.13: Thermal denaturation of the final samples refolded with 0.1 mM oxidised glutathione from solubilisation in guanidine-HCl ± DTT. The $T_m$ calculated of the two samples was 67 °C for the guanidine-HCl solubilised sample and 65 °C for the guanidine-HCl and DTT solubilised sample. The IFN-α2b standard has a $T_m$ is 70 °C. Curve fitted by Origin™ using a Boltzmann function.
The results from this refold using 0.1 mM oxidised glutathione show good refolding of the IFN-α2b especially from the samples solubilised in guanidine-HCl. The far UV CD for these two guanidine-HCl samples show similar signals and intensities at 208 nm and 220 nm (figure 5.10). The barycentric mean for both guanidine-HCl solubilised samples are the same as the folded IFN-α2b standard, see table 5.9. The urea solubilised samples do not give the correct alpha helical structure in the far UV region. The intrinsic fluorescence does not shift to the wavelength 341.6 nm like with the guanidine-HCl solubilised samples. The concentration of the urea samples was too low to measure the near UV (figure 5.11). All samples solubilised in guanidine-HCl even the samples dialysed into urea gave CD signals in the near UV region, the guanidine-HCl solubilised and refolded samples being the best. The samples dialysed into urea after being solubilised into guanidine-HCl show promising results, the far UV does not give as intense signals as the folded IFN-α2b standard however the shape is more correct than the urea solubilised samples. The barycentric mean from the dialysed samples are closer to the standard than the urea solubilised samples. The SDS-PAGE in figure 5.12 show a shift in molecular weight of the refolded samples from guanidine-HCl solubilised inclusion bodies. This shift is seen by the Cobra IFN-α2b reference standard, see chapter 3, figure 4.7. The thermal melts of the samples refolded from guanidine-HCl also give values similar to those of the Cobra IFN-α2b reference standard. Here in figure 5.13 the $T_m$ values are 65 and 67 °C. The standard has a $T_m$ of 70 °C, see chapter 3, figure 3.24.
5.2.4: Addition of 1 mM oxidised glutathione

In this experiment 1 mM oxidised glutathione was added to the refold instead of 0.1 mM. All other steps were kept the same as in section 5.1.2.

Far UV CD results of the refold with 1 mM oxidised glutathione

Figure 5.14: Far UV CD to show the final samples of the refold using 1 mM oxidised glutathione. The far UV shows no alpha helical structure in all six samples.

The result in figure 5.14 indicates that the refolding of IFN-α2b is sensitive to the concentration of oxidised glutathione. The results in all six samples give an unfolded signal in the far UV region. The far UV CD results do however appear to separate into two distinct groups of differing signals. The urea solubilised and refolded samples give a positive signal between 220-230 nm. The characteristic signals of the poly pro (II) helix are a positive signal at 215 and 228 nm and a negative signal at 195 205 nm (Woody, 1992) whereas the remaining samples do not have the positive signal around 228 nm but do show a signal at 205 nm. The values for the poly proline II helix are for a poly proline peptide in aqueous solution and so here the values and positions may differ due to the differing environments, see figure 1.10 from Kelly et al., 2005. The samples were also investigated by SDS-PAGE.
Figure 5.15: Example of the refolded material when 1 mM oxidised glutathione is added to the refold. The samples shown here were solubilised in guanidine-HCl usually the most successful condition. The band splitting indicates incomplete disulphide formation.

The gel shown in figure 5.15 shows the incomplete formation of the disulphide bonds. There is splitting of the IFN-α2b band indicating that some of the IFN-α2b molecules either do not have any correctly formed disulphides or have partially formed disulphides. The first reaction is the formation of the mixed disulphide intermediate (figure 5.16) and the second an intramolecular step in which a second cysteine thiol displaces the mixed disulphide forming the proteins disulphide bond (Creighton, 1992). Oxidised glutathione is an intermolecular disulphide reagent and its concentration can affect the rate at which the protein reacts with the oxidised glutathione producing the mixed disulphide species (Goto and Hamaguchi, 1981). Protein disulphides are formed (and broken) by a two-step sequential thiol disulphide exchange reaction with the disulphide reagent. Work done by Goto and Hamaguchi in 1981 found that the constant fragment of the immunoglobulin light chain (C<sub>L</sub>) which has one disulphide bond forms the disulphide much slower in the absence of urea as the protein adopts the folded conformation burying the cysteine residues. In the presence of urea the reaction was completed in 30 minutes whereas in the absence of urea it took several h however the effectiveness in the formation of the disulphide was better without urea. They also found that at lower concentrations of GSSG the formation of the disulphide increased compared to the accumulation of the mixed disulphide but
with high GSSG concentrations the mixed disulphide accumulated to a higher concentration than the complete disulphide form. They concluded that the formation of the mixed disulphide was dependent on the concentrations of GSSG but that disulphide formation was not. It could be suggested that in the case of the high GSSG concentration the IFN-α2b had been prevented from forming the disulphide. It is not the case that the cysteines are buried within the IFN-α2b structure, the two cysteines are said to be in region of high mobility (Radhaskrisnan et al., 1996). However the cysteines may still be as the mixed disulphide and the refolding was continued too early.

![Diagram of disulphide formation](image)

**Figure 5.16**: Formation of the mixed disulphide intermediate when oxidised glutathione (GSSG) is used as the reagent.

The step shown in figure 5.16 can result in both cysteine residues existing as the mixed disulphide which appears to be the case here with the IFN-α2b. The step which is important to protein folding is the second, the intramolecular step, where the protein disulphide is formed. If the disulphide is not formed two assumptions can be made either, the thiols are unreactive or the conformation of the molecule is keeping the cysteine residues apart (Creighton, 1992) both situations can occur also. If the intermediates accumulate then it is the incorrect conformation which is preventing disulphide formation. The alpha helical structure can be seen to be forming during the dilution step of the refold so conformation should not be the cause of the unsuccessful refold.
5.3: Activity assays
These experiments were carried out to investigate any functional activity of the refolded samples. The samples used for these assays were the samples from the refold where no oxidised glutathione was added. An aliquot of each refolded sample was dialysed into PBS. The experiments were carried out at the National Institute of Biological Standards and Control (NIBSC), Herts, U.K. The IFN-α2b standard 95/566 was provided by NIBSC.

5.3.1: Antiviral assay
The first assay to be carried out was an antiviral assay based on the protection IFN-α2b gives to cells against the virus EMC. Two cell lines were investigated; 2D9 glioblastoma cell line and the less sensitive A549 lung carcinoma cell line. The error bars shown in each activity assay are the standard deviations where n = 3.

Results of the refolded Tris samples with the cell line 2D9

![Graph showing antiviral assay results](image)

Figure 5.17: Results of the antiviral assay using the samples in 20 mM Tris. All samples of IFN provide resistance to the virus EMC. The refolded samples from guanidine-HCl (green and navy lines) have activity closest to the IFN-α2b standard (black line) provided by NIBSC. The error bars shown are the standard deviations where n = 3.
The IFN-α2b reference standard 95/566 shows a sigmoidal relationship between protein concentration and absorbance at 610 nm, see figure 5.17. The majority of the samples show this relationship also which is a good indication that the expressed and refolded IFN-α2b is acting correctly as is the IFN-α2b reference standard. The IFN-α2b standard does not give a complete sigmoidal curve due to lack of data.

**Results of the PBS refolded samples with the cell line 2D9**

![Antiviral assay using 2D9 cell line and PBS refolded samples. All samples of IFN provide resistance to the virus EMC activity. The samples refolded from guanidine-HCl (green and navy lines) have activity similar to the NIBSC standard (black line) and the urea refolded samples (turquoise and pink lines) have the least amount of activity. The error bars shown are the standard deviations where n = 3.](image)

The samples in 20 mM Tris give data which fits a sigmoidal curve better than the samples dialysed into PBS (figure 5.18 and 5.17). PBS is usually the buffer of choice for these experiments.
Results of the PBS refolded samples using the A549 cell line.

For the cell line A549 only the PBS samples were tested.

![Graph showing absorbance at 610 nm for different samples](image)

**Figure 5.19: Data to show the effects of the IFN-α2b on the cell line A549 on infection with EMC virus.** All samples provide resistance from the EMC virus on the less sensitive cell line. The urea refolded samples (turquoise and pink lines) have the least amount of activity and the guanidine-HCl samples (green and navy lines) have the most activity. The error bars shown are the standard deviations where n = 3.

Figure 5.19 show the results from the antiviral assay using the cell line A549, the less sensitive cell line shows good sigmoidal shaped curves similar to the IFN-α2b reference standard 95/599.

### 5.3.2: Reporter gene assay

The second assay to test IFN-α2b function was a reporter gene assay based on a transfected HEK 293 cell line harbouring the secreted alkaline phosphatase cDNA linked to the IFN stimulated response element (IRSE) promotor. This experiment was also only carried out on the PBS samples and as it is a less sensitive assay compared to the antiviral assay the initial dilutions were smaller.
Figure 5.20: **Reporter gene assay results of the PBS samples.** All samples induce expression of alkaline phosphatase leading to some level of substrate breakdown. The sample refolded from guanidine-HCl once dialysed into urea (navy line) shows activity closest to the NIBSC standard (black line). Surprisingly the Cobra standard (red line) is one of the least active samples. The error bars shown are the standard deviations where \( n = 3 \).

The reporter gene assay gave good data for the refolded samples along with the antiviral assay even with the reporter gene assay being less sensitive, figure 5.20. The results from these two assays can be used to calculate specific activity of all the samples relative to the IFN-\( \alpha \)2b standard 95/566.

The 95/566 standard has an assigned potency of 70,000 IU/ml. The estimated IFN-\( \alpha \)2b protein concentration is 500 ng/ml. Therefore the calculated specific activity is 140 IU/ng or \( 1.4 \times 10^8 \) IU/mg.
### A549

#### Specific Activity (IU/mg)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Tris</th>
<th>PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cobra standard</td>
<td></td>
<td>3.3 x 10^8</td>
</tr>
<tr>
<td>Guanidine-HCl DTT</td>
<td></td>
<td>6.4 x 10^7</td>
</tr>
<tr>
<td>Guanidine-HCl dialysed into urea</td>
<td></td>
<td>3.1 x 10^7</td>
</tr>
<tr>
<td>Urea</td>
<td>1.16 x 10^5</td>
<td>2.4 x 10^4</td>
</tr>
<tr>
<td>Urea DTT</td>
<td>1.2 x 10^5</td>
<td>2.5 x 10^5</td>
</tr>
<tr>
<td>Unfolded</td>
<td>8.5 x 10^6</td>
<td>9.6 x 10^6</td>
</tr>
</tbody>
</table>

### 2D9

#### Specific Activity (IU/mg)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Tris</th>
<th>PBS</th>
<th>Reporter gene assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cobra standard</td>
<td>2.11 x 10^8</td>
<td>2.05 x 10^8</td>
<td>1.08 x 10^8</td>
</tr>
<tr>
<td>Guanidine-HCl DTT</td>
<td>1.46 x 10^8</td>
<td>4.8 x 10^8</td>
<td>1.8 x 10^6</td>
</tr>
<tr>
<td>Guanidine-HCl dialysed into urea</td>
<td>10 x 10^7</td>
<td>4.8 x 10^7</td>
<td>1.7 x 10^7</td>
</tr>
<tr>
<td>Urea</td>
<td>2.33 x 10^5</td>
<td>3.96 x 10^5</td>
<td>2.2 x 10^5</td>
</tr>
<tr>
<td>Urea DTT</td>
<td>5.2 x 10^5</td>
<td>2.5 x 10^5</td>
<td>7.8 x 10^5</td>
</tr>
<tr>
<td>Unfolded</td>
<td>15.5 x 10^6</td>
<td>10 x 10^5</td>
<td></td>
</tr>
</tbody>
</table>

**Table 5.11:** Specific activities for the refolded samples from the antiviral assay and the reporter gene assay. The Cobra standard and the guanidine-HCl refolded material give activities good enough for fully folded active IFN-α2b.
The specific activities calculated from the activity assays can be seen in table 5.11. The values generated for the Cobra standard and the guanidine-HCl solubilised samples are what would be expected for properly folded fully active IFN-α2b. There is some variation but that is a reflection on the fact that there is only data from a couple of assays. Manufacturers usually perform at least 5 independent assays (personal communication A. Meager). There is however consistent differences in activity between the guanidine-HCl and urea samples. The fact that the unfolded samples gave activity could be due to the high dilutions the samples undergo as part of the assay which may have lead to some refolding. Examples of values published for the activity of folded and purified IFN-α2b can be seen in table 5.12. These are consistent with the results shown in table 5.12 from the guanidine-HCl solubilised inclusion bodies.

<table>
<thead>
<tr>
<th>Activity (IU/mg)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 x 10^8</td>
<td>Beldarrain et al., 2001</td>
</tr>
<tr>
<td>3 x 10^9</td>
<td>Srivastava et al., 2001</td>
</tr>
<tr>
<td>2.7 x 10^8</td>
<td>Cao et al., 2006</td>
</tr>
<tr>
<td>2 x 10^8</td>
<td>Rabhi-Essati et al., 2007</td>
</tr>
<tr>
<td>1.9 x 10^9</td>
<td>Shi et al., 2007</td>
</tr>
</tbody>
</table>

Table 5.12: Specific activities recorded for different methods of purifying and refolding IFN-α2b. These values are similar to those achieved from the guanidine-HCl refolded samples and also the Cobra standard.

5.4: Ion exchange of the refolded IFN-α2b

Ion exchange was used as a final purification step following the refold. It was clear from the SDS-PAGE analysis that either there was contamination of a higher molecular weight or oligomerisation of the IFN-α2b. But also in the case of the refold where no oxidised glutathione was added the fluorescence of the guanidine-HCl solubilised samples differed from the IFN-α2b standard but the far UV CD gave a very successful result. In order to obtain pure refolded material especially from the refolds from guanidine-HCl ion exchange at pH 8.0 was used.
Ion exchange of the guanidine-HCl solubilised and refolded IFN-α2b

Ion exchange was used to purify the IFN-α2b from the refold without oxidised glutathione from guanidine-HCl solubilised inclusion bodies. The concentrations before and after the ion exchange can be seen in table 5.13.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting concentration post refold</td>
<td>0.14</td>
</tr>
<tr>
<td>Pool post ion exchange</td>
<td>0.025</td>
</tr>
<tr>
<td>Pool post ion exchange concentrated</td>
<td>0.075</td>
</tr>
</tbody>
</table>

Table 5.13: Concentrations of the pools before and after the ion exchange from a guanidine-HCl solubilised and purified inclusion bodies.

Figure 5.21: Ion exchange profile of guanidine-HCl solubilised sample refolded and then purified at pH 8.0. The spectrum shows some loss of protein in the flowthrough. The elution profile shows a prominent peak at 26 ml (NaCl concentration of ~300 mM NaCl), followed by a second peak at 32 ml. The peaks were analysed by fluorescence and far UV CD.
The ion exchange of the final sample from the refold can be seen in figure 5.21. The ion exchange was carried out at pH 8.0. The fractions collected from 20-40 ml were analysed by absorbance at 280 nm and pooled on the basis of their profile as with the initial gel filtration. The samples were too dilute to be analysed by SDS-PAGE. Once the pools had been made they were concentrated using a Vivaspin 5 kDa MWCO concentrator. The fractions used for the pool of the folded protein were made up of fractions taken at 25-29 ml (inclusive).

![Absorbance spectra](figure5_22.png)

**Figure 5.22: Absorbance spectra of the fractions collected during the ion exchange purification of the guanidine-HCl refolded IFN-α2b.** The fractions collected between 25 and 29 ml show similar absorbance spectra to the IFN-α2b standard. The fractions collected later around 30 ml and the flowthrough do not show absorbance spectra similar to the standard indicating purification of a further contaminant.

The fractions collected from the ion exchange at pH 8.0 of the guanidine-HCl refolded IFN-α2b were pooled on the basis of their A$_{280}$ spectra (figure 5.22). The absorbance spectra also highlight the similarities between the material which did
not bind to the resin and was lost in the flowthrough and the material which did bind but eluted later. The differences observed between the fractions at 26 ml and 30 ml indicate that the IFN-α2b has some residual contamination present and/or mis-folded IFN-α2b. Usually a polishing step would be incorporated in an industrial setting and the results here confirm the need for this further purification post refolding.

Intrinsic fluorescence analysis of the refolded and purified IFN-α2b from guanidine-HCl solubilised inclusion bodies.

![Graph](Image)

**Figure 5.23:** Fluorescence results of the ion purified IFN-α2b by ion exchange at pH 8.0, solubilised with guanidine-HCl and refolded. The barycentric mean results in the same wavelength as the IFN-α2b standard.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Barycentric Mean (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Refolded and purified IFN-α2b from guanidine-HCl</td>
<td>341.43</td>
</tr>
<tr>
<td>IFN-α2b standard</td>
<td>341.62</td>
</tr>
</tbody>
</table>

**Table 5.14:** Barycentric mean of the refolded and purified IFN-α2b from guanidine-HCl solubilised inclusion bodies.
The fluorescence emission in figure 5.23 shows tryptophan fluorescence and a barycentric mean which now matches the IFN-α2b standard, see table 5.14.

Far UV CD analysis of the refolded and purified IFN-α2b from guanidine-HCl solubilised inclusion bodies.

![Graph showing Far UV CD analysis](image)

**Figure 5.24: Far UV CD of the guanidine-HCl solubilised, refolded and purified IFN-α2b.** The sample exhibits alpha helical structure similar to that of the IFN-α2b standard suggesting the further purification of the IFN-α2b after refolding was successful.

The far UV CD in figure 5.24 shows alpha helical structure from the guanidine-HCl solubilised, refolded and purified by ion exchange IFN-α2b. The result is not an exact match as with the fluorescence but both show promising and successful purification of the IFN-α2b after refolding. The purification of the IFN-α2b solubilised inclusion bodies at high concentrations of urea by ion exchange was not particularly successful as the IFN-α2b did not appear to bind. This result shows that IFN-α2b does bind to the Q Sepharose™ resin at pH 8.0 providing yet more evidence that the high urea concentrations were the problem during the
initial purification of the IFN-α2b solubilised inclusion bodies, chapter 4, section 4.2.4.

To optimise conditions two further pH values were tested to investigate whether the ion exchange could be improved, possible eliminating any loss of IFN-α2b during in the flowthrough or improvement to the separation. These were carried out at pH 8.4 and 8.6. Once the refolding had ended the samples were dialysed into the appropriate buffers.

![Graph](image)

**Figure 5.25: Ion exchange of refolded guanidine-HCl samples at pH 8.4 and pH 8.6.**

The separation is not as efficient as the ion exchange at pH 8.0. It also shows more unfolded IFN-α2b contaminating the IFN-α2b which elutes at a lower NaCl concentration.

The ion exchange at pH 8.4 and 8.6 shown in figure 5.25 confirm pH 8.0 to be the best condition in which to purify IFN-α2b after refolding. The purification does not result in a clear separation but also increases the amount of protein lost in the flowthrough.
Urea ion exchange

As the ion exchange had been successful in purifying the guanidine-HCl solubilised and refolded inclusion bodies, the urea solubilised material was investigated also.

![Absorbance (mAu) and Conductivity (mSc/cm) graph]

**Figure 5.26**: Ion exchange profile of a refolded sample from urea solubilised inclusion bodies. The peak at 26 ml is present as with the guanidine-HCl solubilised and refolded IFN-α2b however the more dominant peak is at ~33 ml, also the separation of the two species is poor.

The ion exchange results of the urea solubilised and refolded IFN-α2b in figure 5.26 shows a similar separation to the guanidine-HCl solubilised and refolded IFN-α2b however the largest peak comes from the material which elutes later (33 ml). There is protein eluting around 26 ml which was where the correctly refolded IFN-α2b eluted from the guanidine-HCl material. This suggests that the material which elutes above 30 ml is mis-folded IFN-α2b and due to it being refolded from urea there is more mis-folded. The IFN-α2b was purified in the same ways as the guanidine-HCl refolded and purified IFN-α2b.
Figure 5.27: Fluorescence analysis of the ion exchange carried out on the urea refolded material. The fractions collected have a red shifted barycentric mean compared to the IFN-α2b standard whereas the flowthrough has a barycentric mean more blue shifted than the IFN-α2b standard.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Barycentric Mean (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fractions 20-26 ml</td>
<td>346</td>
</tr>
<tr>
<td>Fractions 30-37 ml</td>
<td>345</td>
</tr>
<tr>
<td>Flowthrough</td>
<td>338</td>
</tr>
<tr>
<td>IFN-α2b standard</td>
<td>341</td>
</tr>
</tbody>
</table>

Table 5.15: Barycentric mean of the urea solubilised inclusion bodies post refolding and purification by ion exchange.

The results from the ion exchange from urea solubilised material shows IFN-α2b which does not match the IFN-α2b standard. The fractions collected during the NaCl elution have barycentric means more red shifted than the standard (table
5.15) whereas the flowthrough appears to be blue shifted, see figure 5.27). This suggests the presence of both contamination and mis-folded IFN-α2b. The positive result is that the contamination does appear to be separated from the IFN-α2b in the flowthrough and even the partially and/or mis-folded species appear to elute later.

**Conclusions from the ion exchange**

The ion exchange of both the guanidine-HCl and urea solubilised and refolded material show a need for a purification step once the refold has ended. They both show some degree of contamination which luckily does not bind to the column and is lost in the flowthrough. Both purifications also show the separation of folded and mis-folded IFN-α2b. The guanidine-HCl purification shows that the first peak gives a fluorescence spectrum similar to that of the IFN-α2b standard and an equivalent far UV CD signal. The urea has the same two peaks but the second peak, the one deemed the mis-folded protein from the guanidine-HCl run, is larger indicating more mis-folded species. The first elution peak from the urea run does not give a barycentric mean of the same wavelength to the IFN-α2b standard and is red shifted compared to its original value without ion exchange. However, the mis-folded peak gives a very blue shifted barycentric mean which could have influenced the initial measurement before ion exchange and so the value of 346 nm is a more true value for the urea folded IFN-α2b. In order to improve on the separation (especially the urea) from the ion exchange instead of changing the pH of the running buffers the gradient could be optimised. Due to time restrictions this was not carried out.

**5.5: Investigation of the denatured state of IFN-α2b in solubilised inclusion bodies**

Due to large differences seen in the refolding of the urea and guanidine-HCl solubilised inclusion bodies, their analysis was further investigated. The guanidine-HCl solubilised inclusion bodies which were dialysed into urea were also investigated as they resulted in much better results than the urea solubilised inclusion bodies. The techniques used to analyse the inclusion bodies are ones which are widely available instead of the specialised methods such as NMR and
small angle X-ray scattering which are currently the most commonly used techniques to investigate the presence of structure in denatured proteins.

**Far and near UV CD analysis of the solubilised and purified inclusion bodies**

The far and near UV CD of the solubilised and purified inclusion bodies was investigated. The high concentrations of guanidine-HCl and urea may affect the far UV analysis but was investigated nonetheless.

![Graph](image)

**Figure 5.28: Far UV analysis of the inclusion bodies after solubilisation and purification by gel filtration.** The dialysed samples were initially solubilised in 6 M guanidine-HCl and then dialysed into 8 M urea with or without DTT. Both the dialysed samples (turquoise and pink lines) and the urea solubilised samples (green and blue lines) show some secondary structure which appears very different to the unfolded guanidine-HCl solubilised samples. Data ends before 200 nm due to 600 volts being exceeded due to the absorptive properties of guanidine-HCl and urea.

The far UV of the solubilised and purified inclusion bodies can be seen in figure 5.28. The far UV shows that there is a difference in the secondary structure of
the IFN-α2b under differing denaturant conditions. The most secondary structure appears to be in the samples solubilised in guanidine-HCl and then dialysed into urea. The solubilised urea samples also show some secondary structure, however the guanidine-HCl solubilised samples do not show any secondary structure.

Near UV CD of the solubilised and purified inclusion bodies

![Near UV CD](image)

**Figure 5.29:** Near UV analysis of the inclusion bodies after solubilisation and purification by gel filtration. The dialysed samples were initially solubilised in 6 M guanidine-HCl and then dialysed into 8 M urea with or without DTT. Both urea solubilised samples show some structure which appears very different to the unfolded guanidine-HCl solubilised samples and the dialysed samples.
**Protein concentrations for CD analysis**

<table>
<thead>
<tr>
<th>Sample</th>
<th>A280 Concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>0.32</td>
</tr>
<tr>
<td>Urea DTT</td>
<td>0.3</td>
</tr>
<tr>
<td>Guanidine-HCl</td>
<td>2.4</td>
</tr>
<tr>
<td>Guanidine-HCl DTT</td>
<td>3.8</td>
</tr>
<tr>
<td>Guanidine-HCl dialysed into urea</td>
<td>2.2</td>
</tr>
<tr>
<td>Guanidine-HCl DTT dialysed into urea DTT</td>
<td>2</td>
</tr>
</tbody>
</table>

*Table 5.16: Total protein concentrations of the samples used for far UV CD analysis.*

The near UV CD shown in figure 5.29 does not indicate any native-like tertiary structure in any of the six samples. The two urea solubilised samples do give a large positive signal between 260-290 nm however this is not characteristic of the native IFN-α2b near UV spectrum or the unfolded spectrum seen in figure 3.2. The protein concentrations at which the CD analysis was carried out are shown in table 5.16.
Intrinsic fluorescence analysis

Figure 5.30: Intrinsic fluorescence results of the solubilised inclusion bodies. The results show the difference the denaturant used for solubilisation causes to the fluorescence emission. The most denatured signal comes from the sample solubilised in guanidine-HCl with 1 mM DTT (red line). The sample solubilised in guanidine-HCl and then dialysed into urea (turquoise line) shows the most structure; being the most blue shifted of the samples.

Protein concentrations for fluorescence analysis

<table>
<thead>
<tr>
<th>Sample</th>
<th>A280 Concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>0.1</td>
</tr>
<tr>
<td>Urea DTT</td>
<td>0.11</td>
</tr>
<tr>
<td>Guanidine-HCl</td>
<td>0.11</td>
</tr>
<tr>
<td>Guanidine-HCl DTT</td>
<td>0.11</td>
</tr>
<tr>
<td>Guanidine-HCl dialysed into urea</td>
<td>0.093</td>
</tr>
<tr>
<td>Guanidine-HCl DTT dialysed into urea DTT</td>
<td>0.093</td>
</tr>
</tbody>
</table>

Table 5.17: Concentrations of the solubilised inclusion bodies used for fluorescence analysis.
Barycentric mean analysis of the intrinsic fluorescence

<table>
<thead>
<tr>
<th>Sample</th>
<th>Barycentric Mean (nm) 306-380 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>348</td>
</tr>
<tr>
<td>Urea DTT</td>
<td>348</td>
</tr>
<tr>
<td>Guanidine-HCl</td>
<td>349</td>
</tr>
<tr>
<td>Guanidine-HCl DTT</td>
<td>349</td>
</tr>
<tr>
<td>Guanidine-HCl dialysed into urea</td>
<td>346</td>
</tr>
<tr>
<td>Guanidine-HCl DTT dialysed into urea DTT</td>
<td>347</td>
</tr>
</tbody>
</table>

Table 5.18: Barycentric means of the solubilised inclusion bodies from intrinsic fluorescence analysis. The barycentric means confirms the guanidine-HCl solubilised samples dialysed into urea as showing the most structure.

The fluorescence results in figure 5.30 of the solubilised inclusion bodies all show tryptophan emission. The values for the barycentric means (table 5.18) are all similar to each to other ranging from 346-349 nm. The most blue shifted are the guanidine-HCl solubilised inclusion bodies dialysed into urea and the most red shifted are the guanidine-HCl solubilised samples. The concentrations at which the fluorescence analysis was carried out can be seen in table 5.17.
Results of the ANS binding to the solubilised inclusion bodies

The ANS was added to the above samples at a final concentration of 0.1 mM.

Figure 5.31: ANS fluorescence results of the solubilised inclusion bodies under six different conditions. The results show the difference the denaturant used for solubilisation causes to the fluorescence emission when bound to ANS. The sample with the smallest effect on ANS binding is the samples solubilised in guanidine-HCl, 1 mM DTT. The samples which give the most ANS binding are the two samples that were solubilised initially in guanidine-HCl with or without DTT and then dialysed into urea with or without DTT (turquoise and pink lines), indicating the presence of a molten globule type structure.

The ANS results in figure 5.31 indicated the presence of some structure in the inclusion body samples solubilised in guanidine-HCl and then dialysed into urea. Both the urea and guanidine-HCl solubilised inclusion bodies show similar binding and therefore structure even though there are great differences in their success at refolding. ANS binding results is similar shifts in wavelength (same as tryptophan) in that a blue shift indicates a more hydrophobic environment (Wang and Geng, 2006b).
N-(1-pyrene)maleimide labelling of IFN-α2b’s four cysteines.

N-(1-pyrene)maleimide is a fluorescent probe exhibiting monomer emission peaks at 375 nm and 395 nm when excited at ~345 nm, see figure 5.32. When the pyrene molecules are brought into close proximity, and are excited, they form excited state dimers (excimers) that emit at longer wavelengths usually around 470 nm (Betcher-Lange and Lehrer, 1978).

![Chemical structure of N-(1-pyrene)maleimide](image)

Figure 5.32: Chemical structure of N-(1-pyrene)maleimide. The four benzene rings are the pyrene moiety. Drawn using Chemwindow® 6.

Binding of the N-(1-pyrene)maleimide may give more indication of any structure present in the solubilised inclusion bodies. The N-(1-pyrene)maleimide binds to the cysteine residues elucidating if the structure is compact enough to bring together the cysteine residues therefore forming excimers.

The IFN-α2b samples were reduced using 10 mM TCEP. The N-(1-pyrene)maleimide was added to a 10 fold molar excess and the samples left for 16 h with agitation at room temperature. The excess N-(1-pyrene)maleimide was removed by PD-10 purification using buffers still containing TCEP (Sahoo et al., 2002, Drury and Narayanaswami, 2005). The resulting samples were analysed for their absorbance and fluorescence spectra.
The absorbance spectra shown in figure 5.33 was used to calculate the extent of the $N$-(1-pyrene)maleimide labelling. This was calculated by using the ratio of the peaks at 278 nm and 344 nm of the stock solution and the extinction coefficient of $N$-(1-pyrene)maleimide at 344 nm of $40,000 \text{ M}^{-1} \text{ cm}^{-1}$ (Drury and Narayanaswami, 2005).
Fluorescence analysis of the N-(1-pyrene)maleimide binding

The labelled samples were analysed by fluorescence to investigate the possibility of excimer fluorescence from the N-(1-pyrene)maleimide. The samples were excited at 338 nm and normalised due to concentration differences at 375 nm.

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**Figure 5.34: Fluorescence emission of the N-(1-pyrene)maleimide labelling of the solubilised inclusion bodies.** The samples were excited at 338 nm. The IFN-α2b standard shows excimer fluorescence at 490 nm. The samples solubilised in guanidine-HCl show signs of some excimer emission but it is not as prominent as the IFN-α2b standard.

There is a slight increase in the emission by the guanidine-HCl solubilised inclusion bodies along with the guanidine-HCl solubilised inclusion bodies dialysed into urea indicating that there may be some structure present, but is not sufficient enough to bring the cysteines together causing excimer emission as large as the IFN-α2b standard, see figure 5.34. Thus the partially folded or molten globule states observed are not native like.
SDS-PAGE analysis of the N-(1-pyrene)maleimide labelled samples

Figure 5.35: SDS-PAGE analysis of the labelled inclusion bodies with N-(1-pyrene)maleimide. Figure A: The fluorescently labelled IFN-α2b proteins can be seen in lanes 3, 5, 7 and 9. The molecular weights at 25 and 35 kDa fluoresce also. Figure B: The safe stained SDS-PAGE highlighting the IFN-α2b bands but also shows some dimerisation in all four samples. IFN-α2b standard loaded to 3 µg.

The SDS-PAGE shown in figure 5.35 confirms that all the samples were labelled with the N-(1-pyrene)maleimide.
Figure 5.36: N-(1-pyrene)maleimide fluorescence analysis of the labelled IFN-α2b standard subjected to 6 M guanidine-HCl. The excimer peak seen from the IFN-α2b standard around 490 nm appears to shift to the shorter wavelengths.

Figure 5.36 shows the disruption of the IFN-α2b molecule on addition of guanidine-HCl. This disruption can be seen by the change in pyrene emission indicating the cysteine residues are no longer in proximity to one another.

**Second derivative analysis of the IFN-α2b solubilised inclusion bodies**

Second derivative analysis was introduced to calculate the exposure of the tyrosine residues in the solubilised inclusion bodies. For this, the absorbance spectra of the solubilised inclusion bodies can be used. This technique has the ability to take the overlapping regions of the aromatic residues which bring about the absorbance spectra and separate them into their individual contributions (Ragone *et al.*, 1984).

For the calculation the absorbance spectrum of each sample was measured at similar concentrations, figure 5.37.
Figure 5.37: Absorbance spectra of the solubilised inclusion bodies used for second derivative analysis. All the samples have the same shape however the solubilised inclusion bodies (black, red and green lines) are shifted the shorter wavelengths in comparison to the folded standard (blue line).

The absorbance spectra were converted to their second order derivatives using Origin™ 7 software, the results can be seen in figure 5.38.

Figure 5.38: Second derivative spectra of the solubilised inclusion bodies. All samples give peaks between 287 – 283 nm and 295 – 290.5 nm
The calculation of the percentage of accessible tyrosine residues ($\alpha$) was calculated as described by Ragone et al. (1984) using the following equation 5.1.

$$\alpha = \frac{r_u - r_a}{r_n - r_a}$$  (5.1)

Where $r =$ the ratio between the peak to peak distance of 287 – 283 nm and 295 – 290.5 nm.

$r_n =$ ratio for the native IFN-α2b standard

$r_u =$ ratio for the sample being investigated

$r_a =$ ratio calculated from the Tyr/Trp ratio using the coefficient relative to ethylene glycol given by Ragone et al. (1984).

$r_a$ for IFN-α2b was calculated to be 0.21.

<table>
<thead>
<tr>
<th>Sample</th>
<th>A (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-α2b</td>
<td>39</td>
</tr>
<tr>
<td>Urea solubilised inclusion bodies</td>
<td>100</td>
</tr>
<tr>
<td>Guanidine-HCl solubilised inclusion bodies dialysed into urea</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 5.19: Values for the exposure ($\alpha$) of the tyrosine residues in the solubilised inclusion bodies. Both urea and guanidine samples show complete exposure of their tyrosine residues.

The exposure ($\alpha$) of the IFN-α2b standard was calculated to be 39 %, see table 5.19. The exposure of the urea and guanidine-HCl solubilised inclusion bodies solubilised in urea were over 100 %. These are based on the assumption that the tyrosine residues are completely exposed in the guanidine-HCl denatured sample. There appears to be a large difference between the samples and the IFN-α2b standard, highlighted in both figure 5.37 and 5.38. The IFN-α2b standard shown in figure 5.38 appears to be shifted to the longer wavelengths compared to the samples, which can also be seen in figure 5.33 of the $N$-(1-
pyrene)maleimide experiment, possibly indicating an environmental factor. The consequence of this is that the IFN-\(\alpha2b\) is shifted in figure 5.38. This may affect the calculation of exposure as the ratios are based on the peaks which from figure 5.38 are not all in the identical position.

**Conclusion from the analysis of the solubilised inclusion bodies**

The CD results showed some secondary structure present in the inclusion bodies solubilised in guanidine-HCl followed by dialysis into urea; this was confirmed by the intrinsic fluorescence and the ANS binding. These two samples (with and without DTT) showed fluorescence emission of a shorter wavelength than the other four samples solubilised in guanidine-HCl and urea only. This apparent structure may be due to some refolding of the IFN-\(\alpha2b\) taking place when transferred into the urea by dialysis. Dialysis is a slow process so the partial refolding of the IFN-\(\alpha2b\) may have taken place. The urea solubilised inclusion bodies do not appear to show any structure which was unexpected due to the large differences in the success of the refolding compared to the guanidine-HCl solubilised inclusion bodies. A possible explanation is that the urea solubilised inclusion bodies are locked in a specific unfolded structure which results in a pathway for unsuccessful refolding. The samples solubilised in guanidine-HCl and then dialysed into urea give better refolding results than the urea but not as successful as the inclusion bodies solely solubilised inclusion bodies in guanidine-HCl. The inclusion bodies solubilised in guanidine-HCl are assumed to be completely unfolded and are therefore able to refold successfully, the inclusion bodies then dialysed into urea may have started to refold during the dialysis and are also able to refold better than the urea solubilised inclusion bodies.

The \(N\)-(1-pyrene)maleimide results do not confirm any native like structure in any of the solubilised inclusion body samples. The samples do not appear to have the cysteines in close enough proximity to allow excimer emission, the pyrene molecules must be within 10 Å of each other (Sahoo *et al*., 2002). \(N\)-(1-pyrene)maleimide labelling has been used to investigate the inter- and intramolecular interactions of apolipoprotein E (apoE4), a lipoprotein (Drury and Naravanaswami. 2005). The protein was investigated as lipid free and lipid bound, by labelling of mutated residues for cysteines within specific domains of
the protein. Other proteins investigated include Apolipophorin III (Sahoo et al., 2002) and apomyoglobin (Wang and Geng, 2006b). N-(1-pyrene)maleimide is also used to investigate synthetic polymers due to the excimers long lifetime under non-polar conditions (Gelir et al., 2006), under these highly hydrophobic conditions the ratio of pyrenes emission peaks at 383 and 373 nm increase with increasing hydrophobicity (Gelir et al., 2006; Wang and Geng, 2006b).

The ANS binding showed emission from the guanidine-HCl dialysed into urea samples that may have molten globule-like structure due to the emission being blue shifted compared to the two samples solubilised in guanidine-HCl and urea. Molten globule structures are said to still contain a hydrophobic core but just not as closely packed as the native protein (Ptitsyn, 1992). The lack of definite secondary structure may indicate further that the partial structure formed is again not native.

Second derivative analysis has been used previously to characterise the partially unfolded states of β-lactoglobulin and interferon alpha-2a (Kumar et al., 2005). Instead of absorbance however the tryptophan fluorescence was investigated highlighting changes in the tertiary structure of folding intermediates. From these results the tryptophan residues in the urea solubilised inclusion bodies were calculated to be exposed however, this is based on the assumption that the tryptophan residues present in the guanidine-HCl solubilised inclusion bodies are also completely exposed to the solvent. The results here are inconclusive due to the shifting of the wavelength peaks however previous use of second derivative spectroscopy provides an efficient method of measuring small differences within samples without the need for high concentrations. IFN-α2b has been investigated for the presence of structure as inclusion bodies (Ami et al., 2006). To do this FTIR was used on IFN-α2b expressed using two different levels of expression, high and low. The high producer resulted in more aggregation however both producers showed signs of some native-like secondary structure around 1653 cm⁻¹ however both were shifted to a higher wavenumber compared to the native. The suggestion for this is the possibility of shorter alpha helical segments and/or more flexibility (Ami et al., 2006).
The analysis carried out on the solubilised inclusion bodies highlighted differences between the inclusion bodies solubilised in different denaturants. The implication of structure in these chemically denatured states may explain the differences observed in the refolding. Dill and Shortle, (1991) state that the characterisation of the denatured state is fundamentally important for understanding the proteins stability and folding. The denatured state has been investigated by various techniques; CD (Goto et al., 1990), DSC (Privalov, 1990), NMR (Jonas et al., 1998), small angle x-ray scattering (SAXS) (Konno et al., 1995; Kataoka et al., 1997) and FTIR (From and Bowler, 1998). Ding et al., (2005) devised a computational method to investigate thermally denatured proteins by molecular dynamics simulations. These experiments however tend to be investigating proteins which are not chemically denatured but in pH extremes or heat/cold treated, the chemical denaturants such as guanidine-HCl and urea are usually used as the control for the most unfolded state. The limitation of such techniques is the interference of the denaturants with the analysis due to their own absorptive properties. In order to obtain data for the unfolded sates of proteins from CD the increase in use of synchrotron radiation CD measurements has been introduced allowing data collection at wavelengths of 140 nm being achieved (Matsuo et al., 2007) however still carried out on non-chemically treated proteins.

The random coil was defined by Creighton (1993) as being the conformation of each part of the polypeptide chain which is assumed to be independent of the conformation of the remainder of the polypeptide and by Shortle in 1996 as being a well defined reference state in which no side chain interactions occur. The exploitation of the torsion angles of the peptide backbone has lead to calculation of all the possible conformations of the peptide, see figure 5.39.
In a folded protein the torsion angles have a single conformation and in an unfolded protein they have a distribution for each amino acid residue allowing many conformations (Smith et al., 1996a). The assumption is that the $\Phi$ and $\Psi$ of a random coil are independent of the other residues resulting in no non-local interactions. The values for the $\Phi$ and $\Psi$ torsion angles for each residue has been calculated based on synthetic peptides (Smith et al., 1996a) and are used for the calculation of the torsion angles in specific polypeptides when analysed by methods such as NMR (Smith et al., 1996b) and SAXS (Lattman, 1994). For these types of experiments the need for the two reference states is essential, the native state is required along with the unfolded. These calculations provide a model for the assumed random coil for the specific polypeptide to which the experimental data can be compared. NMR is used to elucidate local characteristics and SAXS is used for more global properties.

Residual structure has however been observed by Bu et al., (2001) using quasielastic neutron scattering (QENS) of $\alpha$-lactalbumin in 9 M urea. NMR has been used to elucidate structure of the 434-repressor in 7 M urea (Neri et al., 1992) and in 6 M guanidine-HCl GED, the assembly domain of dynamin has largely beta-structure measured by multidimensional NMR (Chugh et al., 2007). The occurrence of native like hydrophobic character of staphylococcal nuclease in 8 M urea has been detailed by Shortle and Ackerman, 2001 again using NMR. The resulting structure seen in these cases is not fully stable conformations but
residual structure of a dynamic nature. The techniques used however are specialised and not easily interpreted, the aim here was to use the techniques widely available which do not require high protein concentrations and long periods of time to run and analyse to gain some insight into whether structure is present in the assumed unfolded solubilised inclusion bodies. In this case there may indeed be some small amount of structure present but not necessarily native.

The second aspect influencing this unfolding is also the interaction of the denaturant with the peptide. The large difference observed during the refolding may be structure related and caused by the denaturant itself. Figure 1.5 shows the chemical structure of both urea and guanidine; they are very similar and yet result in very different refolding and unfolding yields of IFN-α2b. The interaction and efficiency of guanidine-HCl is usually focused on the salt effect caused by the HCl group. Urea however poses a different debate. One opinion is that urea interacts favourably with the nonpolar side chains (Nozaki and Tanford, 1963) and the second is that urea interacts with the peptide backbone (Auton et al., 2007; O'Brien et al., 2007). Both could also play a part.
Chapter 6
Chapter 6

Conclusions and Future Work

6.2: Conclusions
The unfolding of IFN-α2b carried out in chapter 3 gave an interesting insight into the behaviour of IFN-α2b in solution. The far and near UV CD was consistent with structural data (Radhakrishnan et al., 1996) but also with the similar study carried out by Beldarrain et al., (2001) who quote maximum stability of IFN-α2b between 0.3-0.5 M phosphate pH 7.0 giving a T_m of 65 °C whereas here the T_m was found to be 70 °C at 0.2 M phosphate pH 7.0. On reduction of IFN-α2b’s two disulphide bonds the protein remained intact, less stable, but still alpha helical, see figure 3.5. A change in pH from pH 7.0 to 9 and 5 shown in figure 3.23 also resulted in very little change in secondary structure. All these results confirmed the stability of IFN-α2b’s structure as a whole. It was however the denaturation of IFN-α2b which lead to unexpected results. The denaturation was carried out with increasing concentrations of guanidine-HCl and urea giving the first insight into the differing behaviour of IFN-α2b during unfolding. The denaturant curves in section 3.2.4 from guanidine-HCl unfolding resulted in cooperative unfolding and the addition of DTT having a destabilising effect on the IFN-α2b molecule. It was the results from the urea denaturation which led to unsuccessful non-cooperative unfolding of IFN-α2b. The data gained from urea unfolding could not therefore be used for any stability determination IFN-α2b.

Once the expression of the IFN-α2b had been initiated and was working efficiently, the solubilisation of the inclusion bodies with both urea and guanidine-HCl was investigated. This was a consequence of the observed differences in unfolding of the IFN-α2b standard, by these two denaturants. As with the unfolding experiments urea was found to be the poorest denaturant, this was shown by the SDS-PAGE analysis in figure 4.9. The resulting solubilised inclusion bodies in guanidine-HCl were however dialysed into urea for anionic ion exchange purification. This purification detailed in
Table 4.1 was unsuccessful and the high concentrations of urea, keeping the IFN-α2b soluble was to blame. The consequent refolding from these solubilisation studies were carried through a refold protocol which resulted in little success due to the interference of Triton X-100 used during the washing of the inclusion bodies. A different method of purification was introduced in section 4.24; gel filtration. This enabled the guanidine-HCl solubilised inclusion bodies to be purified without coming into contact with urea. The refolding from this step was successful and resulted in folded IFN-α2b with a functional activity similar to NIBSC provided WHO international biological reference standard of IFN-α2b (95/566), see section 5.3. The refolding, however successful was only true of the guanidine-HCl solubilised inclusion bodies, the urea solubilised inclusion bodies resulted in activities which were lower than the guanidine-HCl solubilised and both Cobra Biomanufacturing provided standard and the NIBSC reference material.

The differences between the refolding success of the guanidine-HCl and urea solubilised samples led to the investigation of the IFN-α2b solubilised inclusion bodies in both urea and guanidine-HCl, section 5.5. The techniques used were widely available and did not require the high protein concentrations need for techniques such as NMR. The results implicated structure in the urea solubilised samples but not necessarily native. The inclusion bodies which were solubilised in guanidine-HCl and then dialysed into urea showed the most structure out of all the variants, giving both high intensity and blue shifted fluorescence emissions (5.30) but also signal in the far UV region (figure 5.29).

The overall aim of this project was to devise a successful and robust refolding method of IFN-α2b. The refolding of IFN-α2b was successful when the inclusion bodies had been solubilised in guanidine-HCl not urea. The solubilisation of IFN-α2b inclusion bodies with urea is common (Srivastava et al., 2005), this solubilisation is usually followed by ion exchange purification which requires urea rather than guanidine-HCl. However in chapter 4, section 4.2.4 it was found that ion exchange was not successful with urea and so gel filtration became the preferred method of purification. The refold itself was
kept simple using a dilution step followed by two dialysis stages; the first removing the denaturant and the second removing the reducing agent. Valente et al., (2006) also refolded IFN-α2b using a simple two step dialysis, this however was carried out over 9 days. The refold here was carried out over 2 days. In conclusion the refolding method devised was successful and the information gained during the structural analysis of both the folded IFN-α2b (chapter 3, section 3.2.1) and the solubilised inclusion bodies (chapter 5, section 5.5) enabled conclusions to be drawn as to why the refolding had not been successful from urea solubilised inclusion bodies.

6.2: Future Work

The unfolding analysis carried out on the IFN-α2b standard was sufficient enough to provide guidelines for a refolding protocol to be formed. The refolding carried out supported the idea of primarily refolding the majority of both the secondary and tertiary structure under reducing conditions and forming the disulphides during the late stages. For Biomanufacturing the issues of scale up are of great importance and have hopefully been addressed here. The dialysis stages can be replaced with diafiltration coupled with a concentration step. This would reduce both incubation times and volumes however the temperature of 4 °C for this to be carried out may be problematic. The 1 in 10 dilution may also be difficult to overcome but could possibly be achieved by a diafiltration step using a buffer containing low concentrations of denaturant.

The use of guanidine-HCl is not favoured widely by biomanufacturing due to the use of stainless steel tanks during manufacturing, these being susceptible to corrosion by the chloride ions. The washing of the inclusion bodies should therefore be investigated. There is potentially a case for urea to be used during the washing stages due to it having a negative effect on the solubilisation of the IFN-α2b. Pressure could also be investigated as it does not always require urea or guanidine-HCl.

Following the successful refold a polishing step by ion exchange was briefly investigated, firstly to confirm whether the high levels of urea had interfered
with the previous runs but also to further purify the IFN-α2b. This appeared to be successful at pH 8.0 resulting in impurities being lost in the flowthrough and either impurities or mis-folded IFN-α2b eluting later than the correctly folded. This step could be further investigated by increasing the length of the gradient or even incorporating a step elution. With more material the results could be analysed by SDS-PAGE confirming the nature of the impurities but also the purification of the correctly folded IFN-α2b.
References
References


Shortle, D. (1996). The denatured state (the other half of the protein folding equation) and its role in stability. FASEB J., 10, 27-34.


## Appendices

### Appendix 1. Reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Manufacturer</th>
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## Appendix 2. Equipment

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