Disulphide bond formation of nascent proteins within the endoplasmic reticulum

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Abstract

Disulphide bonds form within the endoplasmic reticulum (ER) during the oxidative folding of secretory and membrane proteins. These bonds confer structural stability, bind the subunits of multimeric proteins and can be involved in redox dependent activities.

Our aim was to investigate the biochemical and molecular aspects of ER disulphide formation of nascent proteins. To specifically analyse this process, we developed a modified rabbit reticulocyte lysate (RRL) *in vitro* translation system. Glucose-6-phosphate (G6P) was added to this lysate and was found to prevent the formation of disulphides out with a source of ER by biochemically supporting the thioredoxin reductive pathway. Disulphides were, however, able to form within the ER sources of dog pancreas microsomes (DPMs) or semi permeabilised (SP) cells supplemented to the lysate. Furthermore, the disulphides that formed within these sources closely resembled disulphide formation in cells that has been reported from a pulse-chase study. The G6P supported RRL was thus shown to be a physiologically relevant system for analysing ER disulphide formation.

The enzymatic sources of *de novo* disulphide formation were then investigated by analysing disulphide formation within preprolactin (PPL) in a lysate supplemented with DPMs in which selected enzymes involved in this *de novo* synthesis were inhibited or chemically inactivated. It was found that inhibition of Ero1, but not VKOR caused substantial impairment of disulphide formation if the DPMs were first subjected to a brief reductive challenge. This finding reinforces the perception that Ero1 is a key generator of disulphides but suggest that alternative pathways of PDI oxidation can compensate for its loss. VKOR has been shown previously to contribute to disulphide formation within albumin in human hepatoma cells. The lack of observed contribution to PPL disulphide formation in DPMs therefore suggests that VKOR activity may be limited to particular cell types or substrates.

We also investigated the interaction between PDI and nascent PPL during and following translocation of the protein into the ER. This was assessed by translating PPL, or a translationally stalled PPL, in the presence of SP cells over-expressing a substrate trapping PDI mutant. The translationally stalled PPL was only partially exposed to the ER and thus resembled a partially translocated state. PDI was found to interact with both stalled and non-stalled PPL however the nature of the interaction appeared to be different. These findings suggest the oxidative folding of nascent secretory proteins begins during translocation and that different forms of interaction with PDI may arise during or following translocation into the ER.

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Acknowledgments

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Abbreviations

ATCA: Aurintricarboxylic acid

ATF6: Activating transcription factor 6

BiP: Immunoglobulin heavy-chain binding protein

BPTI: Basic pancreatic trypsin inhibitor

BSA: Bovine serum albumin

CNX: Calnexin

CFPS: Cell-free protein synthesis

CGN: Cis golgi network

CRT: Calreticulin

cVIMP-Cys: Recombinant protein of VIMP containing only the cytosolic region.

DB-PL: Intrachain disulphide bonded prolactin

DB-PPL: Intrachain disulphide bonded preprolactin

DHEA: Dehydroepiandrosterone

DMEM: Dulbecco's modified eagle's medium

DMOS: Dimethyl sulfoxide

DPM: Dog pancreas microsomes

DTT: Dithiothreitol

ECM: Extracellular matrix

ER: Endoplasmic reticulum

ERAD: ER associated degradation

Ero1: Endoplasmic reticulum oxidoreductase 1

G6P: Glucose-6-phosphate

G6PD: Glucose-6-phosphate dehydrogenase

GPx: Glutathione peroxidise

GR: Glutathione reductase

GSH: Reduced glutathione

GSSH: Glutathione disulphide

HA: Haemagglutinin

hVIMP: Human variant of VIMP protein

ICDB PL: Interchain disulphide bonded prolactin

IRE1: Inositol requiring enzyme 1 α

IT1: Haemagglutinin disulphide bonding intermediates 1

IT2: Haemagglutinin disulphide bonding intermediates 2

IT3: Haemagglutinin disulphide bonding intermediates 3

LDLR: Low density lipoprotein receptor

Msr: Methionine sulphoxide

NEF: Nucleotide exchange factor

NEM: N-ethylmaleimide

NT: Native fully oxidized haemagglutinin

OST: Ogosaccharyltransferase

PDI: Protein disulphide isomerise

PERK: pancreatic ER kinase

PPI: Peptidyl prolyl isomerases

PPL: Preprolactin

PL: Prolactin

Prx: Peroxiredoxin

PrxIV: Peroxiredoxin IV

QSOX: Quiescin sulfhydryl oxidase

RER: Rough endoplasmic reticulum

RNR: Ribonucleotide reductase

ROS: Reactive oxygen species

RRL: Rabbit reticulocyte lysate

SER: Smooth endoplasmic reticulum

SP: Semi-permeabilised

SRP: Signal recognition particle

stPDI: Substrate trapping PDI mutant

TGN: Trans golgi network

Trx: Thioredoxin

TrxR: Thioredoxin Reductase

tsPL: Translationally stalled prolactin

tsPPL: Translationally stalled preprolactin

UPR: unfolded protein response

VIMP: VCP-interacting membrane protein

VKOR: Vitamin K epoxide reductase

wtPDI: Wild type PDI

Author's declaration

I declare that, except where explicit reference is made to the contribution of others, that this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Greg Poet

September 2015

Chapter 1: Introduction

1.1 The Secretory Pathway

The secretory pathway is the name given to the specific route that secretory and membrane proteins of eukaryotic cells transverse during their trafficking to the outside of the cell (Karp, 2008). The proteins targeted for the pathway comprise a substantial proportion of the cellular proteome. It has been predicted from sequence analysis that 11% of the proteome are soluble secreted proteins and a further 20% are membrane proteins which are targeted to this pathway (Kanapin et al, 2003). The pathway contains two major cellular organelles, namely the endoplasmic reticulum and the Golgi complex (Fig 1.1). The nascent proteins undergo a range of post-translational modifications as they travel through these organelles, allowing the proteins to develop into their mature forms before either being secreted from the cell or embedded in cellular membranes (Lodish, 2008).

1.1.1 The endoplasmic reticulum

The endoplasmic reticulum (ER) is the first organelle of the secretory pathway. The ER consists of a single continuous membrane which forms a network of flattened enclosed sheets and tubules. These sheets and tubules form layers within the cell (Fig 1.1) which are known as cisternae. The ER is continuous with the nuclear envelope and regions are also closely associated with the plasma membrane as well as other cellular organelles including the Golgi apparatus, vacuoles, mitochondria, peroxisomes, late endosomes and lysosomes (Voeltz et al, 2002).

There are two major structurally and functionally distinct regions of endoplasmic reticulum known as the rough (R) and smooth (S) ER. The most obvious structural difference between the two is the presence of ribosomes on the outer surface of RER membranes but not SER. The cisternae of the SER is also more convoluted than that of the RER while the RER is more granular in texture (Voeltz et al, 2002). Both the SER and RER membranes have a thickness of 6nm (Sadava, 1993) and the cisternae space typically has a diameter of 50nm in animal cells (Westrate et al, 2015).

The primary function of the RER is the maturation of membrane and secretory proteins. This process involves multiple post-translational modifications that occur within the lumen of the organelle. This includes disulphide bond formation, N-linked glycosylation and proline hydroxylation (Lodish, 2008). The SER has a wide range of functional roles that includes drug detoxification, lipid and steroid synthesis and calcium storage and signalling (Voeltz et al, 2002).

The ER provides an environment that is both chemically and enzymatically distinct from the cytosol to facilitate its functions. Notably, the ER is prevalent in a specific subset of enzymes involved in the folding and maturation of secretory proteins. This includes the PDI

oxidoreductase family, BiP chaperone, and the calnexin and calreticulin lectins. In addition, the cellular buffer glutathione is far more oxidised in the ER than the cytosol allowing these enzymes to function optimally for the process of disulphide bond formation (Oka & Bulleid, 2013).

The cellular abundance of RER and SER correlates with the cellular functions. All cells contain at least a discernible amount of RER for the production of basal plasma membrane proteins as well as secreted and extracellular matrix proteins. However the RER is extensive in cell types specialised in the production of secretory proteins such as the cells of the pancreatic islets of Langerhans which synthesize insulin and glucagon (Lodish, 2008). The SER is only abundant in particular cells types such as liver cells and muscle cells. In liver cells extensive SER is required for chemical detoxification processes while muscles cells contain a special type of SER called sarcoplasmic reticulum that is involved in the regulation of intracellular calcium concentrations for the processes of muscle contraction (Voeltz et al, 2002).

1.1.2 Golgi Apparatus

The Golgi apparatus is the second organelle of the eukaryotic secretory pathway. The Golgi has a distinct morphology which is composed of a series of flattened cisternae with dilated rims that are arranged in orderly stacks. These cisternae are also surrounded by vesicles that are also considered part of the organelle. The cisternae of the Golgi apparatus are larger than that of the ER with diameters typically in the range of 0.5 to 1.0µm. Different cell types differ widely in the abundance of the Golgi. Some contain only a few Golgi stacks while others may contain as many as several thousand stacks (Karp, 2008; Lodish, 2008).

There are several functionally distinct types of cisternae in the Golgi apparatus. The cisternae of each Golgi stack are spatial ordered. The cisternae that are closest to the face of the ER and which receive incoming proteins are known as the *cis* cisternae. The cisternae on the exit face of the Golgi stacks are known as the *trans* cisternae and the cisternae that residue between these two groups are known as the *medial* cisternae. Each of these types of cisternae contains different luminal enzymes involved in modifying the maturing secretory and membrane proteins. The maturing proteins progress through each of these cisternae types as they travel through the Golgi apparatus and are modified by the glycosidase and glycosyl transferase enzymes resident at each cisternae. Each protein is modified differently by these enzymes in a manner depending upon the proteins structure and final destination (Karp, 2008; Lodish, 2008).

Two sorting stations composed of vesicles and tubules reside on either side of the cisternae stacks. The vesicles and tubules next to the *cis* face is known as the *cis Golgi network*

(CGN) while those next to the trans side are known as the *trans Golgi network* (TGN). The CGN is involved in the transport of proteins from the ER to Golgi by anterograde (forward-moving) transport vesicles. It is also involved in returning ER components that have been localised to the Golgi via retrograde (backwards-moving) transport vesicles. (Karp, 2008)

The TGN contains three different types of vesicles for transport of proteins from the secretory pathway. These three types are termed exocytotic, secretory and lysosomal vesicles. Exocytotic vesicles are involved in the constitutive secretory pathway and immediately move towards and fuse with the plasma membrane. Secretory vesicles are involved in the regulated secretion process. These vesicles bud off but are not secreted until a particular signalling mechanism is activated. The secretory vesicles that control insulin secretion function in this way. Lysosomal vesicles contain digestive enzymes or other proteins targeted for degradation. These vesicles are first targeted to the late endosome before being transferred to the lysosome. In addition to these functions, the TGN also functions as an acceptor region of endosomal traffic (De Matteis & Luini, 2008; Karp, 2008; Lodish, 2008).

Further to the N-linked glycosylation that occurs in the ER, proteins processed within the Golgi also undergo O-linked glycosylation. This glycosylation is name O-linked because the glycans are attached to the proteins at the hydroxyl (OH) side chain groups of serine and threonine amino acids residues within the target proteins. O-linked glycans are smaller than N-linked glycans and typically contain between one and four sugar residues. Both the N and O-linked glycan groups undergo a diverse range of editing within the Golgi apparatus resulting in a wide range of glycan arrangements in the mature secreted proteins. O-linked glycosylation has a diverse range of functional roles and is involved in processes such as protein localisation and trafficking, recognition of membrane antigens and cell to cell interactions (Karp, 2008; Lodish, 2008).

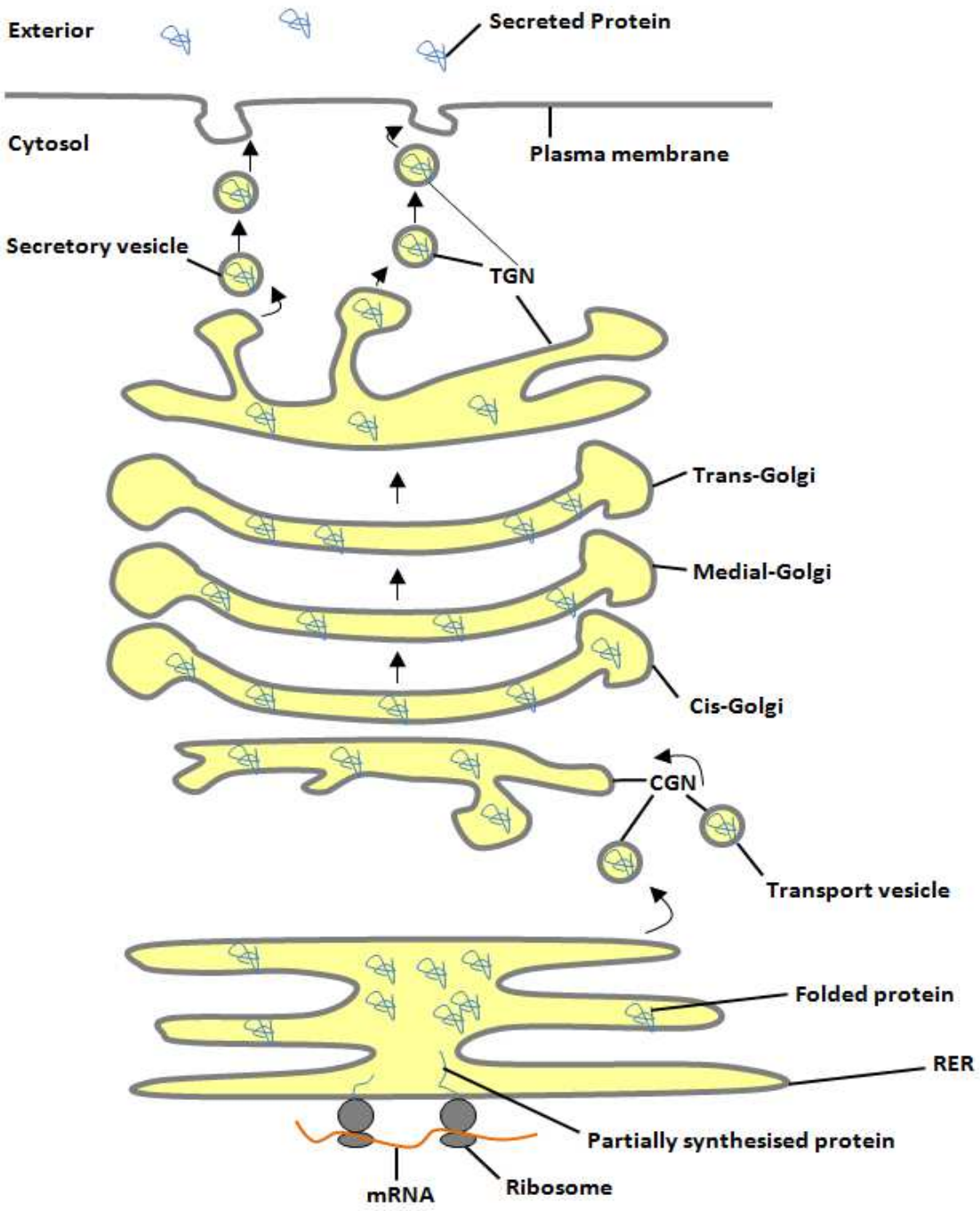

Figure 1.1: The secretory pathway. Nascent secretory and membrane protein are modified within the ER and Golgi apparatus. Transport to and from these organelles is mediated by cellular vesicles. The above figure was adapted from a figure originally shown by Lodish (Lodish, 2000).

1.2 Translocation to the ER

The translocation of nascent membrane and secretory proteins to the ER is directed by a signal sequence which is usually located at the N-terminus of these proteins. The signal sequence is generally between 16 and 30 amino acids in length. This sequence is predominately composed of hydrophobic amino acids, but also contains one or more positively charged residues. A cytosolic ribonucleoprotein called the signal recognition particle (SRP) binds the signal sequence and directs the ribosome/nascent protein complex to the Sec61 translocon channel located in the ER membrane (Fig 1. 2). A cycle of GTP binding and hydrolysis drives the docking and dissociation of the ribosome, SRP and Sec61. The ribosome/nascent protein complex is initially docked and bound to the Sec61 channel by interactions between SRP and Sec61. Subsequently, interactions between the ribosome and Sec61 then bind the two components. The nascent protein then enters and the channel and translocates across the membrane and into the ER. The signal sequence is cleaved from the protein during entry by an ER membrane enzyme called signal peptidase. These translocation events occur while the nascent protein is being synthesised on the ribosome and as such the process is referred to as co-translational translocation (Lodish, 2008; Rapoport, 2008).

There also exists an alternative pathway of translocation to the ER within eukarytoes which occurs post-translationally. This pathway involves an ER membrane channel containing Sec62/63 and may also involve the Sec 61 protein. Translocation is mediated by a ratcheting mechanism with the ER luminal protein BiP serving as the ratchet (Rapoport, 2008). It should be noted however that the majority of secretory and membrane proteins are translocated to the ER by the co-translational pathway (Jan et al, 2014).

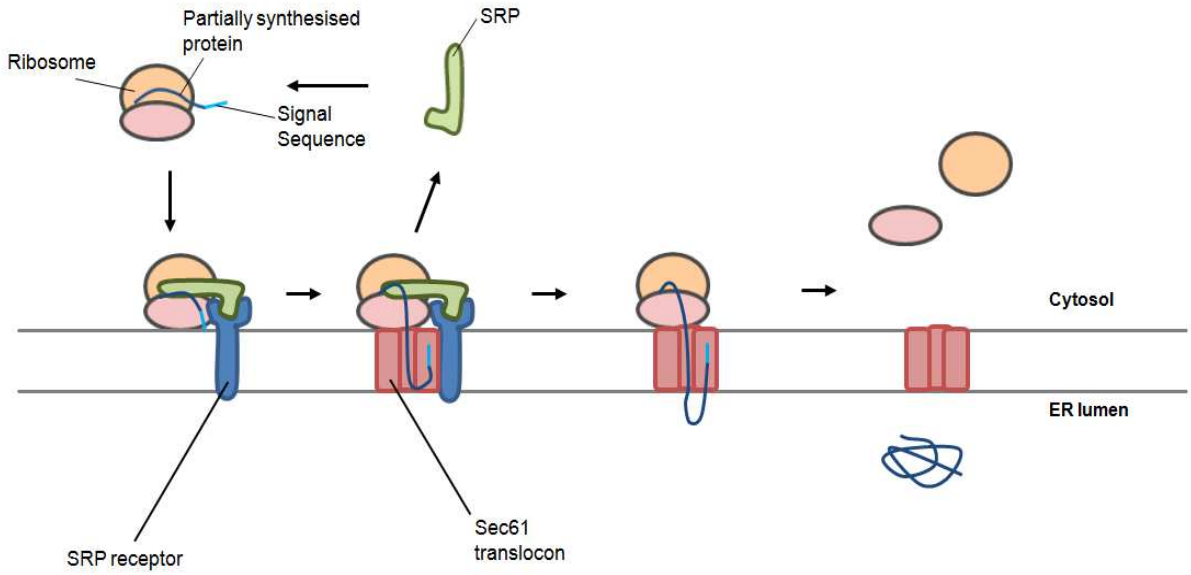

Figure 1.2: Co-translational translocation of nascent proteins to the ER. Co-translational translocation is mediated by SRP binding and docking the nascent protein to the SRP receptor of the ER membrane. This figure was adapated from a figure shown by Rapoport (Rapoport, 2007).

1.3 N-linked glycosylation

N-linked glycosylation is a prominent ER post-translational modification which involves the addition of carbohydrate groups to the amide (NH) group of certain asparagine side chains. This modification occurs to the vast majority of secretory and membrane proteins. N-linked glycosylation has several functions; it promotes proper folding and confers structurally stability and enhances protein solubility. Membrane proteins expressing glycans can also act as antigens and/or be involved in cell-to-cell adhesion (Aebi et al, 2010; Lodish, 2008).

N-linked oligosaccharide synthesis beings with the formation of an oligosaccharide precursor on the cytosolic surface of the rough ER membrane (Fig 1.3). This precursor forms upon a dolichol phospholipid located in the ER membrane. Dolichol phosphate phospholipids are strongly hydrophobic. The precursor is composed of glucose, mannose and N-acetyl glucosamine sugar residues which are attached to the dolichol phosphate by a pyrophosphate linkage. After the addition of the first 7 sugar residues the oligosaccharide precursor is flipped to face the luminal side of the ER membrane before a further 7 sugar residues are added. The oligosaccharide may then be transferred from the dolichol carrier to asparagine residues of a nascent protein entering the ER lumen. This transfer reaction is catalysed by an ER membrane protein complex called oligosaccharyltransferase (OST). The oligosaccharide may only be attached to asparagines residues in a three amino acid consensus sequence consisting of of Asn-X-Ser or Asn-X-Thr (where X is any non-proline amino acid). Not all of these consensus sites are glycosylated as rapid folding of protein segments containing these amino acid sequences can prevent their glycosylation.

Addition of the completed oligosaccharide presents a site in which the ER lectin chaperones chaperones calnexin (CNX) and calreticulin (CRT) can bind and assist in folding of the protein. These two chaperones bind to glycan groups in only one of the three terminal glucose residues are present. Removal of this final glucose residue causes release from these lectins. The removal of these three residues and the readdition of the third most terminal residues are catalysed by α-glucosidase I and II and UGT1. The nascent protein exists in a flux state that may pass through cycles of CNX/CRT binding during the folding process (Aebi et al, 2010; Lodish, 2008; Tannous et al, 2014).

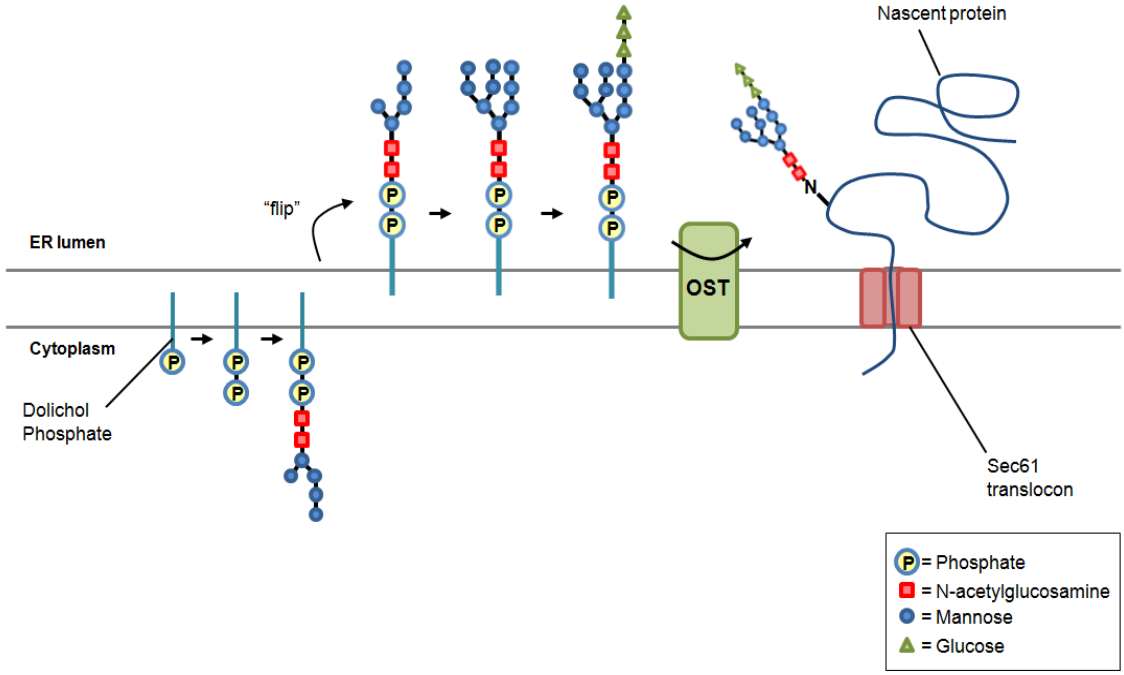

Figure 1.3: Synthesis and addition of N-glycans to nascent proteins. The figure shown was adapated from a figure shown by Lodish (Lodish, 2008) and a figure on the website of Prof William Clemons (California Institute of Technology) (http://clemonslab.caltech.edu/n-linked-glycosylation.html).

1.4 Protein folding and quality control in the ER

The folding of secretory protein begins as soon as they are synthesised. Initially folding within the ribosome and Sec61 translocon only allows the adoption of α-helical conformations as the space available within tunnels of these structures is limited. The range of potential conformations greatly expands upon entry of the secretory proteins to the ER lumen (Braakman & Bulleid, 2011).

Within the ER lumen protein folding is assisted by folding factors and chaperones. This includes the PDI oxidioreductases, peptidyl prolyl isomerases, calnexin and calreticulin and BiP. The protein disulphide isomerises (PDIs) are a family of enzymes that catalyse the formation, removal and rearrangement of disulphide bonds in nascent substrates. The peptidyl prolyl isomerases (PPIs) catalyse the rotation of peptidyl bonds of proline residues between *cis* and *trans* conformations (Fig 1.4). There are eight PPIs resident in the ER. Both the formation of disulphides and the rotation of proline peptidyl bonds are rate-limiting steps in protein folding and as such these enzymes greatly accelerate the folding process. Calnexin/calreticulin and BiP are key enzymes of the two primary chaperone systems of the ER. Calnexin and calreticulin are lectins (carbohydrate binding proteins) that bind to the glycan groups of unfolded proteins. BiP (immunoglobulin heavy-chain binding protein) on the other hand is an Hsp70 chaperone that binds to hydrophobic regions of proteins in an ATP dependent manner (Behnke et al, 2015; Tannous et al, 2014).

The folding factors and chaperones of the ER act in combination to facilitate the correct folding of nascent proteins within the ER. However, as protein folding is a stochastic process correct folding does not always occur. As such, the ER contains machinery to distinguish between correctly folded and misfolded proteins and to process these proteins accordingly. Proteins that fold correctly may progress to the Golgi by anterograde transport. Misfolded proteins are sequestered within the ER by interactions with chaperones and are ultimately degraded if native folding cannot be achieved (Fig 1.5) (Braakman & Bulleid, 2011; Tannous et al, 2014).

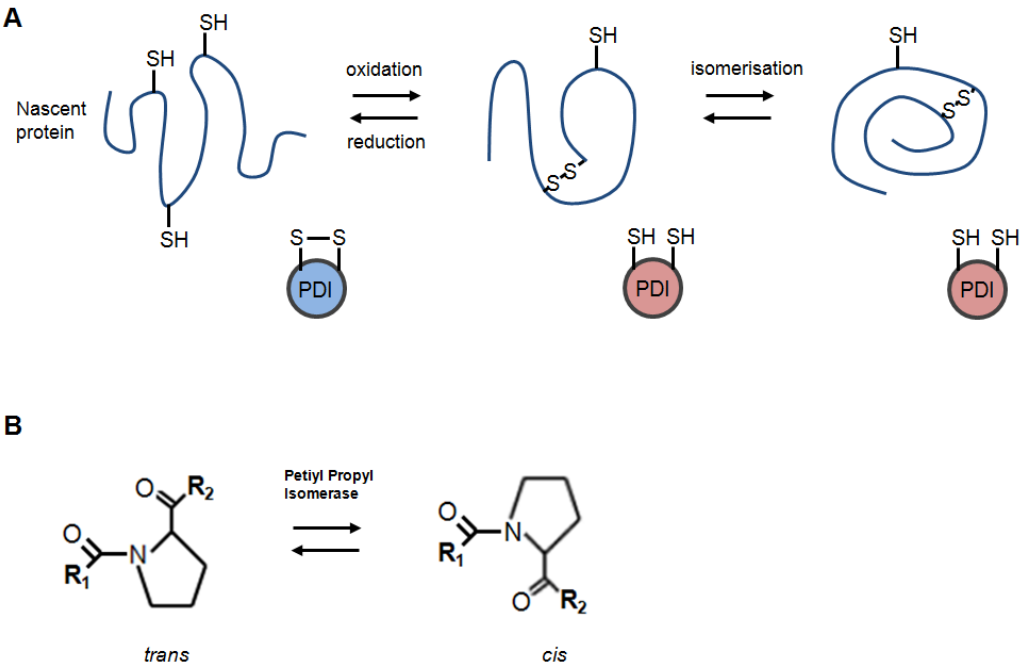

Figure 1.4: Disulphide formation and proly-peptidyl rotation. The rate-limiting steps of disulphide bond formation **(A)** and rotation or peptidyl bonds of proline residues **(B)** are catalysed by the PDI family and PPI family respectively. This figure was adapated from a figure by Tannous (Tannous et al, 2014).

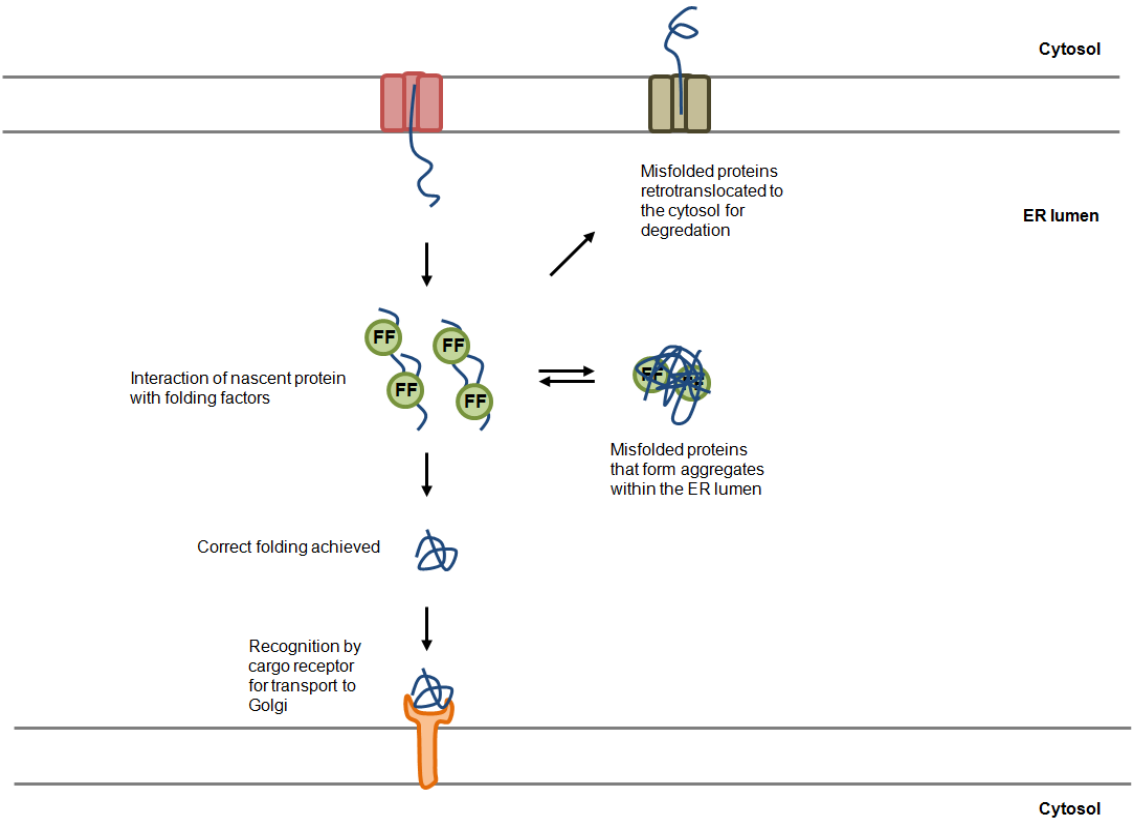

Figure 1.5: Pathways of protein folding and misfolding in the ER. Proteins that fold correctly are recognised by ER cargo receptor proteins and are transported to the Golgi in anterograde vesicles. Misfolded proteins are either exported from the ER to be degraded in the proteosome or may form aggregates which are later destroyed in autophagic processes. This figure was adapated from a figure by Braakman and Bulleid (Braakman & Bulleid, 2011).

1.4.1 Calnexin/Calreticulin Cycle

Calnexin (CNX) and calreticulin (CRT) bind to the newly synthesised glycan groups shortly after their addition to nascent proteins (Fig 1.6). The binding of these two lectins to nascent glycoproteins is dependent upon the number of terminal glucose residues exposed on each glycan group. The use of this glycan code allows CNX and CRT to distinguish between proteins that have fully folded and those which are unfolded or misfolded (Tannous et al, 2014).

Binding of CNX and CRT has several functional purposes. Firstly it influences protein folding and prevents aggregation by shielding regions of the unfolded protein. Secondly, CNX and CRT are also associated with ERp57 (a PDI family member) and as such binding of these lectins facilitates interaction of the nascent protein with this enzyme. This binding allows ERp57 mediated disulphide formation and rearrangement of disulphides to occur. And

thirdly, CNX and CRT sequester nascent proteins that have not completed folding within the ER (Tamura et al, 2010).

Proteins that become terminally misfolded eventually exit the CNX/CRT cycle by undergoing trimming of mannose residues from their glycans. This mannose trimming identifies these proteins as targets for degradation (Benyair et al, 2015).

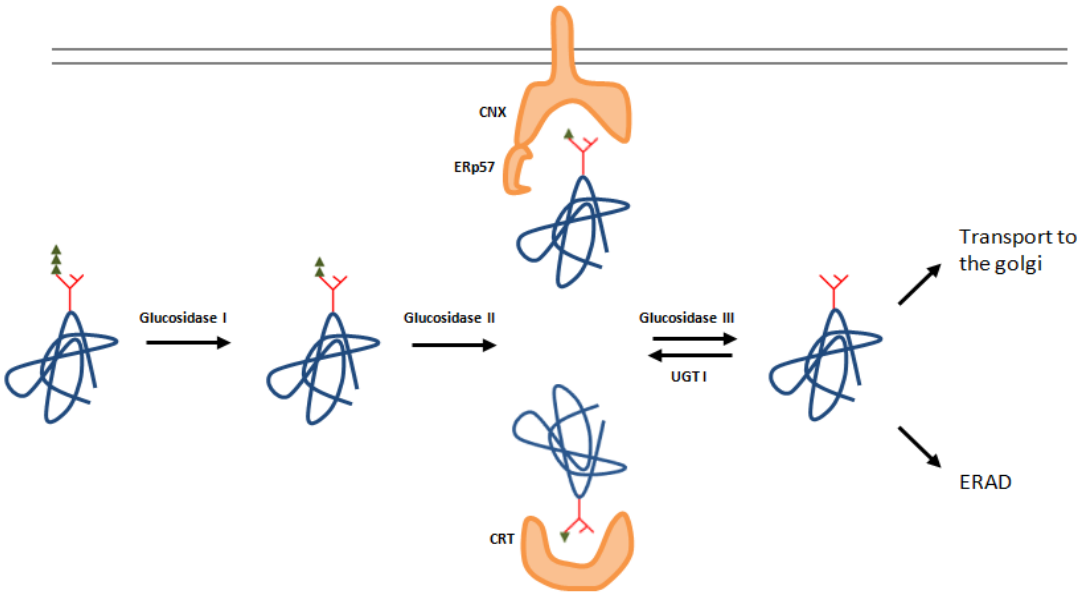

Figure 1.6: The calnexin/calreticulin cycle. The terminal two glucose residues of glycans are removed by glucosidases I and II which allows binding of CNX and CRT. Cleavage of the third terminal glucose reside by glucosidase II causes release from the lectins. UGT1 can however reattach glucose reside to glycan groups near exposed hydrophobic regions. This causes rebinding of CNX and CRT to proteins that have not achieved native folding. This figure was adapated and modified from a figure by Tamura *et al* (Tamura et al, 2010).

1.4.2 BiP chaperone system

Similarly to CNX and CRT BiP binds to nascent or unfolded proteins but unlike these lectin chaperones BiP does not bind to proteins via the glycan groups but rather binds to hydrophobic regions of the folding polypeptide. BiP contains a substrate binding domain and a nucleotide binding domain which are joined via a linker sequence. The activity of BiP is regulated by a cycle of ATP binding, hydrolysis and nucleotide exchange and also interaction with co-factors (Fig 1.7) (Behnke et al, 2015)

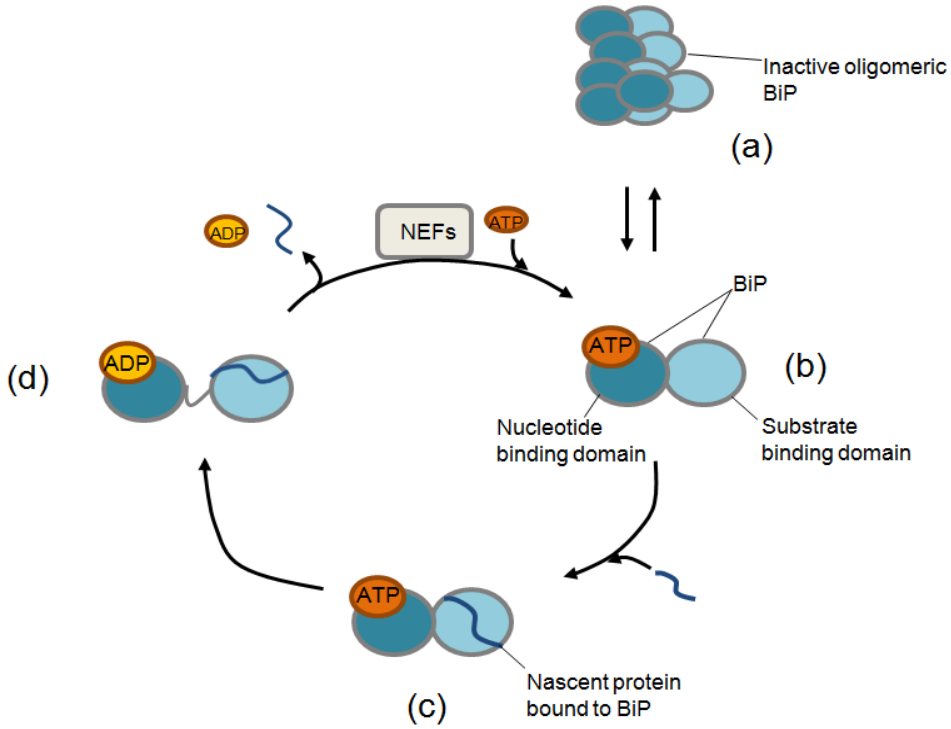

Figure 1.7: BiP interaction with unfolded protein substrates. (a) When levels of unfolded protein are low a large proportion of BiP exists as inactive oligomers that are ADP-ribosylated and phosphorylated. **(b)** In the presence of its substrates BiP becomes ATP bound and induced into a monomeric active state. The substrate binding domain adopts an open conformation allowing interaction with the substrate. **(c)** Substrate proteins are targeted to BiP from multiple co-chaperone enzymes that contain a DnaJ domain. **(d)** Hydrolysis of ATP to ADP causes BiP to enter a more stable bound state with its substrate. The conformation adopted by BiP in this state differs between elongated and globular substrates. Exchange of ADP for ATP causes release of the substrate and allows BiP to become available for subsequent rounds of substrate binding. The exchange of ADP for ATP is facilitated by the nucleotide exchange factors (NEFs) Grp170 and Sil1. Note that the substrate binding domain of BiP is shown in blue and nucleotide binding domain is shown in maroon. This figure was adapated from a figure by Behnke *et al* (Behnke et al, 2015).

1.5 ER associated degradation

Proteins that fail to fold correctly are retrotranslocated from the ER to the cytosol and targeted to the proteasome for degradation in a process known as ER associated degradation (ERAD) (Fig 1.8). Misfolded proteins can be recognised by ER chaperones and lectins by several distinctive motifs. Mannose trimmed glycan groups is one of these motifs and other include hydrophobic patches, unpaired sulphydryls and exposed sequences that would normally be processed. The type of misfolding motif expressed on each protein correlates with the protein that identifies the misfolded protein and also the type of ERAD protein complexes encounter by these proteins on the ER membrane (Hegde & Ploegh, 2010; Lemus & Goder, 2014).

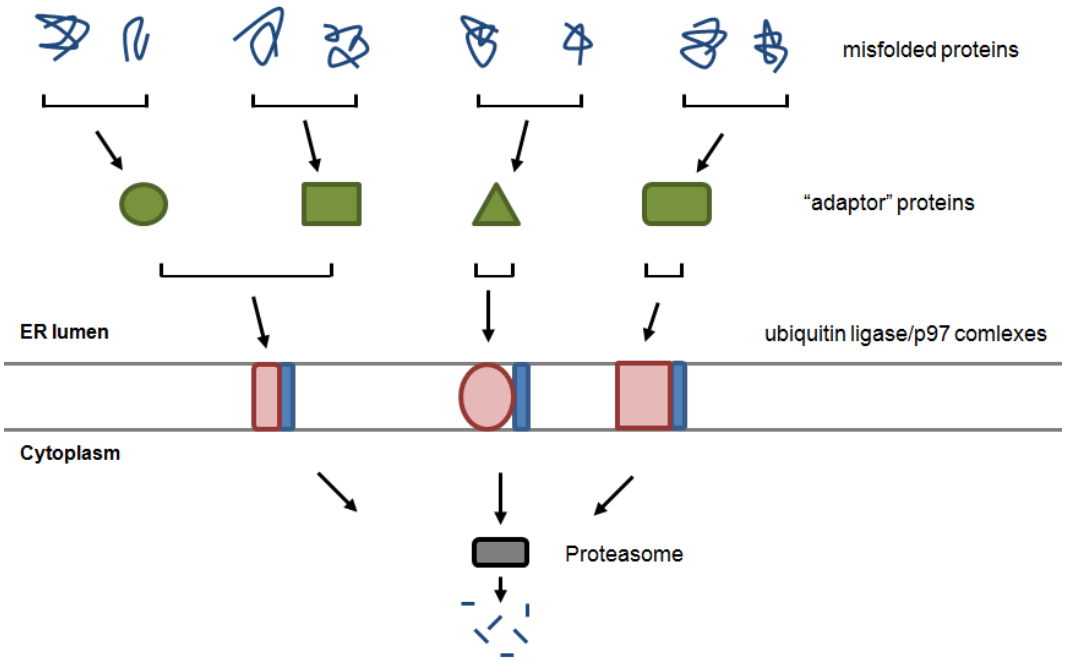

Figure 1.8: Functional overview of ERAD. Misfolded protein substrates are recognised by "adaptor" proteins which are lectins and chaperones such as PDI. Adaptors and their protein substrates are then targeted to membrane embedded complexes containing an ubiquitin ligase embedded in the ER membrane. p97 is also recruited to the complex. The substrates are then retrotranslocated with the ATPase activity of p97 supplying the energy for the process. The substrates are ubiquitinated on the cytosolic face of the membrane during this process. Lastly the protein substrates are transferred to the proteasome in the cytosol for degredation. This figure was adapated from a figure by Hedge and Ploegh (Hegde & Ploegh, 2010).

1.6 Unfolded Protein Response

The quality control mechanisms of the ER and the processes of ERAD enable eukaryotic cells to identify and degrade misfolded proteins which commonly form within the organelle. In some instances however stress conditions can cause the protein folding load of the ER to exceed the folding capacity. This results in the toxic accumulation and aggregation of misfolded proteins within the organelle. This type of ER dysfunction can be caused by a variety of cellular stresses including viral infection, glucose deprivation, redox imbalance and hypoxia. It is also common in many disease states. Under such circumstances the unfolded protein response (UPR) pathway is activated which enables the ER to adapt to the stress conditions and match protein folding capacity to secretory demand (Chakrabarti et al, 2011; Maly & Papa, 2014).

Signalling of the UPR is mediated by three ER membrane proteins, namely pancreatic ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol requiring enzyme 1 α (IRE1) (Fig 1.9). Each of these receptors is capable of sensing misfolded proteins through their association with BiP. In each case the receptors are inactive when bound to BiP but become activated following dissociation from BiP. This mechanism allows the receptors to only signal when the level of misfolded proteins exceeds a critical threshold in which much of the BiP protein is bound to the exposed hydrophobic regions of these proteins and not to these three receptors. In addition, misfolded proteins may themselves also act as 'activating ligands' of IRE1 and PERK by direct binding to the luminal regions of the receptors (Chakrabarti et al, 2011; Senft & Ronai, 2015).

Activated PERK, ATF6 and IRE1 each have complex downstream signalling mechanisms. Collectively they invoke an adaptive response to ER stress to restore secretory homeostasis. Activate PERK results in a global reduction in protein synthesis. This reduces the protein folding load of the ER by decreasing the influx of newly synthesized proteins and allowing time for existing proteins within the ER to be correctly folded or degraded. The activation of both ATF6 and IRE1 results in up regulation of the expression of ER chaperones as well as protein involved in ERAD, thus increasing the capacity of the ER to fold as well as degrade proteins. However, in circumstances in which ER stress is particularly high or is chronic the UPR can also lead to the activation of mitochondrial apoptotic pathways resulting in cellular destruction (Chakrabarti et al, 2011; Maly & Papa, 2014).

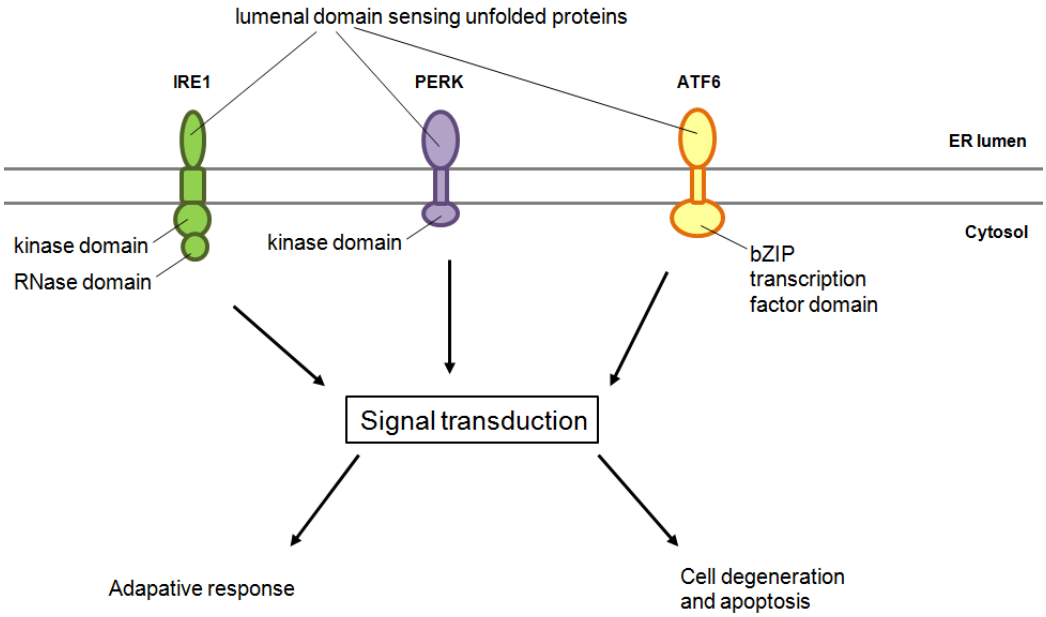

Figure 1.9. Overview of the unfolded protein response. UPR signalling is initiated by the ER membrane receptors IRE, PERK and ATF6. Accumulation of unfolded proteins within the ER is detected by sensing mechanisms in the luminal domain of these receptors and results in signal transduction via their cytosolic effector domains. Signal transduction either results in an adaptive response to restore protein homeostasis or to initiation of cell degeneration and apoptotic pathways. This figure was adapated from a figure by Maly and Papa (Maly & Papa, 2014).

1.7 Disulphide bond formation

Disulphide bonds form in nascent membrane and secretory proteins following entry of these proteins to the lumen of the rough ER. The formation of disulphide bonds is an important event in the maturation of these proteins and constitutes part of the protein folding process. In addition, disulphides confer structural stability and also bind together the subunits of mulitmeric proteins (Lodish, 2008). Disulphides are introduced to nascent proteins in the ER by thiol-disulphide exchange reactions with the PDI family of oxidoreductases (Fig 1.10) (Tavender & Bulleid, 2010a).

1.7.1 The PDI family

The mammalian PDI family is made of up of twenty different enzymes located within the ER (Fig 1.11). In addition to their cellular location the family members are defined by the presence of at least one domain that contains a thioredoxin-like structural fold. This feature is an αβ fold that contains a mixed β-sheet core (Fig 1.12). The thioredoxin-like domains of the PDI family members can be categorised depending on whether they are catalytically active or not. The catalytically active domains contain a CXXC motif involved in thiol-disulphide exchange reactions (Hatahet & Ruddock, 2007; Kozlov et al, 2010). Domains containing a CXXS or SXXC may also be considered as catalytically active as mixed disulphide species have been detected for family members containing these motifs, such as PDILT (van Lith et al, 2005). The catalytically inactive domains of the PDI family members are often involved in substrate binding and chaperone activities. These non-catalytic domains have a greater diversity in sequence and structure than the catalytic domains (Kozlov et al, 2010).

A current research goal is to understand the functional roles of the PDI family members (Kozlov et al, 2010). Emerging evidence suggests that different family members may have different niche roles within this process. One difference identified between the family members is in their substrate specificities. ERp57 for example has been found to preferentially interact with substrates that are glycosylated and requires the assistant of calnexin or calreticulin for these interactions (Jessop et al, 2009a). Another known difference between the family members is in their preference to oxidation of thiol groups or reduction of disulphides. Most of the PDI family members appear to favour oxididation of thiols, however, certain PDI family members, particularly ERdj5, favour action as a reductase (Oka & Bulleid, 2013; Oka et al, 2013).

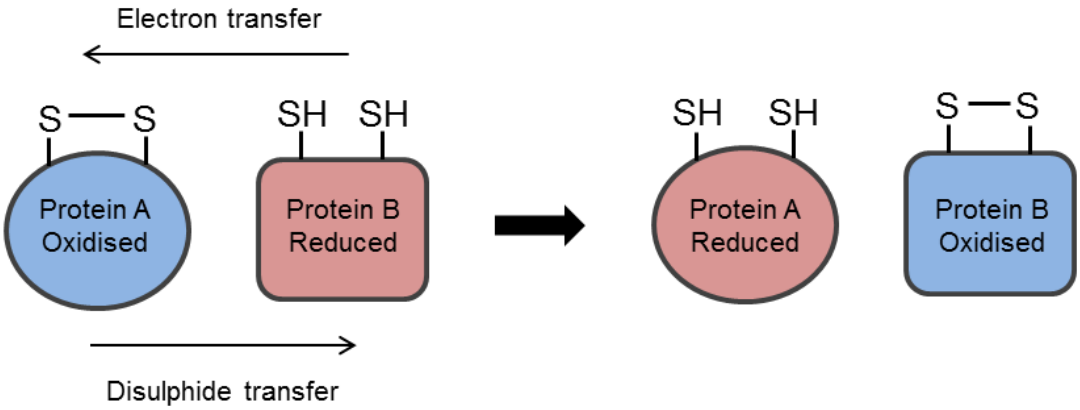

Figure 1.10: Thiol-disulphide exchange reactions. Disulphide bonds can be exchange between proteins in thiol-disulphide exchange reactions. In these reactions a disulphide bond is effectively donated from one protein to another causing the two cysteines of the target protein to become oxidised to a disulphide while the disulphide of the donating protein becomes reduced to a thiol group. Disulphide bonds are introduced into nascent proteins by thiol-disulphide exchange reactions with the PDI family members. This figure was adapated from a figure originally shown by Tavender and Bulleid (Tavender & Bulleid, 2010a).

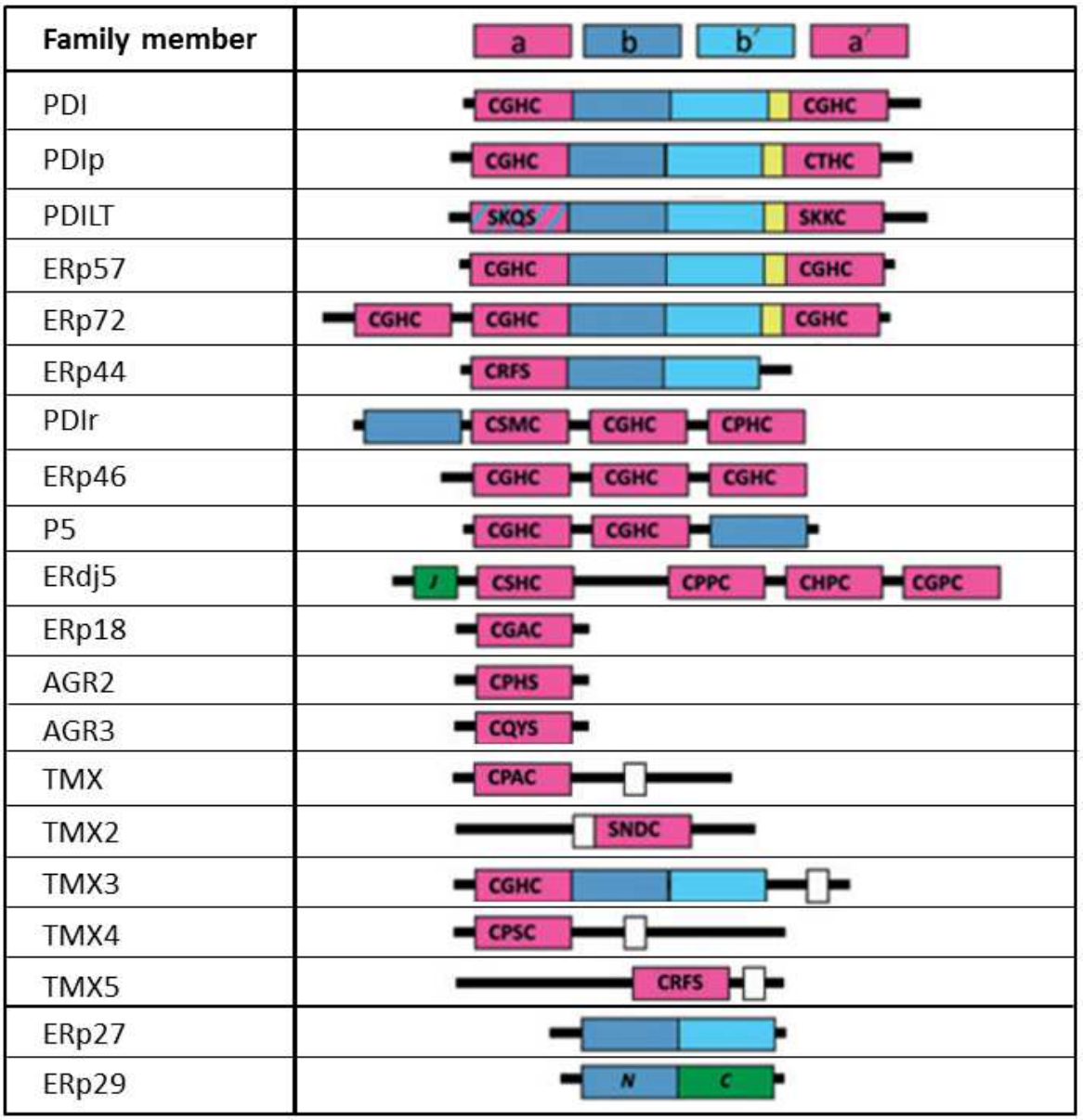

Figure 1.11: Domain structure of the PDI family. The catalytic thioredoxin-like a domains are shown in pink while non-catalytic b domains are shown in blue and light blue. The only exception to this is the a domain of PDILT which is not catalytically active however is hatched as it has a strong similarity to the a domain of PDI. X-linker regions are shown in yellow. PDI family members embedded in the ER membrane have the TMX designation and their transmembrane domains are shown by white boxes. Non-standard PDI domains such as the DnaJ domain of ERdj5 and the helical domain of ERp29 are shown in green. ERp27 and ERp29 are shown in a separate division of the table as they do not contain catalytically active thioreodoxin-like domains. This figure was adapted from a figure originally shown by Kozlov et al. The domain structural diagrams are as originally shown by Kozlov et al (Kozlov et al, 2010).

Figure 1.12: Structure of human thioredoxin 1. The image shown was produced using DeepView and represents the structure of reduced human thioredoxin 1 (3TRX). The ribbon form of the protein backbone is shown in light blue and the two catalytic cysteines (C32 and C35) of the CXXC motfi are shown in red. The secondary structure of this protein is comprised of a αβ fold that contains a mixed β-sheet core surrounded α helices. This structural motif is a common within the thioredoxin superfamily which includes the PDI family of oxidoreductases. The structure shown was originally solved by Forman-Kay *et al* by NMR spectroscopy (Forman-Kay et al, 1991)

1.7.2 *De novo* sources of disulphides

In order for the PDI family to continuously introduce disulphide bonds into substrates the catalytic disulphides of the PDI enzymes must themselves be regenerated. Endoplasmic reticulum oxidoreductase 1 (Ero1) is an enzyme commonly regarded as being the primary *de novo* enzymatic generator of ER disulphides (Tavender & Bulleid, 2010a). Peroxiredoxin IV (PrxIV) is also well established as an additional enzymatic contributor to PDI oxidation (Tavender & Bulleid, 2010b). Furthermore, several other enzymes; namely VKOR, QSOX and GPx7/8 have been identified as potential contributors to disulphide bond formation within the ER (Fig 1.13) (Bulleid & Ellgaard, 2011).

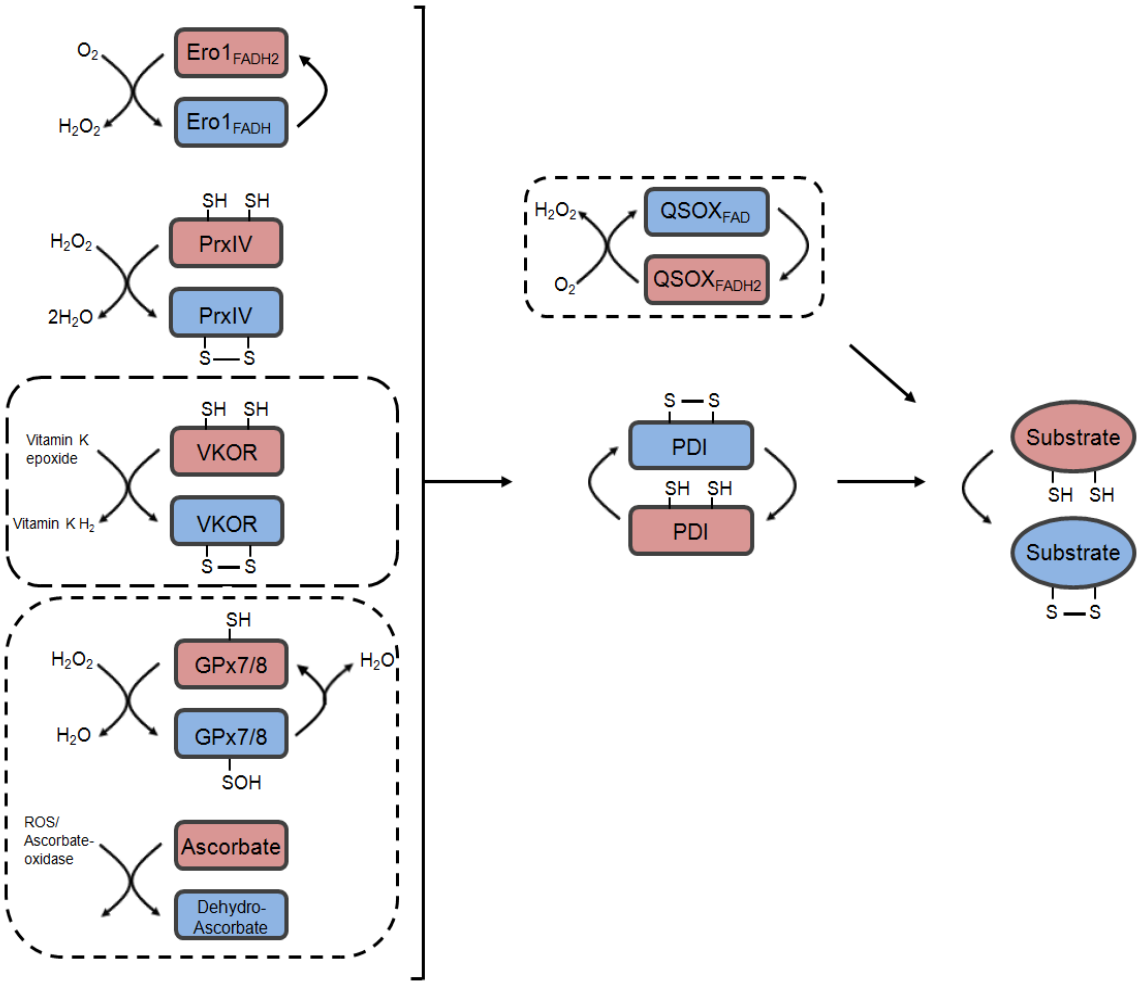

Figure 1.13: Known and hypothesised sources of *de novo* disulphide bond formation within the ER. Disulphide bonds are introduced into nascent proteins within the ER by thiol-disulphide exchange reactions with members of the PDI family. The catalytic disulphides of PDI are themselves regenerated by thiol-disulphide exchange reactions with enzymes capable of *de novo* generation of disulphides. Ero1 and PrxIV are established *de novo* generators of ER disulphides with strong *in vitro* and *in vivo* evidence supporting their involvement. The enzymes and chemicals shown in dashed boxes are hypothesised contributors to this process. In most cases their contribution to this process is supported by *in vitro* but not *in vivo* evidence. VKOR is the exception to this as there is *in vivo* data supporting evidence for VKOR contribution to disulphide formation, however this evidence comes from a single study using a hepatoma cell line (Rutkevich and Williams, 2012). As such it is unkown if VKOR contributes to disulphide formation in other tissues. To distinguish VKOR from the other hypothesised disulphide generators it is shown in a box with longer dashes. QSOX is shown in a separate position from the other disulphide generators as it has been shown *in vitro* to be both capable of disulphide generation and transfer of disulphides to nascent proteins without the need for interaction with PDI. The reduced and oxidised form of each member of these redox pathways is

shown in red and blue respectively. The figure was adapated and modified from a figure shown by Bulleid and Ellgaard (Bulleid & Ellgaard, 2011).

1.7.3 Endoplasmic reticulum oxidoreductase 1

Ero1 is a flavoenzyme that couples the oxidation of the catalytic cysteines of PDI to the reduction of molecular oxygen to hydrogen peroxide. The flavine adenine dinucleotide (FAD) moiety of Ero1 is involved in the transfer of electrons to oxygen during this reaction. The enzymatic activity of Ero1 is tightly regulated by an intricate mechanism involving the formation/breakage of regulatory disulphide bonds which effectively switches the molecule into an active or inactive catalytic state in response to the redox conditions of the ER. Functionally, this regulatory mechanism appears to exist to prevent excessive generation of hydrogen peroxide and thus prevent oxidative stress (Tavender & Bulleid, 2010a). This is of importance as the activity of Ero1 produces one molecule of hydrogen peroxide for every disulphide it introduces. It has been estimated that Ero1 activity accounts for 25% of the hydrogen peroxide produced during cellular protein synthesis (Tu & Weissman, 2004).

Ero1 is the most evolutionary conserved of the PDI oxidants. It is essential for disulphide formation in simple eukaryotes such as yeast and worms but not more complex eukaryotes such as mammals in which alternative oxidases exist. Two isoforms of Ero1 exist in vertebrates designated Ero1α and Ero1β. Ero1α is expressed throughout the body however expression of Ero1β is enriched in secretory cells. The activity of β is also greater than α as β does not contain the regulatory disulphides found in α (Zito, 2015).

1.7.4 Peroxiredoxin IV

PrxIV is an ER resident protein that exists as a ring shaped disulphide bonded homo decamer. The peroxiredoxins are a family of highly expressed antioxidant enzymes that metabolise hydrogen peroxide. There are six members of the family in humans, however, PrxIV is the only member that resides within the ER. PrxIV has an intriguing functional mechanism by which it reduces hydrogen peroxide to water with the concurrent oxidation of PDI, P5 or ERp46. This utilisation of hydrogen peroxide by PrxIV as a reactant lessens the oxidative stress causes by the activity of Ero1. In addition, its activity in combination with that of Ero1 potentially allows two disulphide bonds to be formed following the reduction of one molecule of oxygen to water (Cao et al, 2014; Tavender & Bulleid, 2010b; Tavender et al, 2008; Zito et al, 2010b).

1.7.5 Vitamin K epoxide reductase

Vitamin K epoxide reductase (VKOR) is an ER membrane protein that plays a key role in the vitamin K cycle and blood coagulation. VKOR enzymatically reduces vitamin K epoxide to vitamin K hydroquinone, however, during this reaction key catalytic cysteines of a CXXC motif of VKOR become oxidised forming a disulphide (Van Horn, 2013). It is currently unknown how this disulphide is reduced to allow further catalytic cycles. However, a study involving over expression of a CXXA substrate trapping form of VKOR found that VKOR can form mixed disulphides with the membrane bound PDI family members TMX, and TMX4 as well as the luminal PDI family member ERp18. The mixed disulphide interaction with TMX was found to be particularly strong (Schulman et al, 2010). This indicates that VKOR may be involved in redox reactions with these PDI family members which are oxidised while the disulphide of VKOR is reduced. Interestingly, the same study reported that Ero1α interacts poorly with the membrane bound PDI family members suggesting that Ero1 and VKOR may act as oxidases for different subsets of the PDI family (Schulman et al, 2010). Analysis of VKOR mRNA expression in various human tissues has indicated that VKOR is ubiquitously expressed, but is particularly abundant in liver cells (Wang et al, 2005). This suggests that the involvement of VKOR in ER disulphide bond formation may be particularly prominent in liver cells.

1.7.6 Quiescin sulfhydryl oxidase

Quiescin sulfhydryl oxidase (QSOX), like Ero1, is a flavoprotein capable of catalysing disulphide bond formation with the concurrent reduction of oxygen to hydrogen peroxide (Kodali & Thorpe, 2010). There are two isoforms of QSOX expressed in mammalian cells; namely QSOX1 and QSOX2. QSOX1 is expressed at higher levels in most tissues than QSOX2 (Coppock & Thorpe, 2006) and has so far been the focus of the majority of QSOX research (Kodali & Thorpe, 2010). QSOX has been shown *in vitro* to be capable of introducing disulphide bonds into various substrates (Hoober et al, 1999; Hoober & Thorpe, 1999). In addition, in yeast over expression of QSOX1 has been found to suppress the lethality of Ero1 deletion suggesting the two enzymes share functional roles (Chakravarthi et al, 2007). However, QSOX is predominantly located in the Golgi apparatus and also cellular secretions suggesting that its primary role is not in the ER (Mairet-Coello et al, 2004; Ostrowski & Kistler, 1980). Recently, QSOX1 depleted cell cultures have been shown to have a defective extracellular matrix (ECM). Experimental evidence strongly suggests that this defect is due to a lack of QSOX1 catalysed disulphide bond formation of the α4 laminin and other components of the ECM (Ilani et al, 2013). This evidence now suggests that the functional activity of QSOX is primarily extracellular.

1.7.7 Glutathione Peroxidases 7 and 8

Glutathione peroxidises (GPx) 7 and 8 are ER resident enzymes that despite their names are inefficient enzymes for the reduction of oxidised glutathione. GPx7 and 8 have been shown *in vitro* to increase the rate of oxidative refolding of denatured protein by approximately 2 fold in the presence of PDI and a hydrogen peroxide generating system (Nguyen et al, 2011). Recently reports using pulse chase methodology indicate that over expression of GPx7 accelerates disulphide formation *in vivo* following DTT treatment (Wang et al, 2014). The current model of GPx7/8 activity is that these enzymes assist oxidative folding by catalysing the oxidation of PDI disulphides with the concurrent reduction of Ero1 generated hydrogen peroxide. The catalytic rate of GPx7/8 in these reactions however appears slow (Nguyen et al, 2011), and therefore these reactions perhaps make only a minor contribution to ER disulphide formation.

1.8 Thiol-dependent redox systems

Mammalian cells contain two major thiol-dependent redox systems, namely the glutathione and thioredoxin systems. Both of these systems are key factors in cellular thiol and disulphide redox biology.

1.8.1 Glutathione

Glutathione (GSH) is a ubiquitous cellular tripeptide molecule that is composed of the amino acids, glutamate, cysteine and glycine. This molecule is present at millimolar concentrations (up to 10mM) within the cell and has several functions including protecting against oxidative stress (Anderson, 1998; Chakravarthi et al, 2006).

GSH is synthesised in two ATP dependent reactions. Firstly, a dipeptide is formed between glutamate and cysteine. The γ-glutamylcysteine produced then reacts with glycine at the C-terminus to produce GSH. These two reactions are catalysed by the enzymes γ-glutamyl cysteine synthetase and glutathione synthetase respectively. The GSH tripeptide produced contains an unusual γ-peptide bond between the amine group of cysteine and the carboxylic acid group of the side chain of the glutamate. The presence of this γ peptide bond greatly reduces glutathione degradation by cellular peptidases (Anderson, 1998; Lehninger et al, 2005).

Glutathione can exist in both a reduced and oxidised state. Loss of an electron by oxidation causes GSH to readily react with another oxidised GSH molecule to form glutathione disulphide (GSSG) (Lehninger et al, 2005). Due to the high concentration of glutathione and its ability to exist in these two states under physiological conditions glutathione is a major cellular redox buffer (Hwang et al, 1992). The high concentration of cellular GSH allows it to

react non-enzymatically with reactive oxygen species (ROS) as part of the cellular ROS scavenging mechanisms. This causes neutralisation of the ROS and the formation of GSSG (Apel & Hirt, 2004). GSH can also reduce the ROS hydrogen peroxide to water enzymatically in a reaction catalysed by the glutathione peroxidise. Despite this and other mechanisms that lead to GSSG production, the concentrations of GSSG within the cytosol are maintained at low levels by the action of the enzyme glutathione reductase which continuously converts GSSG back to GSH (Fig 1.14) (Lehninger et al, 2005).

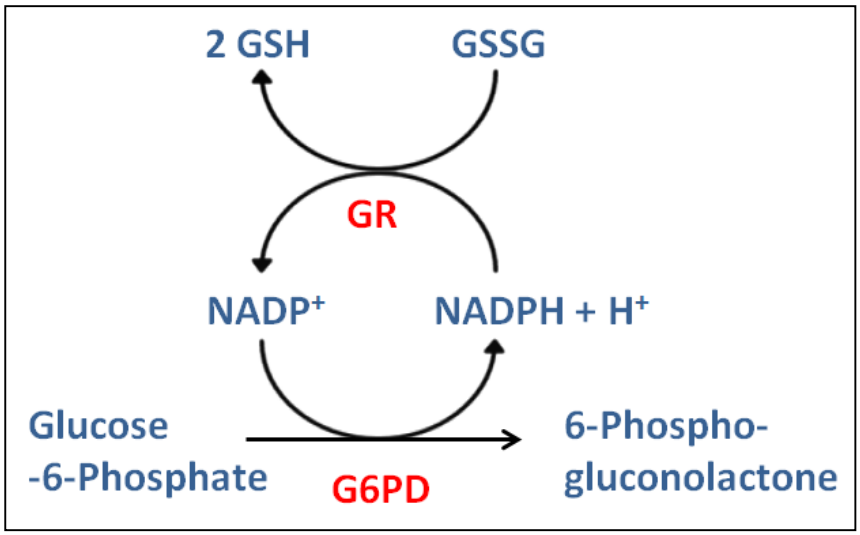

Figure 1.14: Cytosolic glutathione is maintained in a reduced state. GSSG produced in the cytosol is converted back to GSH by the action of glutathione reductase. NAPDH produced during the oxidation of glucose-6-phosphate acts a cofactor in this reaction. GR: glutathione reductase, G6PD: glucose-6-phosphate dehydrogenase (Lehninger et al, 2005).

The ratios of concentration of GSH to GSSG varies between different compartments within the cell. Within the cytosol the GSH/GSSG ratio is very high and has been found to range from 30:1 to 100:1. Within the organelles of the secretory pathway however the GSH/GSSG is far lower and has been found to range from 1:1 to 3:1 (Go & Jones, 2008). As such, the luminal environment of the ER and Golgi is more oxidising than that of the cytosol. Both the metabolism of GSH/GSSG within the ER and its transport to and from the organelle are poorly understood. Key questions remain regarding how the oxidised GSH/GSSG is maintained in the ER, whether GSSG can be reduced to GSH by an ER resident enzyme and how GSH/GSSG is transported across the ER membrane (Chakravarthi et al, 2006)?

For many years glutathione (in addition to thioredoxin) has been regarded as being involved in maintaining thiol groups within a reduced state within the cytosol (Lopez-Mirabal & Winther, 2008). This paradigm has been challenged in recent years as it has been reported that thiol-redox control is unaffected in yeast cells depleted of, or expressing toxic levels of

glutathione (Kumar et al, 2011). Glutathione is, however, widely regarded as playing an important role in the oxidative folding process within the ER (Fig 1.15) (Chakravarthi et al, 2006). This perception is based on several findings. The oxidising GSH/GSSG ratio within the ER appears to be optimal for the enzymatic introduction of disulphide bonds to nascent proteins by PDI (Lyles & Gilbert, 1991). GSH is also thought to be a key component of the disulphide reduction pathway within the ER and there is evidence that GSH is involved in the reduction of non-native disulphides (Chakravarthi & Bulleid, 2004; Molteni et al, 2004).

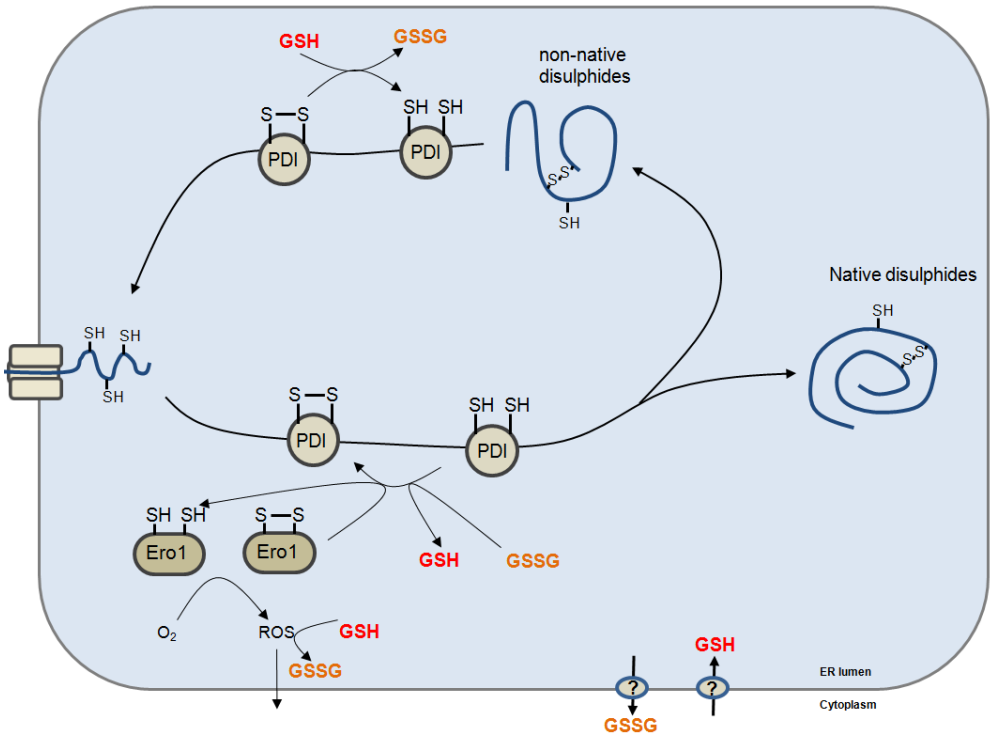

Figure 1.15: Model of glutathione metabolism within the ER. ER luminal glutathione is thought to be involved in several redox reactions. These include reduction and oxidisation of PDI and neutralisation of luminal ROS. The luminal GSH/GSSG could be maintained by either flux from the cytosol via unidentified glutathione transporters or by conversion of GSSH to GSH by an unidentified ER resident glutathione reductase enzyme. The figure was adapated from a figure shown by Chakravarthi *et al* (Chakravarthi et al, 2006).

1.8.2 Thioredoxin

The thioredoxin system, like the glutathione system, is a major regulator of cellular redox homeostasis and antioxidant defence. The components of this system are NADPH, thioredoxin reductase and thioredoxin itself. These components are involved in a redox pathway that causes the reduction of disulphide bonds in the target proteins and molecules of thioredoxin. Thioredoxins are small, ubiquitous enzymes of about 12kDa that catalyse disulphide reduction. The thioredoxins are maintained in a reduced state by thioredoxin

reductase which in turn has its catalytic site reduced by NADPH (Fig 1.16) (Lu & Holmgren, 2014).

There are two isoforms of thioredoxin (Trx) within mammalian cells termed Trx1 and Trx2. Trx1 is mainly a cytosolic enzyme, but, under certain circumstances it can also be translocated to the nucleus or secreted from the cell. Trx2 is located within the mitochondria (Lu & Holmgren, 2014). Thioredoxin reductase (TrxR) is a selenium containing enzymes responsible for maintaining the cellular pool of reduced thioredoxin. There are three isoforms of TrxR in mammalian cells; TrxR1 which is located in the cytosol, TrxR2 which is located in the mitochondira and TGR, a testis-specific thioredoxin glutathione reductase. Both the TrxR1 and TrxR2 isoforms also have several splice variants. Two of the TrxR2 splice variants do not contain mitochondrial targeting sequences and consequently these variants are located in the cytosol. Both Trx1 and Trx2 enzymes operate as homodimeric enzymes in their respective cellular compartments. These enzymes have overall structures similar to glutathione reductase and contain interface domains as well as FAD and NADPH binding domains (Lu & Holmgren, 2014).

The thioredoxin system has a broad range of downstream targets and reduces several key redox enzymatic systems within this cell (Fig 1.17). This includes the peroxiredoxins (Prx), methionine sulphoxide (Msr), and ribonucleotide reductase (RNR) enzymes. These target enzymes are responsible for reduction of hydrogen peroxide to water, reduction of methionine sulphoxide to methionine and converting ribonucleotides to deoxyribonucleotides respectively. The thioredoxin system also supports the reduction of several redox sensitive molecules within the cell including ASK1, Txnip and PTEN. ASK1 and Txnip are involved in apoptotic signalling while PTEN is involved in the opposing anti-apoptotic Akt signalling pathway. Due to the hierarchical nature of the redox homeostasis of these enzymes and molecules that is mediated by the thioredoxin, the thioredoxin system plays a major role in maintaining reducing conditions within the cell and protecting the cell from oxidative stress that could lead to apoptosis (Lee et al, 2013).

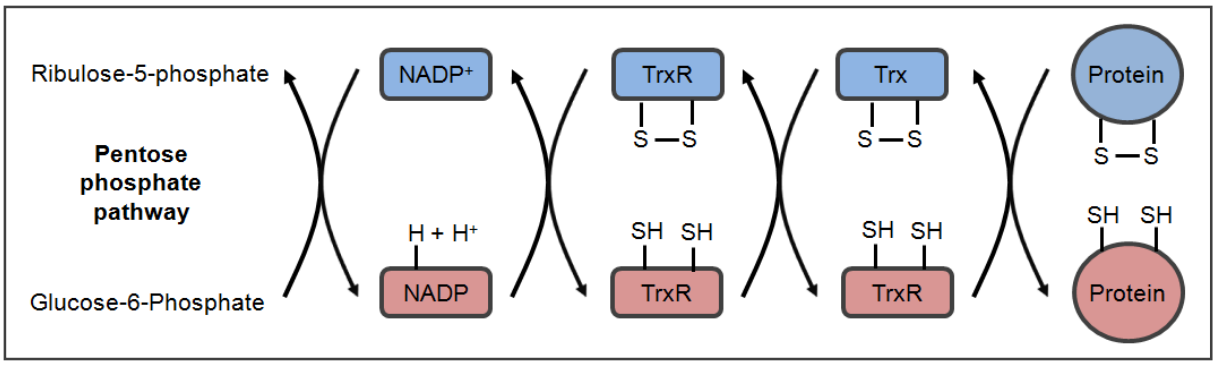

Figure 1.16: Disulphide bonds are reduced within the cytoplasm by the thioredoxin redox system. A redox relay pathway catalyses disulphide reduction. The above figure is above was adapated from a figure shown by Lee *et al* (Lee et al, 2013).

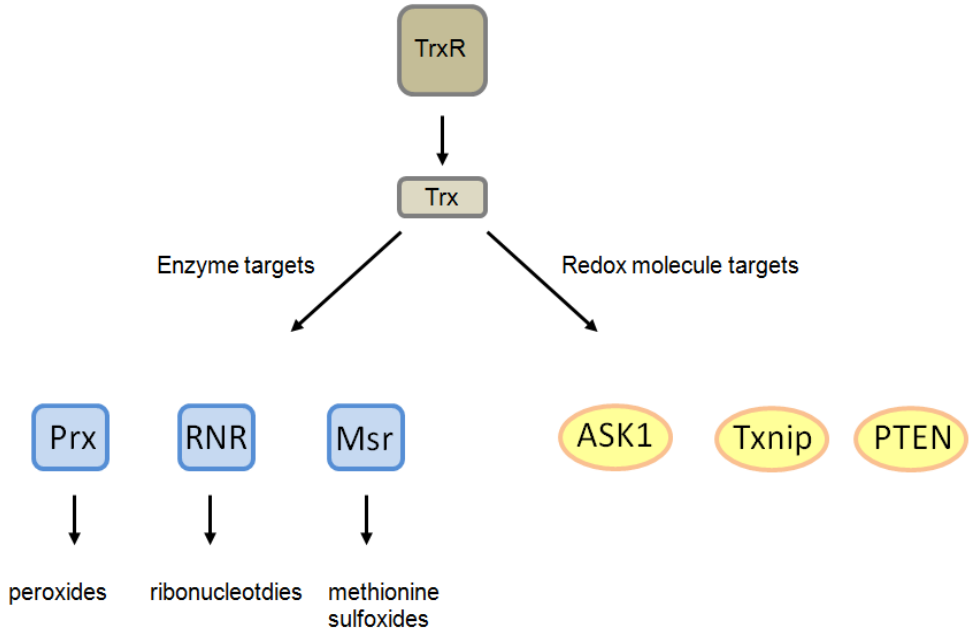

Figure 1.17: Thioredoxin reduces the disulphides bonds of a broad range of substrate enzymes and molecules. Thioredoxin has a prominent position in the cellular redox hierarchy. As such the thioredoxin reductive pathway is important for numerous cellular processes. This figure was adapated from a figure originally shown by Lee *et al* (Lee et al, 2013).

1.9 *In Vitro* Translation

The experiments described in this thesis were conducted using a rabbit reticulocyte lysate translation system. This system was supplemented with either microsomes or semi-permeabilised cells for the majority of experiments which acted as a source of ER and enabled ER processing events to occur to the proteins synthesised within the lysate.

1.9.1 Rabbit Reticulocyte Lysate

The rabbit reticulocyte lysate (RRL) translation system is one of most commonly used cell free protein synthesis systems in use. This system has been used in a wide range of investigations including analysis of transcriptional and translational control, targeting of nascent proteins to the ER and processing events within the ER. The latter two of these processes have been investigated by the addition of microsomal membranes or SP cells to the lysate (Chong, 2014; Titus & Promega Corporation., 1991).

The reticulocytes from which RRL is derived are immature red blood cells (Weissbach & Pestka, 1977). These cells were chosen as a source of lysate as they are a type of mammalian cell which have the desirable property of having particularly low ribonuclease activity (Hulea & Arnstein, 1977). Therefore, there will be little degradation of mRNA added to the lysate during *in vitro* translation reactions.

The RRL translation system currently used is largely unchanged from the system developed by Pelham and Jackson in 1976 (Pelham & Jackson, 1976). The lysate of this system comes from the reticulocyte cells of New Zealand White rabbits (Titus & Promega Corporation., 1991). The procedure for producing rabbit reticulocyte involves first making rabbits anaemic. One method of doing this is to inject the rabbits with acetylphenylhydrazine which causes the destruction of erythrocytes (Stewart et al, 1953). The rabbit are then bled a few days after the treatment. As the rabbits are recovering their blood contains a high proportion of reticulocytes. The reticulocytes are lysed by osmotic lysis and the lysate is centrifuged at 30,000 xg for 15 min and the supernatant removed and the cellular debris discarded (Hunt & Jackson, 1974). The lysate is usually treated with micrococal nuclease to degrade endogenous mRNA within the lysate, thereby reducing background translation (Titus & Promega Corporation., 1991). The lysate is then stored at -70°C until required for experimental use.

The Promega Corporation is a major supplier of commercially available RRL. The RRL contains the components required for protein synthesis such as ribosomes, tRNA, amino acids and initiation, elongation and termination factors. The Promega RRL has been optimised for *in vitro* translation by the addition of several components. This includes hemin which is added to prevent inhibition of initiation factor eIF-2α, a phosphocreatine kinase and

phosphocreatine energy generating system, and additional tRNAs from calf liver to supplement the endogenous tRNAs within the lysate. A “Flexi RRL” is also available in which any additional Mg^{2+} K^{+} or DTT that is required to be added to the system is added separately by the user (Titus & Promega Corporation., 1991). This can be very useful as the Mg^{2+} and K^{+} concentration often have to be optimised for translation of each mRNA to maximise translation efficiency. It is also useful for the addition of DTT to be optional if disulphide bond formation is being analysed in the translation reaction as DTT will interfere with this process. All the translation reactions described in this thesis are carried out using a Flexi lysate system purchased from Promega.

1.9.2 Microsomes

Microsomes are vesicle fragments of the rough ER that are produced when cells are homogenised (Lodish, 2008). These vesicles are about 100nm in diameter (Avers, 1986). Microsomes can be isolated from the other cellular components of cellular homogenates by equilibrium density gradient centrifugation (Sadava, 1993). The ribosomes that are bound to the outer surface of microsomes derived from the rough ER which gives these microsomes a high density (Avers, 1986). Aside from existing as vesicles rather than as a reticulum the microsomes are structurally and functionally very similar to that of the ER. Despite the breakage and formation of vesicles the interior of microsomes remains equivalent to the ER lumen (Avers, 1986). Due to these properties microsomes are a valuable research tool for researching the ER. Microsomes are often supplemented to cell-free protein synthesis (CFPS) systems to act as a source of ER to allow post-translational modifications to occur within secretory proteins synthesised within these systems (Titus & Promega Corporation., 1991).

Microsomes are commonly purified from dog pancreas as the pancreas tissue has high secretory output and as such the cells of this tissue has a well-developed secretory pathway allowing for high yields of microsomes from this tissue. In addition, dog pancreas tissue also has the desirable property of having high levels of endogenous ribonuclease inhibitor which preserves the integrity of the polysomes bound to the microsomes (Kaderbhai et al, 1995; Walter & Blobel, 1983).

1.9.3 Semi-permeabilised cells

Semi-permeabilised cells (SP cells) were first developed to act as a source of partially isolated ER in which the ER remains intact in the form of a reticular network as it is found in the cell. The original purpose of this was to act as a tool for investigating vesicle trafficking between the ER and the Golgi apparatus. Becker *et al* first generated SP cells by treating cells with the mild detergent digitonin which selectively permeabilised the cholesterol rich

plasma membrane but not the membranes of the ER and the Golgi (Beckers et al, 1987). This treatment caused the loss of the cytosolic components from the cells but retention of the morphologically intact and functional ER and Golgi. Becker *et al* used the SP cells to investigate the influence of cytosolic factors upon ER and Golgi transport. Using this approach, they discovered that transport between the ER and Golgi requires cytosolic proteins as well as energy in the form of ATP (Beckers et al, 1987).

SP cells were later adapted for use in CFPS systems to act as a source of ER in a similar manner to the use of microsomes in such systems (Wilson et al, 1995). Initial characterisation of SP cells within RRL and wheat germ CFPS systems found that SP cells were capable of translocation, disulphide formation, hydroxylation and glycosylation of nascent membrane and soluble secretory proteins synthesised within these lysates (Wilson et al, 1995).

SP cells have several benefits over the use of microsomes within CFPS systems. They can be readily produced from tissue culture while purification of microsomes from tissues (von Jagow et al, 1965) or from cell culture (Sukhodub & Burchell, 2005) is a more laborious process. The most significant advantage is that the ER of SP cells contain any manipulations in protein expression that is present in the cell line from which they are derived. This allows for a greater range of experimental manipulations for the investigation of particular proteins in ER biological processes. SP cells have been used in this way in a range of studies (Jessop et al, 2009b; Lang et al, 2012).

1.10 Translation substrates

mRNA encoding sequences of the haemagglutinin, preprolactin and cVIMP-Cys were used as translation substrates in this thesis.

1.10.1 Haemagglutinin

Haemagglutinin (HA) is one of the three proteins expressed on the surface of influenza A virus particles. The other two are neuraminidase and the M2 proton channel (Russell et al, 2008). HA has two functions in the life cycle of an influenza virus. The first is to bind sialylated glycoprotein receptors on the target cells of the virus. The second is involved in the fusion of virus particles with the cell membranes following uptake of the virus into a cell by endocytosis (Skehel & Wiley, 2000).

An HA monomer typically contains 540 to 550 amino acids and contains a N-terminal ER signal sequence and a C-terminal membrane anchor. The mature HA protein is a trimer made up of three identical HA subunits (Fig 1.18). HA maturation and formation of the trimer occurs within the ER of a host cell (Webster et al, 2013).

Sixteen different subtype of HA have been identified. These are distinguished from one and other by their structure and antigenicity (Webster et al, 2013). The subtype of HA used in this thesis is from the Japan strain of H2N2 Influenza A virus which contains 562 amino acids (Gething et al, 1980). Based upon sequence analysis and comparison with other HA subtypes it has been inferred that this subtype contains 7 N-linked glycosylation sites and six intra-chain disulphide bonds and has a signal peptide length of 15 amino acids. The six intra-chain disulphides are formed between cysteines 19-477, 57-288, 70-82, 105-149, 292-316 and 484-488 (uniprot).

HA has been used as model protein for investigation of oxidative folding in both pulse chase studies and using RRL *in vitro* translation systems (Braakman et al, 1991; Marquardt et al, 1993). The extensive glycosylation which HA undergoes allows the HA that has been processed by the ER to be easily distinguished. In addition, as HA forms multiple intra-chains disulphide bonds it passes through several disulphide bonded intermediates before forming a native oxidised form. Due to these properties HA is an effective substrate for investigations requiring a substrate with a complex oxidative folding pathway.

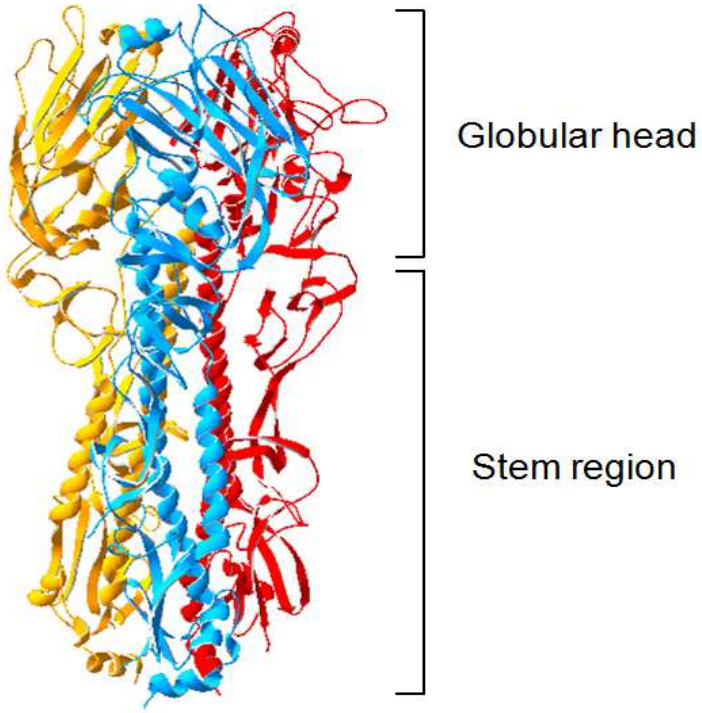

Figure 1.18: Structure of a haemagglutinin trimer. The image shown was produced using DeepView and represents the ribbon form of structure of H2 haemagglutinin (3KU5). The monomer units that make up the trimer are shown in yellow, light blue and red respectively. The globular head contain the receptor binding region while the lower proportion of the stem region contains the transmembrane region of the protein. The structure shown was originally solved by Xu et al by X-ray crystallography (Xu et al, 2010).

1.10.2 Preprolactin

Prolactin, along with the closely related growth hormone, placental lactogen, is part of a family of long-chain cytokines. Prolactin is a multifaceted hormone that is found in all vertebrates and which has over three hundred separate known functions within these species. The major functions are the stimulation of growth and differentiation of the mammary epithelia and stimulation of lactation. Prolactin is primarily secreted from the anterior pituitary gland but can also be secreted from other cells and tissues. Prolactin secreted by the pituitary acts via a classic endocrine pathway in which it is secreted from the gland, travels through the circulatory system and acts on target cells by the activation of specific receptors on the plasma membrane. The prolactin secreted from cells and tissues other than the pituitary acts in an autocrine or paracrine fashion where it may function in roles including acting as a growth factor or an immune-modulator (Bole-Feysot et al, 1998).

Preprolactin contains a signal sequence which targets the nascent protein to the endoplasmic reticulum. In human preprolactin this signal sequence comprises the first 28 amino acids of the protein while the signal sequence of bovine preprolactin comprises the first 30 amino acids (Cooke et al, 1981; Graf et al, 1970). In both human and bovine prolactin the corresponding cysteines residues and disulphide bonds of the mature proteins are in the same positions. If the signal sequence is omitted from the numbering then in both cases the disulphides are formed between cysteines 4-11, 58-174 and 191-199. The mature protein itself has a molecular weight of 23kDa. Overall the sequences of prolactin between humans and bovine species are highly conserved with a sequence identity of 74% being shared between the two species (Miller et al, 1981).

The structure of human prolactin in solution has been solved by NMR (Fig 1.19) (Teilum et al, 2005). The most obvious structural features of prolactin are its four major α-helices. These four α-helices are composed of two antiparallel helix pairs. The first and fourth helices form a pair, as do the second and third helix. Each of the helices is packed more closely to its pairing helix then to the other two helices. The fourth helix is bound to the linker region of the first and second helix disulphide bond between cysteines 58 and cysteine 174. The N-terminus and C-terminus also both contain short-ranged disulphide bonds that are formed between residues 4 and 11, and 191 and 199 respectively. The C-terminal disulphide causes the last five residues to pack closely to the fourth helix (Teilum et al, 2005).

Bovine preprolactin has been used as a model protein for investigation of disulphide bond formation within the ER. A historical reason for this is that PPL could be readily isolated in high yields from the bovine pituitary gland and thus provided a convenient source of mRNA for a translation substrate before *in vitro* transcription became a common place laboratory

technique (Nilson et al, 1979). Bovine PPL also has two other properties that make it model protein for investigation of disulphide formation. Firstly, bovine PPL unlike many other species of PPL is not glycosylated and thus the disulphide bond formation of bovine PPL is simplified as the addition of glycan groups is not a factor. And secondly, the disulphide bond formation within prolactin causes a clear and obvious shift in electrophoretic mobility making this modification easy to detect (Kaderbhai & Austen, 1985).

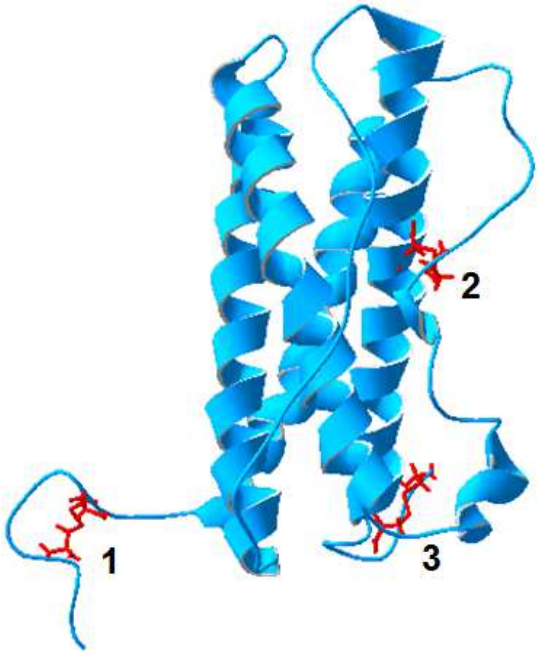

Figure 1.19: Structure of human prolactin. The image shown was produced using DeepView and represents the structure of human prolactin (IRW5). The ribbon form of the protein backbone is shown in light blue. The four α helices of the protein can be clearly seen. The six disulphide paining cysteine residues and the three disulphide bonds they form are highlighted in red and are numbered by their positions in the protein sequence. These three disulphides are formed between cysteines 4-11, 58-174 and 191-199 respectively. Disulphide one and three are short range and can be seen to affect the local structure. Disulphide two is a long range disulphide and connects the linker sequence between the first and second α helices with the backbone of the fourth α helix. The structure shown was originally solved by Teilum *et al* by NMR spectroscopy (Teilum et al, 2005).

1.10.3 cVIMP-Cys

VIMP (VCP-interacting membrane protein) also known as SelS is a selenoprotein located in the ER membrane. VIMP is involved in linking microtubules to the ER and thus is important for formation and maintenance of the intracellular structure of the ER (Noda et al, 2014). VIMP has also been hypothesised to act as a reductase of disulphide bonds in proteins

retrotranslocated from the ER lumen to the cytosol during the process of ER associated degradation (Christensen et al, 2012). Structurally VIMP contains both a short region exposed to the ER lumen and a longer region exposed to the cytosol (Fig 1.20). The ER transmembrane domain lies between these two regions. The cytosolic region contains a selenocysteine at the penultimate C-terminal amino acid residue.

cVIMP-Cys is a recombinant protein that is a shortened version of the human VIMP protein. This recombinant protein was created by the laboratory of Lars Ellgaard (University of Copenhagen) as a tool to investigate the structure and function of human VIMP (Christensen et al, 2012). cVIMP-Cys contains only the cytosolic region of VIMP. In addition, the selenocysteine of VIMP has been replaced with a standard cysteine residue. cVIMP-Cys, therefore, contains two cysteine residues at positions 174 and 188 which may form a single intra-chain disulphide bond. It was shown by Christensen et al that the formation this intra-chain disulphide causes a small but detectable shift in electrophoretic mobility when analysed by SDS-PAGE electrophoresis. Structural analysis of cVIMP-Cys indicates that the N-terminal half is comprised of two α-helices, while its C-terminal region is intrinsically disordered (Christensen et al, 2012).

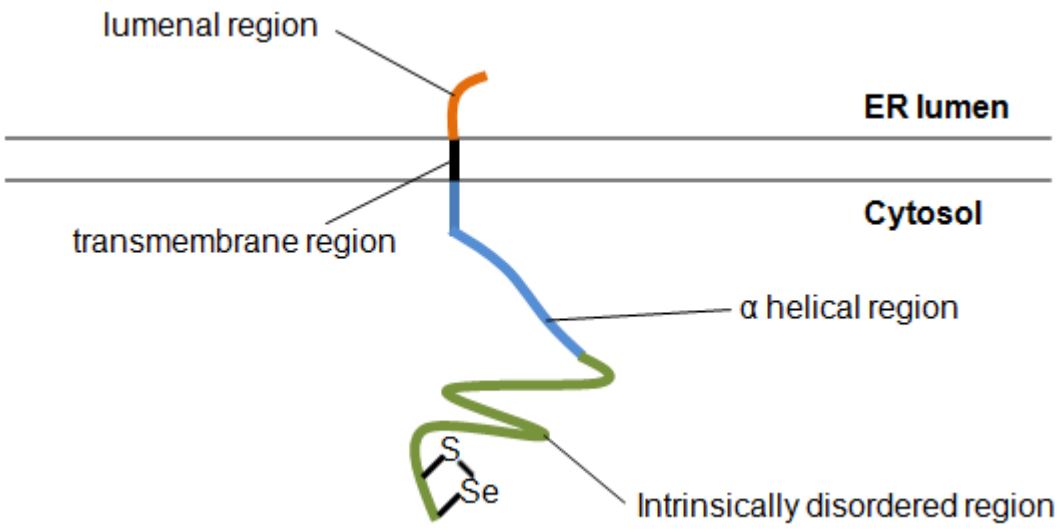

Figure 1.20: Structural model of human VIMP: Structural model of VIMP based upon findings from NMR and CD spectroscopy (Christensen et al, 2012). The above figure was adapated from a figure shown by Christensen et al (Christensen et al, 2012). N.B. The cVIMP-Cys recombinant protein differes from the VIMP model shown; it does not contain the ER luminal or transmembrane region and the selium cysteine is replaced with a sulphur cysteine residue.

1.11 Thesis aims

The overall goal of this thesis is to advance the understanding of the biochemical and molecular aspects of disulphide bond formation and oxidative protein folding within the ER. The research described in each of the three results chapters (chapters 3-5) had a specific aim to further this goal:

1. Develop a modified RRL *in vitro* translation system capable of specifically analysing nascent protein disulphide formation within a source of ER (chapter 3).
2. Assess the contribution of known and potential ER enzymatic *de* novo sources of disulphides to disulphide formation in nascent secretory proteins (chapter 4).
3. Analyse the interactions of nascent proteins with PDI during and following translocation to the ER lumen (chapter 5).

Chapter 2: Materials and Methods

2.1 Cell lines

Semi-permeabilised cells (SP cells) were generated from cell cultures of either wild-type HT1080 human fibrosarcoma cells, H1080 cells over expressing V5-tagged PDI, or HT1080 cells over expressing V5-tagged PDI in which both active sites of the enzyme have had their CXXC active site mutated to CXXA (stPDI). Both of the PDI overexpressing cell lines were generated by Catherine Jessop and have been described previously (Jessop et al, 2009b).

2.2 Generation of semi-permeabilised cells

SP cells were generated using a previously described protocol (Wilson et al, 1995). A confluent T75 flask of cells was washed with phosphate buffered saline (PBS) and trypsinised. The cells were then resuspended in 8 ml KHM buffer (20 mM HEPES buffer pH 7.2, containing 110 mM potassium acetate, 2 mM magnesium acetate and 100 µg/ml soybean trypsin inhibitor). The cells were centrifuged at 460 xg for 3 min and the pelleted cells were resuspended in 6 ml KHM containing 40 µg/ml digitonin and incubated for 5 min. A further 8 ml of KHM buffer was added before isolating the cells again by centrifugation and resuspending in HEPES buffer (50 mM HEPES, pH 7.2, 90 mM potassium acetate) for 10 min. The cells were next isolated by centrifugation and resuspended in 100µl KHM buffer. The cells were incubated at room temperature for 12 min in the presence of 1 mM CaCl and 10 µg/ml staphylococcal nuclease in order to degrade endogenous mRNA. EGTA was added to a concentration of 4 mM and the cells isolated by centrifugation and resupended in 100 µl KHM to be used in translation reactions. The cells were kept on ice throughout the procedure except during incubation with CaCl and staphylococcal nuclease.

2.3 Preparation of rough dog pancreas microsomes

Rough dog pancreas microsomes (DPMs) were prepared by established methodology (Austen et al, 1984; Scheele et al, 1980; Walter & Blobel, 1983; Yang & Braciale, 1995). Dog pancreases were washed twice in homogenisation buffer (50 mM Tris buffer pH 7.5, containing 0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$.6H20 and 200 µM phenylmethanesulfonylfluoride [PMSF]) and minced before being forced through a tissue press (Biospec products). The pancreas was then homogenised by eight passes through a Potter Elvejham homogeniser in a volume of homogenising buffer equal to the wet weight of the pancreas. The resulting homogenate was then centrifuged at 13,000 xg for 10 min, the pellet and any floating lipid was discarded. The supernatant was layered over a sucrose step gradient of 1.65 M and 2.1 M sucrose dissolved in TMK buffer (50 mM Tris pH 7.5, 5 mM MgCl2.6H20, and 25 mM KCl) and centrifuged at 200,000 xg for 3.5 h. The DPMs were recovered from the interface of the 1.65 M and 2.1 M Sucrose TMK buffers using a wide bore syringe. The DPMs were then diluted with 3 times volume of TMK buffer. The DPMs

were then pelleted by centrifugation at 20,000 xg for 1 hour and resuspended in buffer B (20 mM HEPES, pH 7.5, 0.25 M sucrose) at a concentration with an optical density of 50 units/ml at 260 nm. The DPMs were nuclease treated prior to use in translation reactions by incubation with 1 mM CaCl and 10 µg/ml staphylococcal nuclease for 12 min at room temperature. EGTA was then added at a concentration of 4mM. The microsomes were centrifuged at 150,000g for 10min and resuspended in buffer B. The DPMs were kept on ice throughout the procedure except during incubation with CaCl and staphylococcal nuclease.

2.4 mRNA sequences used for *in vitro* translation

mRNA encoding influenza virus haemagglutinin (A/Japan/305/57 H2N2 strain), bovine preprolacin (Uniprot entry: P01239) and cVIMP-cys was synthesised from plasmid vectors. The plasmid vector encoding the haemagglutinin, bovine preprolactin cVIMP-cys were gifts from Mary Jane Gething (University of Melbourne), Stephen High (University of Manchester) and Lars Ellgaard (University of Copenhagen) respectively. The haemagglutinin and cVIMP-cys sequences have been described previously (Christensen et al, 2012; Gething et al, 1980). In addition to these 3 sequences we also engineered a DNA sequence of preprolactin that lacked a stop codon and as such would become stalled during translation (tsPPL). As with the other sequences mRNA was generated from this sequence by *in vitro* transcription. We termed this mRNA sequence the tsPPL sequence.

2.5 Generation of translationally stalled PPL mRNA

The traslationally stalled preprolactin (tsPPL) construct was generated as follows. A pGEM plasmid in which the bovine preprolactin gene was inserted (PPLpGEM) was used as a template for a PCR reaction with a forward primer annealing to a T7 promoter region upstream of the PPL sequence (T7 primer: 5'-TAATACGACTCACTATAGGG-3') and a reverse promoter annealing to the 3' end of the PPL sequence up to but not including the termination codon (Reverse primer:5'-GCAGTTGTTGTTGTAGATGATTCTG-3'). This PCR reaction produced a DNA sequence of bovine preprolactin that lacked a stop codon. This sequence was used as template for *in vitro* transcription to produce tsPPL mRNA.

2.6 Rabbit reticulocyte lysate translation reactions

A volume of 16.5 µl Flexi RRL (Promega) was aliquoted and the following reagents added to a final volume of 25 µl at the indicated final concentrations: KCL at 40 µM, amino acids minus methionine (Promega) at 20 µM and EasyTagTM EXPRESS^{35}S Protein Labelling Mix (PerkinElmer)**,** at 16.3 kBq/µl. Except where stated otherwise, the lysate mixture was also supplemented with glucose-6-phosphate (G6P) at a final concentration of 0.5 mM. mRNA (1µl) encoding the sequence of the protein to be translated was added to initiate translation. For reactions with SP-cells, 4.6 µl of the 100 µl produced during the generation of SP cells

procedure (approximately 150,000 cells) were used for each 25 µl translation reaction. For reactions involving DPMs the total 25 µl reaction volume contained 1.0 µl of DPMs with an absorbance at 260 nm of 50 units/ml. The only exception to this was for the experiments in which the luminally depleted DPMs were used and experiments in which *de novo* sources of disulphide bond formation were inhibited. For these experiments the 1 µl volume of DPMs added to the lysate was 5 times the concentration of the stock DPMs. H_2O was added to the translation mixture to bring the total volume of the mixture up to 25µl.

All translation reactions were incubated at 30°C in a water bath for the times indicated. Reactions were terminated by the addition of the thiol alkylating agent N-ethylmaleimide (NEM) (Thermo Scientific) to a final concentration of 50 mM (Braakman et al, 1992). For RRL samples containing no SP cells or DPMs, 2 ul of the lysate sample was added to SDS-PAGE loading buffer. Samples containing DPMs were centrifuged at 12,470 xg for 10 min and the supernatant removed before adding SDS-PAGE loading buffer to the samples. Samples containing SP cells were pulse centrifuged at 12,470 xg for a few second to pellet the cells. The lysate was then removed and 100 µl of KHM buffer was added. The pulse centrifugation was repeated and the KHM buffer was removed and SDS-PAGE loading buffer was added. Dithiothreitol (DTT) was added to a final concentration of 50 mM to indicated samples and all samples were heated to 105°C for 5 min. The samples were then run on a SDS-PAGE gel. The percentage of acrylamide used in the resolving regions of each SDS-PAGE was varied depending on the mRNA sequence used in the translation reaction: 7.5% acryladmide was use to resolve samples in which haemagglutinin was translated while 15% acrylamide was used for samples were preprolactin or cVIMP-cys was translated. The gels were then incubated with 10% acetic acid and 10% methanol solution for 20 minutes, dried and exposed to either a a phosphorimager plate or a BioMax MR film (Kodak). Lastly, the images captured using a phosphorimager plate were scaned using a FLA-7000 bioimager (Fujifilm).

2.7 Time synchronised translations

Aurintricarboxylic acid (ATCA) was added to the translation mixture of indicated reactions at a final concentration of 75 µM 5 min after incubation of the mixture in the 30°C water bath. ATCA prevents the re-initiation of translation, thus allowing the progression of disulphide bond formation in a synchronized set of nascent polypeptides to be analyzed (Stewart et al, 1971).

2.8 Inhibition of glucose-6-phosphate dehydrogenase, thioredoxin reductase and gluatathione reductase

The enzymes glucose-6-phosphate dehydrogenase, thioredoxin reductase and glutathione reductase were inhibited in the rabbit reticulocyte lysate by addition of the inhibitors dehydroepiandrosterone (DHEA), auranofin and carmustine respectively. Stock solutions of DHEA (Sigma-Aldrich) and auranofin (Sigma-Aldrich) were made up in dimethyl sulfoxide (DMS0) while stock solutions of carmustin (Sigma-Aldrich) were made up in ethanol. A volume of 2 ul of DHEA (3.4 mM) was added to RRL. A range of stock concentrations of auranofin and carmustine were made up and in each case 1 µl of the stock contributed to the 25 µl translation mixture volume. The translation mixture was incubated for 10 min on ice before translation reactions were initiated by adding mRNA and incubating the mixture at 30°C in a water bath. The translation reactions were then processed as described above.

2.9 Luminal depletion of DPMs

Crude saponin purchased from Signal-Aldrich was dissolved in buffer B (20 mM HEPES buffer pH 7.5 containing 0.25 M sucrose). Positively and negatively charged constituents were removed by passing the saponin mixture through SP and Q sepharose cation and anion exchange columns. The purified saponin was used to deplete the DPMs of luminal proteins by a methodology similar to previously described reports (Bulleid & Freedman, 1990). The DPMs were incubated on ice with 0.5% w/v of purified saponin for 10 min. The DPMs were then layered over a 0.5 M sucrose cushion and isolated by centrifugation at 109,000 xg for 10 min. The DPM pellet was resuspended in buffer B at one fifth their volume prior to depletion. The luminally depleted DPMs were added to the 25µl translation mixtures.

2.10 Determination of DPM luminal depletion by western blotting and coomassie blue staining

In order to determine the effectiveness of luminal depletion of DPMs by saponin treatment, supernatant (sn) and cell pellet (p) fractions of these depletion steps were retained for western blotting and Coomassie blue staining. The following primary antibodies were used for western blotting: calnexin rabbit polyclonal antibody (Sigma), PDI rabbit polyclonal (described previously) (John et al, 1993) and anti-ERp57 antibody (gifted from Dr. T. Wileman, University of East Angelia). In all cases the primary antibodies were incubated with the samples overnight. Anti-rabbit 800 nm fluorescent secondary antibody (Licor) was then used and the target proteins were detected using an Odyssey Sa Infrared Imaging System (Licor). Samples of sn and p fractions as well as untreated DPMs were separated by SDS PAGE electrophoresis. The SDS PAGE gel was then stained overnight in Coomassie blue and destained in water.

2.11 Inhibition of the *de novo* sources of disulphide bond formation in DPMs

The Ero1 inhibitor, QM295 was purchased from the ChemBridge Corporation in 10 mM stock solutions dissolved in DMSO. Stock solutions (200 µM) of the VKOR inhibitor warfarin were made up in DMSO. Where indicated QM295, and/or warfarin were added to DPMs at concentrations of 50µM each. The samples were mixed by gently pipetting and incubated on ice for 5 min. Due to the DMSO present in the warfarin and QM295 stock the DPMs resided in 25.5% (v/v) DMSO and 74.5%(v/v) buffer B (25mM HEPES pH 7.5, 50 mM KCL and 0.25 M Sucrose) during this incubation step. DPM samples that were not incubated with QM295 or Warfarin were instead incubated with 25.5% (v/v) DMSO and 74.5% (v/v) buffer B.

DTT (10 mM) was added to the indicated samples followed by incubating on ice for a further 10 min. Samples that were not subjected to the DTT treatment were incubated on ice without the addition of the DTT. The DPMs were then layered over a 0.5 M sucrose cushion and isolated by centrifugation at 109,000 xg for 10 min before being resuspended in buffer B at 5 times their original concentration. 1µl of the treated DPMs were then added to each 25µl translation mixture. QM295 and warfarin were also added to the translation mixture at a final concentration of 50 µM. Due to the presence of DMSO in inhibitor stock solutions the translation mixtures contained a final concentration 4% DMSO. DMSO was also added to a final concentration of 4% to DPM samples to which QM295 and warfarin were not added. Translation reactions and processing of samples then proceeded as described above.

2.12 Inhibition of the *de novo* sources of disulphide bond formation in SP cells

QM295 and warfarin were added as indicated indicated to SP cells at a concentration of 50 µM each. The SP cells were then incubated on ice for 5 min. DTT was added as indicated to samples of SP cells on ice at a final concentration of 10 mM for 10 min. SP cells were isolated by pulse centrifugation 12,470 xg for few second. The SP cells were resuspended in KHM buffer at their original volume. The SP cells were then added to translation mixtures.

2.13 Determination of PDI expression by western blotting

PDI expression was compared between untransfected H1080 cells (UT) and HT1080 cells over expressing the substrate trapping (ST) V5-tagged PDI mutant. Samples of WT and ST cells were loaded in duplicate on a SDS-PAGE gel. The samples were transferred to a nitrocellulose membrane and the membrane was cut to allow each of the duplicate samples to be probed separately with either anti-PDI antibody or anti-V5 antibody. The anti-V5 antibody was used to detect the exogenously expressed V5-tagged PDI. Each of the two nitrocellulose membranes was cut again using the molecular weight markers as a guide. The lower half of the nitrocellulose membranes was then probed for GAPDH which was used as loading control. Anti-PDI was detected with an anti-rabbit secondary antibody while anti-V5

and anti-GAPDH were detected with anti-mouse secondary antibodies. The 800nm fluorescence of the bound secondary antibodies was then detected by scanning with an Odyssey Sa Infrared Imaging System (Licor).

2.14 Release of translationally stalled prolactin

Prolactin synthesised from the translationally stalled (ts) PPL mRNA sequence is not released from the ribosome due to the lack of a stop codon in the sequence. The stalled prolactin nascent chain was released by treatment with the enzyme RNase A. The RNase A digested the ribosome causing release of the stalled peptide. RNase A (1µl of the 1mg/ml dissolved in water) was added to a 25µl RRL translation mixture programmed with tsPPL mRNA 20 min after the mixture was incubated at 30°C in a water bath. The mixture was gently mixed by pipetting and the incubation was continued for a further 20 min. The sample was then NEM treated and processed as described above.

2.15 Immunoisolation of prolactin bound to the substrate trapping PDI mutant

Translation reactions programmed with prolactin mRNA were conducted in the presence of HT080 cells overexpressing V5 tagged substrate trapping (st) PDI in which both PDI activate sites had CXXA mutations. Following completion of the translation reactions the samples were NEM-treated and the SP cells were isolated by centrifugation at 12,470 xg for a few seconds. The supernatant was removed and KHM buffer was added. The centrifugation was repeated and the KHM buffer was removed. IP buffer (500 µl) (50 mM Tris buffer pH 7.5, containing 1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 0.5 mM PMSF and 0.02% Na-azide) was then added to the pellet of SP cells. The samples were incubated in the IP buffer for 1 h on ice with occasional vortexing. This caused most of the SP cell pellet to become dissolved into the IP buffer. The samples were then centrifuged at 12,470 xg for 10 min and the supernatant was transferred to a fresh tube. A 10% suspension of protein A Sepharose was incubated for 30 min with 1% (w/v) BSA dissolved in PBS. The samples of dissolved SP cells were then incubated with the BSA treated protein A Sepharose for 30 min at 4°C on a rock and roller. The protein A Sepharose was then isolated by centrifugation at 1540 xg for 3 min. The supernatant was transferred to fresh tubes containing 50 µl of 10% protein sepharose that had also been pretreated with 1% BSA. The SP cell samples were then incubated overnight with V5 antibody (1 µl). The samples were then put through 3 wash cycles in which the protein A Sepharose was pelleted by centrifugation at 1540 xg for 3 min, the supernatant removed, and 1ml of IP buffer added and the sample mixed by vortex. Following the final wash the protein A Sepharose was pelleted and SDS-PAGE loading buffer was added to the sample. The sample was then heated for 10 min on a heating block at 105°C. Samples were split in two for reduced and non-reduced conditions. DTT was

added to a final concentration of 50 mM in samples that were to be reduced. Both the reduced and non-reduced samples were then returned to the heating block for a further 5 min. The samples were then processed by SDS-PAGE electrophoresis and the gels processed in the same way as described above.

Note that where indicated RIPA buffer (50mM Tris buffer pH 7.5, containing 150mM NaCl, 1% Triton, 1% deoxycholic acid and 0.1% SDS) was used instead of IP buffer for antibody incubation and/or washing steps.

Chapter 3: Results 1: A rabbit reticulocyte lysate translation system optimised for investigation of disulphide bond formation within the endoplasmic reticulum

3.1 Summary

The use of a cell lysate from rabbit reticulocytes for conducting *in vitro* translation reactions is a well established research method that is highly amenable to experimental manipulation of its biochemical and molecular components. This system has been used for investigation of a variety of cellular processes, including protein translation, protein targeting, and disulphide bond formation. The system however has limitations in investigation of redox-dependent processes such as ER localised disulphide bond formation as the system becomes oxidised during translation reactions. This oxidation causes disulphide bonds to form within newly synthesised protein even in the absence of a supplemented source of ER. This problem has commonly been circumvented by the addition of dithiothreitol (DTT) to the lysate at millimolar concentrations. The reducing agent prevents oxidation in the system however it is not an ideal solution as the action of the reductant differs from cellular redox homeostasis in several ways and limits certain experimental manipulations. We sought to develop a more effective RRL translation system for investigation of ER disulphide bond formation and disulphide interactions. We found that addition of glucose-6-phosphate (G6P) at a concentration of 0.5mM to the lysate prevented disulphide bond formation from occurring out with a source of ER but allows disulphide bonds to form within a source of ER in the form of SP cells. We found that under these conditions ER disulphide bond formation resembles cellular disulphide bond formation reported from pulse chase experimentation. Furthermore, we show that G6P mediates the prevention of disulphide bond formation out with the ER in the lysate by supporting the thioredoxin reductive pathway.

3.2 Introduction

In vitro translation systems are effective tools for the investigation of a variety of cellular processes including protein translation, targeting of proteins to the ER and disulphide bond formation. Theoretically the lysate of any cell type could be used for such systems however a few particular cell types have been optimised and are commonly used in scientific studies . The lysates of these systems provide a quasi cellular environment for protein synthesis and maturation. A key benefit of using an *in vitro* translation system is the components of the system are highly amenable to manipulation, thus allowing the involvement of specific molecular or biochemical components in biological processes to be investigated (Chong, 2014; Titus & Promega Corporation., 1991).

The RRL system is commonly chosen for studies investigating mammalian biological processes. Much of the development and optimisation of RRL translation systems was carried out during the late 1970s and early 1980s (Jackson et al, 1983a; Pelham & Jackson, 1976; Wu, 1981). Early studies found that lysate translation systems could be used to

investigate processing of nascent proteins in the ER if the system was supplemented with microsomes (Shields & Blobel, 1978). It was later found that SP cells derived from tissue culture could also be used an alternative source of ER material (Wilson et al, 1995).

Investigations into the RRL translation system by Richard Jackson, Tim Hunt, and others provided great insight into the biochemical requirements of the lysate (Hunt et al, 1983; Jackson et al, 1983a; Jackson et al, 1983b). A key finding of these papers was that in order for protein synthesis to be maintained, the redox systems of the lysate needed to be adequately supported (Jackson et al, 1983a; Jackson et al, 1983b)**.** It was found that this could be achieved by addition of DTT or certain sugar phosphates capable of generating NADPH (Jackson et al, 1983a). DTT has since been commonly added to RRL reactions at millimolar concentrations for this purpose (Marquardt et al, 1993). DTT however also causes reduction of the luminal proteins of microsomes (Marquardt et al, 1993). As disulphide bonds are redox sensitive they cannot be maintained in proteins in the presence of a strong reductant such as DTT (Cleland, 1964). Thus it is not possible to analyse disulphide bond formation in nascent proteins in the lysate under such conditions. This problem has been circumvented by also adding millimolar concentrations of GSSG to the lysate which allow disulphide bonds to form and be maintained even in the presence of millimolar concentrations of DTT (Scheele & Jacoby, 1982). The DTT/GSSG system has since been commonly used in RRL translation reactions in experiments supplemented with microsomes in which disulphide bond formation in the nascent translated proteins is being analysed (Kaderbhai & Austen, 1985; Marquardt et al, 1993).

The DTT/GSSG RRL system however has several problems, particularly in the analysis of disulphide bond formation. Firstly, it is known that oxidised glutathione induces disulphide bond formation in purified proteins in the absence of oxidoreductase enzymes (Kaderbhai & Austen, 1985) . Therefore it should be considered that the disulphide bond formation in microsomes in RRL supplemented with GSSG may be partially attributed to the oxidising effect of GSSG in the absence of PDI and not just to the enzymatic action of PDI family members. Secondly, as the GSSG is present throughout the lysate and not localised to supplemented ER luminal components it may also induce intra and intermolecular disulphide bonds prior to the nascent peptides being translocated into the ER luminal component. This also deviates from the current *in vivo* model of ER disulphide bonds formation where the disulphides are predicted to be introduced co-translationally during translocation to the ER (Chen et al, 1995; Molinari & Helenius, 1999). Thirdly, this method relies on millimolar concentrations of DTT being present during the reaction. DTT is a non-physiological chemical and is also known to cause reduction of the ER lumen and thus also perturb the system (Marquardt et al, 1993). Furthermore DTT also causes hydrogen peroxide to be

produced as it is a substrate of Ero1 (Gross et al, 2006). This also perturbs the redox conditions of the system. These three points indicate that using DTT/GSSG in RRL is likely to result in a system of disulphide bond formation that is distorted from how this process occurs *in vivo*. A forth criticism is that use of the DTT/GSSG also limits the range of biochemical and molecular experimental manipulations available for investigation. For example, it would be problematic to investigate the influence of particular chemicals or enzymes on disulphide bond formation using this system. This is because the presence of GSSG and particularly DTT greatly perturb the disulphide bond formation of the system and therefore would reduce the biological validity of the findings.

Considering these problems, some investigations have not used the DTT/GSSG system in studies using RRL to investigate protein disulphide formation/interactions (Alvares et al, 1999; Chambers et al, 2008). However, this can result in aberrant disulphide bond formation due to the oxidising conditions of the lysate during translation reactions. For example, one investigation in which the DTT/GSSG system was not used, a substantial proportion of the translated protein formed multiple high molecular weight mixed disulphide species under non-reducing conditions (Chambers et al, 2008). The authors of this investigation acknowledge that the disulphide interactions identified in the investigation were likely to have been influenced by the oxidising conditions of the lysate (Chambers et al, 2008). This further stresses the problems of using the RRL system for investigation of disulphide bond interactions.

The early papers by Tim Hunt, Richard Jackson and others provided important indications of a more elegant method of supporting the redox system of RRL during translation reactions. They found that the addition of NADPH generating metabolites to the lysate, particularly G6P appeared to support the thioredoxin redox system of the lysate and found that this may reduce aberrant disulphide bond formation in the lysate (Jackson et al, 1983a). Indications that G6P prevented disulphide bond formation came from two experiments using RRL filtered of low molecular weight components. Firstly, it was found that when the filtered lysate was treated with radiolabelled (C14) N-ethylmaleimide (NEM) following a translation reaction in which oxidised insulin was also added, a greater amount of C14 NEM covalently modified the insulin if 0.2mM G6P was also present during the reaction (Jackson et al, 1983b). NEM reacts with reduced thiols, but not those which have formed disulphides (Winther & Thorpe, 2014). Therefore, this suggested that the thiol groups of the insulin become more reduced during incubation of the lysate with G6P (Jackson et al, 1983b). Similarly, a second C14 incorporation experiment found that an unidentified 24kDa protein that is present in the lysate has a lower propensity to form disulphide bonds during lysate translation reactions if G6P is present (Jackson et al, 1983b). Thus it was shown that G6P supports the redox

system of the lysate, both in its ability to remove disulphides from oxidised protein and prevent disulphide formation in reduced protein (Jackson et al, 1983b).

While the indications of these early experiments from Hunt, Jackson and others are insightful, subsequent experimental improvements now allow us to make a more direct analysis of the effect of G6P on disulphide bond formation on a RRL translation system. The results in this chapter clearly show that when used in RRL translation reactions at a concentration of 0.5 mM, G6P specifically prevents the formation of disulphide bonds in nascent protein out with the ER, while allowing disulphide bonds to form within the ER. Furthermore, it is shown that the arrangements of disulphide bonds that form within translocated proteins are consistent with previously reported findings using cells. Evidence is also presented indicating that prevention of disulphide bonds out with the ER by G6P is mediated by biochemically supporting the thioredoxin reductive pathway thus indicating support of the RRL redox system by G6P. These findings are of importance as they show that the G6P-optimised RRL system is a superior method for investigation of disulphide bond formation and protein disulphide interactions than the previously reported RRL systems.

3.3 Results

3.3.1 Investigation of disulphide bond formation within the ER using a RRL translation system is problematic as disulphide bonds form in proteins synthesised in the absence of a source of ER

Previous investigations have indicated that disulphide bond formation during RRL translation reactions could be prevented be the addition of certain metabolites capable of generating NADPH within the lysate (Jackson et al, 1983b). In particularly, G6P has been indicated to be especially effective in this role (Jackson et al, 1983b). As a preliminary experiment we translated mRNA encoding HA in the presence of 0.2mM G6P (Fig 3.1). This G6P concentration had been used in a previous study by Jackson *et al* and it was indicated that less disulphide bond formation occurs during translation reactions using gel-filtered RRL if this concentration of G6P was present (Jackson et al, 1983b).

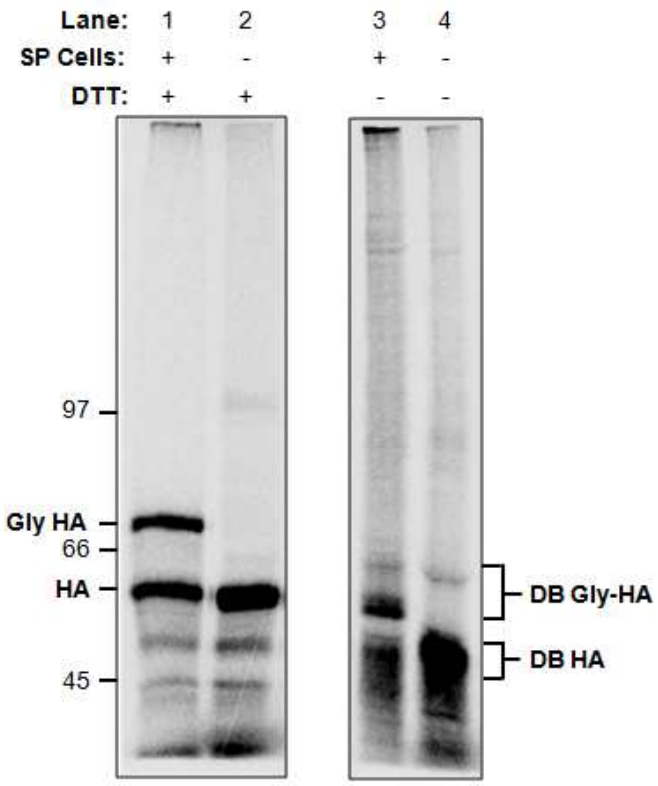

Figure 3.1: HA synthesised in a RRL translation system undergoes extensive disulphide bond formation even in the absence of a source of ER. mRNA encoding influenza HA was translated for 60 min using a RRL translation system. SP cells were present in the indicated samples while G6P was present in all samples at a concentration of 0.2mM. The reducing agent dithioltheriotol (DTT) was added to the loading buffer of the indicated samples before running on the SDS-PAGE gel. DTT abolishes the disulphide bonds presents within the proteins of the sample. As such, comparison between samples with and without DTT allows the disulphide dependent gel mobility shifts to be observed. It can be seen that HA only becomes glycosylated in the presence of SP cells. However, unlike what occurs *in vivo* it can be seen that disulphide bonds are formed even in the absence of the source of ER provided by the SP cells. The formation of disulphide bonds out with the ER interferes with the specific analysis of ER disulphide bond formation. N.B.: All lanes shown are from the same SDS-PAGE gel. HA: unglycosylated reduced hemagglutinin, Gly HA: glycosylated reduced hemagglutinin, DB Gly-HA: disulphide bonded glycosylated hemagglutinin, DB HA: disulphide bonded unglycosylated hemagglutinin.

When translated in the absence of an added source of ER, HA was synthesised as a single major translation product (Fig. 3.1, lane 2). In the presence of SP cells an additional higher molecular weight was observed (Fig 3.1, lanes 1). This higher molecular weight band has previously been observed (Marquardt et al, 1993) and is due to the addition of 7 glycans to HA that is processed in the ER. HA also contains 6 disulphide bonds. It is known that

formation of disulphides results in a reduction of the hydrodynamic radius of a protein resulting in faster gel mobility (Wilkins et al, 1999). It was however seen that disulphide bonds formed in the presence of 0.2mM G6P even in the absence of SP cells (Fig 3.1, lane 4). This result showed that the formation of disulphide bonds out with the ER had not been prevented and indicated that conditions of the lysate during the reaction were oxidising.

3.3.2 G6P prevents the formation of disulphide bonds out with the ER in proteins synthesised with a RRL translation system

As disulphide bonds formed in our preliminary experiment using 0.2mM G6P we hypothesised that the concentration used may not be optimal to completely prevent disulphide bond formation. We therefore added a range of G6P concentrations to determine if disulphides are prevented by G6P at higher concentrations.

We carried out RRL translation reactions for the substrates HA, PPL and cVIMP-cys, in which G6P was titrated at various concentrations (Fig 3.2). Each of these proteins has different properties and disulphide arrangement. All three of these proteins are targeted to the ER, however only PPL is soluble within the lumen as HA and cVIMP-cys both contain transmembrane domains (Christensen et al, 2012; Teilum et al, 2005; Webster et al, 2013). Furthermore, of these transmembrane proteins only the cysteine residues of HA are exposed to the ER lumen as the two cysteine residues of cVIMP-cys are exposed to the cytosol. The substrates also differ in the number of intrachain disulphides they form with HA, PPL and cVIMP-cys forming six, three and one intrachain disulphides respectively. Due to these differences the use of these three substrates allowed for a thorough analysis of the effect of G6P on disulphide formation.

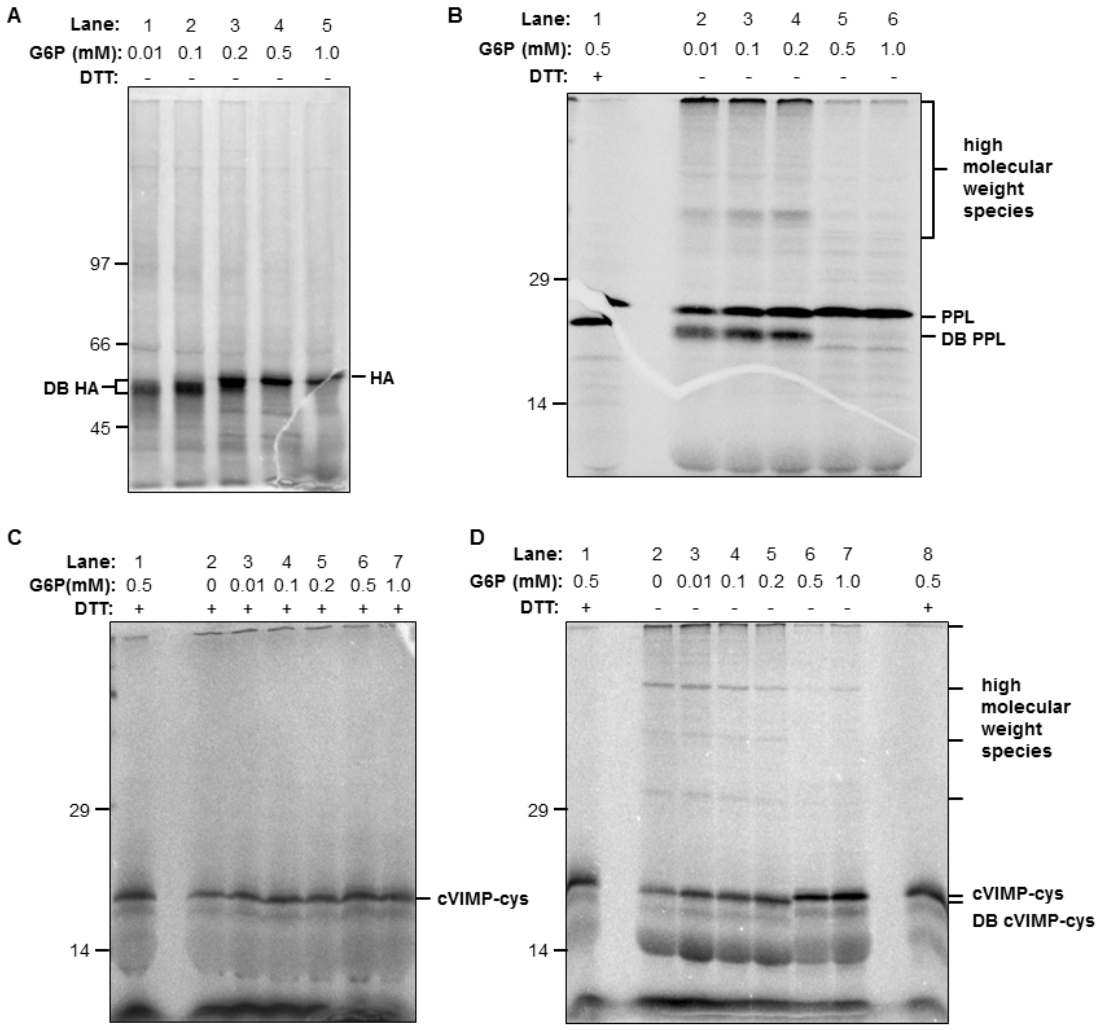

Figure 3.2: The addition of G6P prevents the formation of disulphide bonds out with the ER in proteins synthesised with a RRL translation system. Translation reactions were carried out for 60 min with mRNA encoding HA **(A)**, PPL **(B)** and cVIMP-cys **(C, D)** in the presence of the indicated concentrations of G6P. No source of ER was present. It can be seen that a concentration of 0.5mM G6P prevents the formation of disulphide bonds in each of the substrates. HA: non-disulphide bonded haemagglutinin, DB HA: disulphide bonded haemagglutinin, PPL: non-disulphide bonded preprolactin, DB-PPL: disulphide bonded preprolactin, cVIMP-cys: non-disulphide bonded cVIMP-cys, DB: disulphide bonded cVIMP-cys.

Analysis of the gel mobility of the translated proteins under non-reducing conditions indicates that each of these substrates formed disulphide bonds at low concentrations of G6P. Formation of these disulphides was however prevented in all three cases by a G6P concentration of 0.5mM.

In the case of HA the disulphides that formed with low G6P concentrations appeared to be predominantly intramolecular as evident from the formation of smeared bands of slightly faster mobility than full length HA (Fig 3.2A). This indicated that at low G6P concentrations

the HA of these samples were made up of a mixed population of HA molecules with varying numbers and/or arrangements of internal disulphide bonds. Interestingly, the extent of HA intrachain disulphides can be seen to gradually decrease as concentrations of G6P increased from 0.01-0.5mM. This suggested that formation of the disulphides in nascent HA is highly sensitive to the oxidation conditions of the RRL. It also indicated that the banding pattern of nascent HA could be used as a reporter of the oxidising conditions of the lysate.

It can be seen that when translated at low G6P concentrations that PPL formed three populations which could be categorised by the type of disulphides (Fig 3.2B, lanes 2-4). One population consisted of a large proportion of the synthesised PPL with equivalent mobility to the reduced form of the protein (PPL). A second population was of faster mobility which correlated with the previously reported mobility of intrachain disulphide bonded prolactin (DB-PPL) (Kaderbhai & Austen, 1985) which most likely indicated that a long range disulphide had formed in the protein (though not necessarily the C88 to C204 long range disulphide of native bovine prolactin) (Wallis, 1974). A third population consisted of several higher molecular species indicating that the PPL had formed various interchain disulphide bonds either between PPL molecules and/or between PPL and other proteins of the lysate. Both the intrachain and interchain disulphide bonds were abolished at a G6P concentration of 0.5mM G6P with the disappearance of these disulphides occurring abruptly between concentrations of 0.2mM and 0.5mM rather than gradually over a larger concentration range as had been observed with HA.

The third substrate we used was cVIMP-cys (Fig 3.2 C-D). cVIMP-cys is a variant of the ER membrane protein VIMP that was created by the research group of Lars Ellgaard (University of Copenhagen) (Christensen et al, 2012). The cVIMP-cys construct contains only the cytosolic region of VIMP and contains one disulphide bond. The gel shift caused by the formation of the intrachain disulphide in purified cVIMP-cys has been reported (Christensen et al, 2012) and matches the corresponding shift for intrachain disulphide bonded cVIMP-cys observed with our assay. When translated in our RRL system under varying concentrations of G6P it can be seen that under non-reducing conditions at G6P concentrations less than 0.5mM all of cVIMP-cys is either intrachain disulphide bonded or interchain disulphide bonded (Fig 3.2D, lanes 2-5). Unlike PPL and HA none of the cVIMP-cys remains in the reduced state suggesting the two cysteines of cVIMP-cys are particularly reactive. Similarly to PPL there is an abrupt difference in the disulphide bonding profile of cVIMP-cys between the G6P concentrations of 0.2 and 0.5mM. The entire population of cVIMP-cys is disulphide-bonded at 0.2mM G6P while almost none of the protein is disulphide bonded at 0.5mM.

These findings indicate that the addition of G6P to RRL prevents the formation of intra and interchain disulphide bonds in proteins translated with the system.

3.3.3 Complex sequences of ER localised disulphide bond formation can be observed using the G6P optimised RRL translation system

Having prevented the formation of disulphide bonds in the translation system when a source of ER was absent, our next objective was to determine if disulphide bond formation would occur in the presence of ER and determine if these disulphides would form as they do in intact cells. For this experiment we again used the model substrate influenza HA as a report is available of disulphide bond formation in HA from pulse chase experimentation which we could use as a comparison with our results (Braakman et al, 1991). As before it could be seen that under reducing conditions a higher molecular weight band representing glycosylated HA was observed only when SP cells were present (Fig 3.3, lanes 1 and 2 and Fig 3.1, lanes 1 and 2). When HA was translated in the presence of 0.5 mM G6P and separated under non-reducing conditions, four disulphide-bonded forms of the glycosylated product were observed (Fig 3.3, lanes 3 to 8).

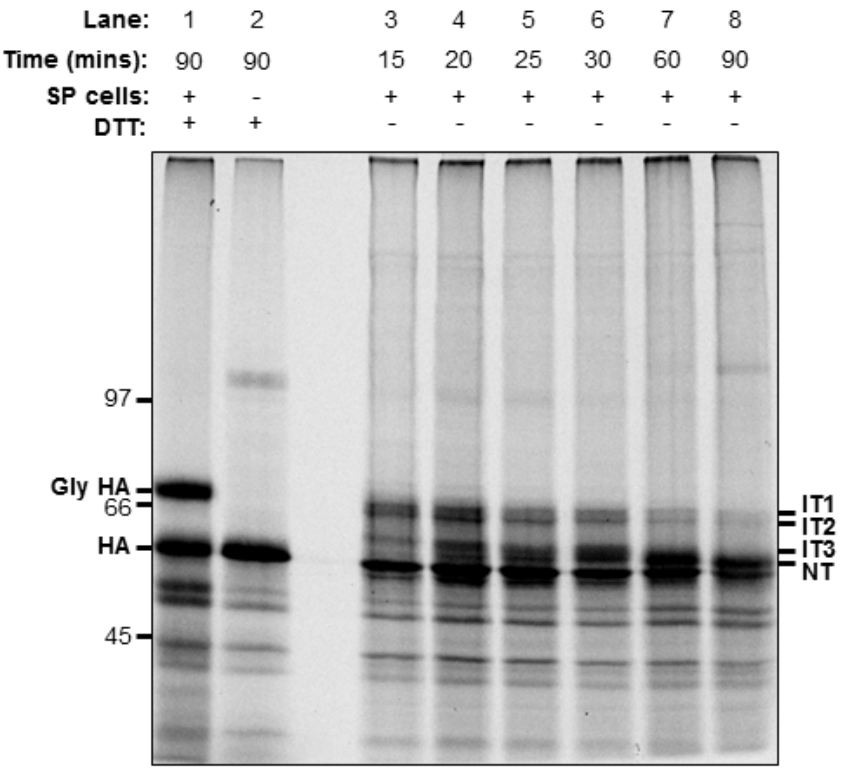

Figure 3.3: ER localised disulphide bond formation of HA can be observed using a G6P optimised RRL translation system. Translation reactions programmed using mRNA encoding influenza HA was conducted in the presence 0.5mM G6P. Reactions proceeded for the indicated times and in the presence of SP cells where noted. ATCA was added to a concentration of 75 µM 5 min after initiating translation to prevent further translation initiation, thereby allowing distinct analysis of disulphide bond formation within a time synchronised set of nascent proteins. Four disulphide bonded

intermediates of glycosylated HA can be observed (IT1-3 and NT). The fastest mobility form of these intermediates (NT) accumulates over the course of the reaction. HA: unglycosylated reduced hemagglutinin, Gly HA: glycosylated reduced hemagglutinin, IT1-IT3: glycosylated hemagglutinin disulphide bonded intermediates 1 to 3, NT: native disulphide bonded hemagglutinin.

A previously study investigated the disulphide bond formation of HA using a pulse chase assay with 2 different HA sequences and several different mammalian cell lines (Braakman et al, 1991). In this study three different disulphide bonded HA forms where identified that were designated as IT1 and IT2 (disulphide bonding intermediates 1 and 2) and NT (native fully oxidized form) (Braakman et al, 1991). Consistent with this previous nomenclature we have designated the 4 disulphide bonded forms identified in our assay as IT1-3 and NT. The presence of four disulphide bonded forms within our assay would perhaps be expected as the Japan strain of HA which we use contains 4 long range disulphides (C19-C477, C57-C288, C105-C149 and C292-C316) and two short range disulphides (C70-C82 and C484-C488) (Gething et al, 1980). Long range disulphides generally cause greater changes in the hydrodynamic volume and hence gel mobility of proteins while short range disulphides have little effect on gel mobility and usually cannot be detected (McManaman & Bain, 2002).

It can be seen from the time course shown in figure 3.3 that the low mobility disulphide bonded forms IT1 and IT2 are the most prominent forms at the earlier time points. However, these two forms gradually disappears over time while, simultaneous, the faster mobility forms of IT3 and NT become more prominent. At the final 90 min time point the majority of the disulphide bonded HA is in the fastest mobility NT disulphide bonded form. As the accumulation of the faster mobility forms is concurrent with the disappearance of the lower mobility forms it would appear that either formation of new disulphides and/or isomerization of disulphides over the time course are occurring and thus converting the slower mobility forms into the faster mobility forms. As the NT forms is the final form that the HA eventually reaches it would appear that this represents the mature disulphide bonded form.

It is interesting to note that while the study by Ineke Braakman *et al* identified 3 HA disulphide bonded forms in each of their HA pulse chase assays the relative mobility of what they called the IT2 form varied between the cell types used in their assay (Braakman et al, 1991). In experiments using either HeLa or CHO cell IT2 was found to run at mobility slightly faster than IT1, consistent with the mobility of IT2 in our assay. In other experiments using CV-1 and 3T3 cells IT2 was observed to run closer to the NT form, consistent with the mobility of IT3 in our assay. Both of these pulse chase experiments used the same Japan strain of the HA sequence that we have used for our study and therefore sequence difference cannot account for the differences in disulphide bonding pattern. The

inconsistency in the number of HA disulphide bonded forms detected by Braakman *et al* (1991) and ourselves must therefore either be due the different cell types used between the two studies or differences in the assays used. As no completely comparable experiment has been conducted (such as using the same HA sequence, the same cell type and normalising certain variables between each assay) this cannot be determined at this point. Never the less both sets of results report disulphide bonded forms of HA with consistent mobilities that in both cases accumulate in the NT form.

We conclude from this finding that disulphide bond formation of complex substrates such as HA within our G6P optimised RRL assay does appear to closely resemble cellular disulphide bond formation of the same substrate as detected by pulse chase assay.

3.3.4 G6P prevents the formation of disulphides out with the ER by supporting the thioredoxin reductive pathway

Our next objective was to determine the biochemical mechanism by which G6P prevents the formation of disulphide bonds out with the ER. We hypothesised that metabolism of G6P within the lysate was generating NADPH which was then supporting either the thioredoxin and/or the glutathione reductive pathways. One or both of these reductive pathways were in turn expected to be responsible for maintaining the thiol groups of the proteins synthesised in the lysate in a reduced state (Fig 3.4A). In order to test this hypothesis we sought to inhibit key enzymes supporting these reductive pathways. Namely, we choose to inhibit the enzymes G6P dehydrogenase (G6PD), thioredoxin reductase (TrxR) and glutathione reductase (GR) by using the inhibitors dehydroepiandrosterone (DHEA), auranofin and carmustine (also known as BCNU) respectively (Fig 3.4B). DHEA is an uncompetitive inhibitor of G6PD, auranofin appears to act as a non-competitive inhibitor of TrxR, while carmustine is an irreversible inhibitor of GR (Go et al, 2013; Liu & Sturla, 2009; Shantz et al, 1989). Therefore, if the inhibition of these enzymes caused disulphide bonds to form out with the ER even in the presence of G6P then this would indicate that these enzymes were required components of the biochemical pathway utilised by G6P to prevent the formation of these bonds.

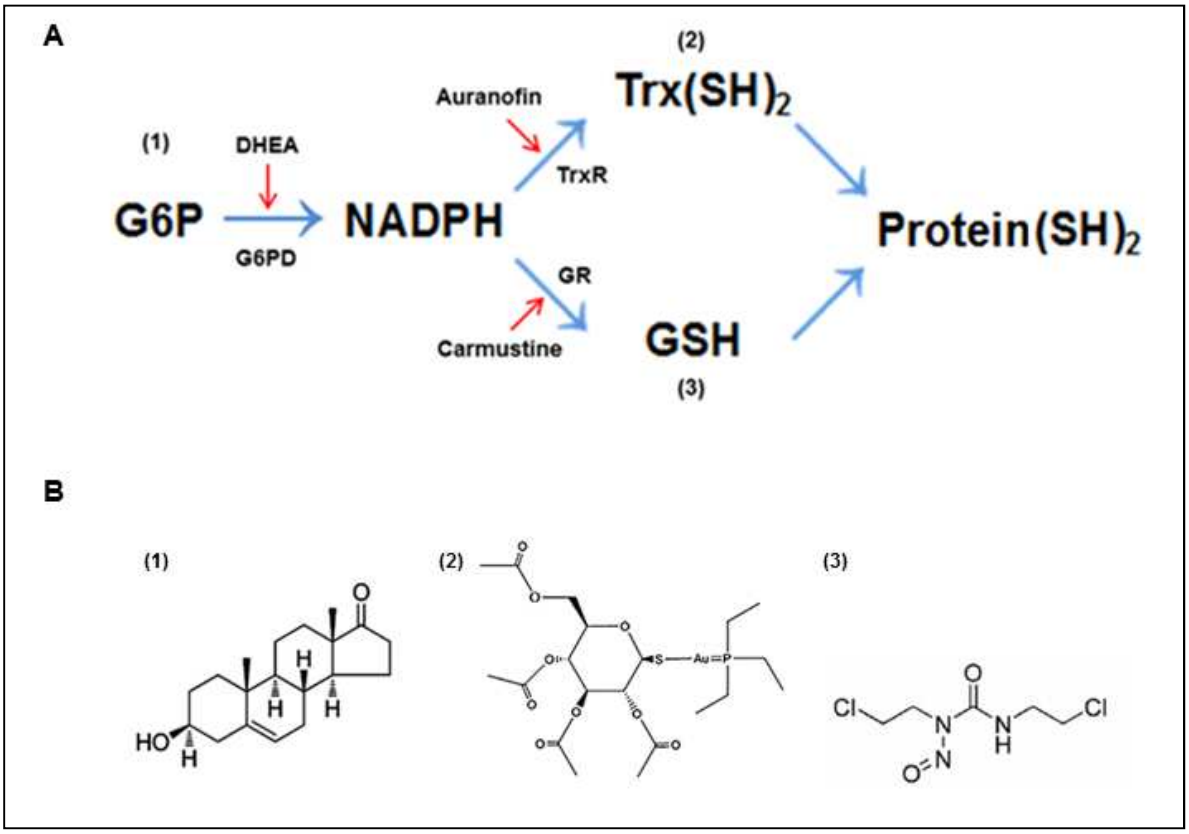

Figure 3.4: G6P prevents the formation of disulphides in nascent non-translocated proteins by supporting either the thioredoxin and/or glutathione reductive pathways. Inhibitors that target key enzymes of these pathways can be used to determine the enzymes involved in this reaction. **(A)** The conversion of G6P to 6-phophogluconolactone by the enzyme glucose-6-phosphate dehydrogenase (G6PD) is the first step of the pentose phosphate pathway and results in the generation of NADPH from NADP (1) (Berg et al, 2007). The NADPH generated may then in turn support the maintaince of reduced thiol groups within nascent non-translocated proteins by supporting either the thioredoxin or glutathione reductive pathways. NAPDH maintains thiredoxin in a reduced state by biochemically supporting the action of thioredoxin reductase (TrxR). The reduced thioredoxin in turn may maintan the thiol groups of target proteins in a reduced state (2) (Fig 1.16) (Holmgren & Lu, 2010). Similarly, NADPH supports the conversion of glutathione disulphide to reduced glutathione (GSH) by the enzyme glutathione reductase (GR) (Fig 1.14). GSH is hypothesised to maintain protein thiol groups in a reduced state (3) (Lopez-Mirabal & Winther, 2008). **(B)** The key enzymes of these pathways, namely; G6PD, TrxR and GR are the targets of the inhibitors DHEA (1), auranofin (2) and carmustine (3) respectively (Frischer & Ahmad, 1977; Go et al, 2013; Gordon et al, 1995). The structures of DHEA, auranofin and carmustine are as presented by Hamilton *et al* (2012), Roder and Thomson (2015), and Liu and Sturla (2009) respectively (Hamilton et al, 2012; Liu & Sturla, 2009; Roder & Thomson, 2015).

It can be seen that inhibition of G6PD by DHEA caused disulphide bonds to form in HA even in the presence of 0.5mM G6P (Fig 3.5, lane 8). This supported the hypothesis that the NADPH generated by G6PD during the metabolism of G6P was required to support the reductive pathways of the lysate. While, it can be seen that disulphides form when DHEA is added the extent of disulphide formation was not as great as when G6P was absent (Fig 3.5, compare lanes 7 and 8). This is because DHEA is an uncompetitive inhibitor (Shantz et al, 1989) and as such its addition will lower but not block the activity of the enzyme (Lehninger et al, 2005).

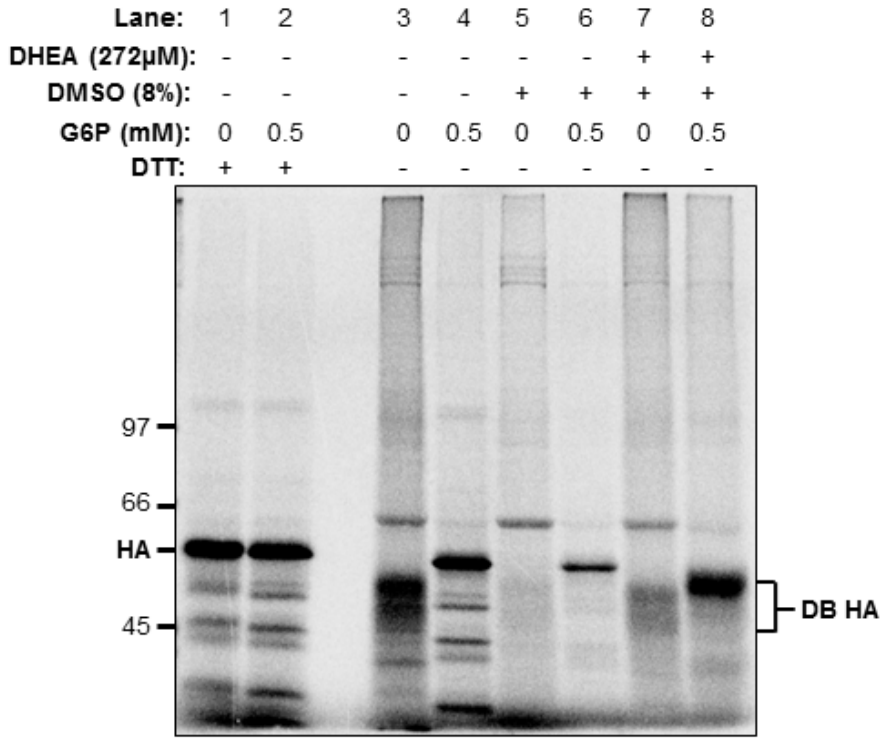

Figure 3.5: G6PD is required to maintain nascent protein thiol groups in a reduced state during G6P optimised RRL translation reactions. Translation reactions programmed with influenza HA were carried out for 60 min with either 0mM or 0.5mM G6P. DHEA dissolved in DMSO were added to the indicated samples to a final concentration of 272µM. The DMSO of samples in which DHEA was added made up 8% (v/v) of the total reaction volume. 8% DMSO was also added to the indicated samples in the absence of DHEA. It can be seen that disulphide bonds formed in the presence of G6P if DHEA was also added. This indicates the G6PD is required to mediate the prevention of disulphide bond formation in the presence of G6P. The radiolabeled signal of the sample in which DMSO was added is reduced suggesting addition of the solvent reduced translation efficiency. The presence of DMSO in the absence of DHEA did not however have any effect on disulphide bond formation. HA: reduced hemagglutinin, DB HA: disulphide bonded hemagglutinin.

We next targeted the enzyme TrxR for inhibition using the gold containing compound auranofin (Fig 3.6). A previous study has shown that incubation of purified TrxR with equimolar concentrations of auranofin causes near complete inhibition of the activity of the enzyme (Go et al, 2013). This thus indicates that auranofin acts as a non-competitive inhibitor of TrxR. Treatment of the RRL with auranofin caused disulphides to form in the presence of G6P, with the maximal levels of disulphide bonds forming at concentrations of 20µM and above (Fig 3.6). This finding suggests that thioredoxin is responsible for maintaining the reduced state of nascent protein thiol groups within the RRL system in the presence of G6P. However, it has also been reported that auranofin can cause the inhibition of GR when used at micromolar concentrations (Gromer et al, 1998). The concentration of TrxR and GR in the RRL is unknown as neither the concentration of these enzymes within reticulocyte cells, nor the concentration of lysed cells per volume of lysate known. Therefore, it is not possible to estimate the expected concentration of auranofin required to inhibit the thioredoxin reductase of the lysate without causing inhibition of glutathione reductase. It therefore remained ambiguous whether the effect of auranofin on disulphide bond formation in the presence of G6P was due to inhibition of TrxR or GR.

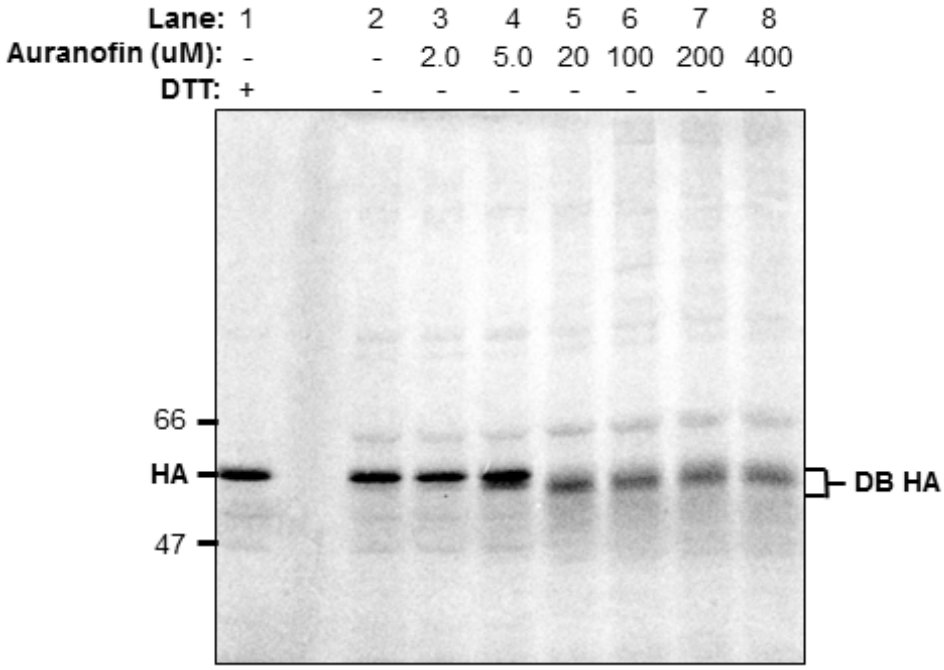

Figure 3.6: The TrxR inhibitor auranofin causes disulphide bonds to form during G6P optimised translation reactions. Translation reactions programmed with HA were conducted for 60 min in the presence of 0.5mM G6P. Auronofin was titrated with the reaction mixture at increasing concentrations. 4% of the total reaction volume of all samples was DMSO which was present to maintain the solubility of auranofin. It can be seen that disulphide bonds can observed even in the presence of 0.5mM G6P at concentrations of auranofin of 5µM and above. This suggests that TrxR is

required to mediate the prevention of disulphide formation in nascent proteins in RRL in which G6P is present. HA: reduced hemagglutinin, DB HA: disulphide bonded hemagglutinin.

To resolve the ambiguity we next targeted GR for inhibition by carmustine. Carmustine is a potent inhibitor of GR and has been shown to prevent the reduction of GSSG to GSH (Ma et al, 2003). Therefore, if the effect of auranofin on disulphide bond formation was caused by inhibition of GR rather than TrxR we would expect to see a similar effect with carmustine. However, it can be seen that carmustine only had an effect on disulphide bond formation when used at a concentration of 3.66 mM (Fig 3.7). This concentration is far higher than the concentrations at which such an inhibitor would be expected to cause an effect (N.B. severely impaired cellular response to oxidative stress in cells have been detected following the addition of 120µM carmustine implying it is effective at this concentration) (Ma et al, 2003). Furthermore, 3.66 mM concentration is also higher than the concentration (0.5 mM) of the G6P metabolite used in this reaction. This carmustine concentration is also close to the experimental limits as the inhibitor requires to be dissolved in ethanol at a concentration near its solubility limits (19.6 mg/ml) before being added to the reaction. As ethanol causes increasing inhibition of RRL translation (Wu, 1981) as it is added at higher concentrations it is not feasible to add large volumes of carmustine dissolved in the ethanol inhibitor to the reaction mixture. These results suggest that the effect carmustine had on disulphide bond formation at 3.66 mM was not due to inhibition of glutathione reductase but is most likely due to an effect of the carmustine chemical itself on disulphide formation when used at high concentrations. These findings also indicate that the effect on disulphide bond formation caused by auranofin treatment was due to auranofin inhibiting TrxR and not due to auranofin inhibiting GR.

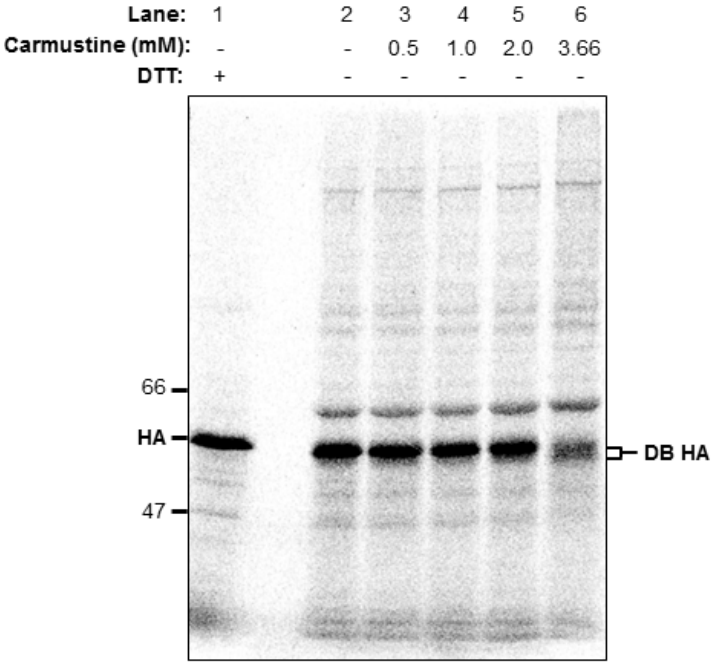

Figure 3.7: The GR inhibitor carmustine only causes disulphide bonds to form during G6P optimised translation reaction reactions when present at milli molar concentrations. Translation reactions programmed with HA were conducted for 60 min in the presence of 0.5mM G6P. Carmustine was titrated at increasing concentrations. 4% of the total reaction volume of all samples was ethanol which was present to maintain the solubility of the inhibitor. Carmustine can be seen to only have an effect on the disulphide bond formation at HA when present at a concentration of 3.66mM. HA: reduced hemagglutinin, DB HA: disulphide bonded hemagglutinin.

In order to further verify our findings we tested the effect of combined inhibition of TrxR and GR (Fig 3.8). We hypothesised that if both the thioredoxin and glutathione pathways were involved in maintaining thiol groups in a reduced state in the presence of G6P then inhibition of both of these pathways would have a greater effect on disulphide bond formation than inhibition of each of these pathways alone. Once again it could be seen that auranofin had a clear effect on disulphide bond formation (Fig 3.8, lane 4) however, no enhanced effect was seen when both auranofin and carmustine were used in combination at concentrations of 40µM and 1.83mM respectively (Fig 3.8, lane 6).

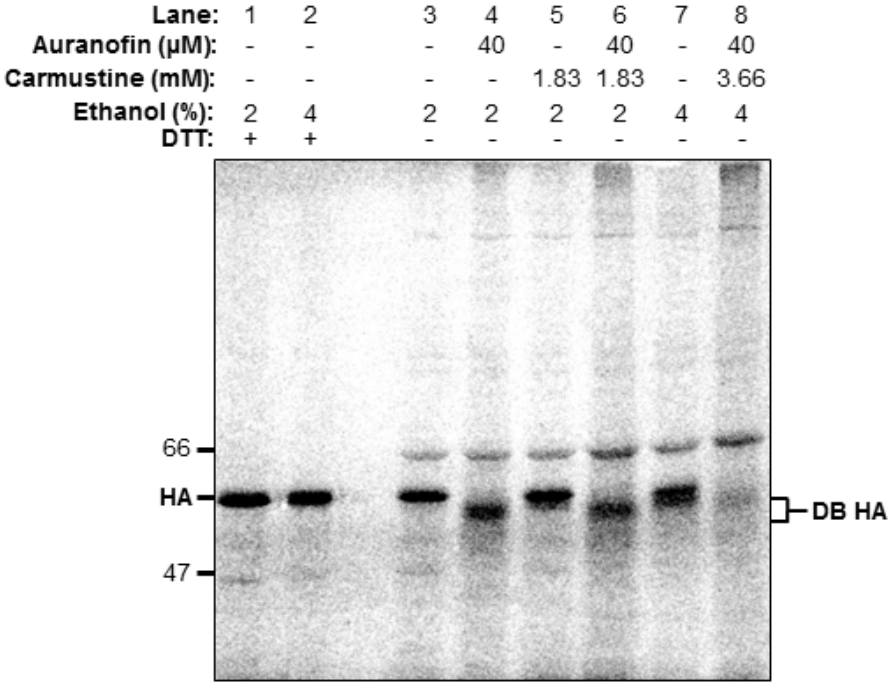

Figure 3.8: Combined inhibition of TrxR and GR in G6P optimised translation reactions by the addition of auranofin and carmustine does not cause greater formation of disulphide bonds than inhibition with these inhibitors individually. Translation reactions programmed with HA were conducted for 60 min in the presence of 0.5mM G6P. The solvents DMSO and ethanol were added to each sample to maintain solubility of the inhibitors auranofin and camustine. DMSO made up 2% of the reaction volume of each sample. The volume percentage made up of ethanol in each sample is indicated. Auranofin and carmustine were present in the samples at the concentrations indicated. It can be seen that auranofin and to a lesser extent carmustine cause disulphide bonds to form in the presence of G6P however this effect is not enhanced by addition of these inhibitors in combination. HA: reduced hemagglutinin, DB HA: disulphide bonded hemagglutinin.

Our previous results had shown that the maximum effect of auranofin on disulphide bond formation of HA is achieved at 20μM (Fig 3.6). Therefore, the 40μM used for this experiment is in excess of that required to achieve maximal effect. Due to the aforementioned experimental limitations with the concentration at which we could add carmustine it was difficult to add carmustine at the concentrations at which it has an effect on disulphide bond formation in combination with auranofin. This is because auranofin required to be added dissolved in ethanol while carmustine must be added dissolved in DMSO and both ethanol and DMSO reduced translation efficiency when added at high concentrations to the translation reactions. Despite this we attempted to add carmustine and auronfin together at concentrations of 40μM and 3.66mM respectively. However as anticipated the translation

efficiency of this reaction was poor due to the high concentrations of both DMSO and ethanol present which resulted in a low intensity radiolabelled signal (Fig 3.8, lane 8). Never the less, it can be seen from the low intensity signal that the extent of disulphide bond formation in the present of 40 µM auranofin and 3.66 mM carmustine was not greater than when 40 µM auranofin was used alone (Fig 3.8, compare lanes 4 and 8).

The results of these experiments consistently indicate that the TrxR pathway is solely responsible for maintaining the thiol groups of HA in a reduced state during reticulocyte lysate translation reactions in the presence of G6P. The glutathione reductive pathway does not appear to make a contribution even when the thioredoxin pathway is inactivated.

3.4 Discussion

3.4.1 A modified RRL system for investigating disulphide formation

In this chapter we have described a modified RRL translation system that can be used to investigate ER specific disulphide bond formation and disulphide interactions within nascent proteins in the ER. The addition of G6P to this system stabilises the lysate redox conditions and prevents the formation of disulphide bonds out with the ER. In the two subsequent results chapter this system is utilised to investigate two aspects of ER disulphide formation. In chapter 4 the contribution of enzymatic sources of *de novo* disulphide formation are analysed by inhibiting key enzymes involved in this process. In chapter 5 the disulphide interaction of PDI with nascent proteins within the ER is investigated. These subsequent investigations (and future studies investigating aspects of ER disulphide formation with a RRL system) can be conducted more effectively than would have been possible with a DTT/GSSG supplemented RRL system. This is primarily because the redox conditions of the G6P supplement RRL is stabilised in a physiological manner by supporting the reductive pathways of the lysate rather than the more artificial stabilisation achieved by the addition of DTT and GSSG.

3.4.2 Use of DHEA, auranofin and carmustine inhibitors with a RRL system

In this study we also investigated the biochemical route that G6P mediated the prevention of non ER disulphide bonds within a RRL translation system using the inhibitors DHEA, auranofin and carmustine. To our knowledge, this is the first such study to use these inhibitors with an *in vitro* translation system. As such the most relevant reports for comparison of the effects of the inhibitors come from studies in which they were used against purified proteins or cells.

DHEA has been found to inhibit partially purified G6PD (5.1µg) in an uncompetitive manner with Ki values of approximately 15 to 20 µM depending on whether the NADPH or G6P

substrate concentrations were varied (Shantz et al, 1989). Furthermore, treatment of 3T3-L1 mouse embryonic fibroblast cells with 250 µM has been found to decrease levels of 6-phosphogluconate (a metabolite of pentose phosphate pathway), thus indicating successful inhibition of G6PD (Shantz et al, 1989). Our finding that treatment of RRL with a DHEA concentration of 272 µM resulted in a clear effect suggests that DHEA may have similar effective concentrations in lysate as with cells.

It has been shown that incubating TrxR with auranofin at a 1:1 concentration ratio blocks the activity of the enzyme suggesting the compound acts a non-competitive inhibitor of the enzyme (Go et al, 2013). It has also been found that treatment of cells with auranofin in a concentration range between 0 and 100µM affects the oxidative state of thioredoxin in a dose dependent manner (Go et al, 2013). This is despite the fact that the cellular concentrations of TrxR are approximately 1uM (Go et al, 2013). This indicates the effective concentration for inhibition of TrxR by auranofin is different when the TrxR exists as a purified protein from when it is present in a cellular environment (or presumably in a quasi cellular environment such as RRL). It has been suggested that the difference between the effective concentrations of auranofin with purified proteins and in cells is due to the non-specific binding of auranofin to glutathione and other thiols (Gromer et al, 1998). As such the concentration of free auranofin will be lower when used with cells than used with purified proteins (Gromer et al, 1998). We found in this investigation that treatment with RRL with 20µM auranofin allowed disulphide bond formation to occur in the presence of G6P. However, as the concentrations of free auranofin are likely to be low in RRL (as with cells) this suggests that the lysate concentration of TrxR is lower than 20µM.

Auranofin has also been found to inhibit purified GR at micromolar concentrations. However direct analysis of the effect of auranofin on purified thioredoxin reductase and glutathione reductase found the effective concentration of auranofin for inhibition of GR was approximately three orders of magnitude greater than that of TrxR (Gromer et al, 1998). This further supports our conclusion that effects detected by auranofin addition in our assay are due to TrxR inhibition and not GR inhibition. Furthermore it has also been found that addition of 20µM auranofin to cells caused only a small effect on GSH/GSSG ratio (Go et al, 2013). This suggests that addition of 20µM auranofin also would only have a small effect on lysate GSH/GSSG ratio.

Carmustine has been shown to inhibit GR activity *in vitro* with 5nM of the enzyme being inhibited by the compound with an IC50 of 71µM (Liu & Sturla, 2009). Carmustine has also been found to inhibit GR activity and reduced GSH levels when injected into rats (Becker & Schirmer, 1995). Addition of 120µM carmustine to cells has been shown to dramatically reduce cell viability in following an oxidative challenge suggesting the glutathione reductive

pathway is compromised at this concentration (Ma et al, 2003). By contrast, we did not detect any effect of carmustine on the disulphide formation of HA unless added at 3.66mM which is far greater than the µM effective concentrations range used in these previous *in vitro* and *in vivo* studies. Both the addition of DHEA and auranofin caused effects within the RRL at similar concentrations to those reported in cellular studies. This suggests that the target enzymes of these inhibitors are present at comparable concentrations to those in cells. Therefore, the lack of an effect by carmustine within effective cellular concentrations further suggests that GR was not involved in preventing disulphide formation within the HA substrate.

3.4.3 The influence of glutathione and thioredoxin on maintaining reduced thiols

There has been considerable speculation and research effort in recent years in determining the functional roles of both the thioredoxin and glutathione redox systems within eukaryotic cells (Kojer & Riemer, 2014). Until recently it has been thought that both glutathione and thioredoxin play a role in the maintenance of thiol groups within the cytosol in a reduced state. (Lopez-Mirabal & Winther, 2008). A fairly recent paper in which yeast cells were either deprived of glutathione or exposed to toxic levels of glutathione found that under both conditions the cells suffered from impaired iron sulphur cluster synthesis but maintained thiol redox control (Kumar et al, 2011). This suggested that the primary role of the glutathione redox system within mammalian cells is in supporting iron sulphur cluster biogenesis and may play a secondary role to the thioredoxin redox system in maintaining thiol-redox control. Consistent with the finding, the evidence reported in this chapter suggests that the thioredoxin redox system is responsible for preventing disulphide formation within a G6P optimised RRL.

Chapter 4: Results 2: The enzymatic sources of *de novo* disulphide bond formation within the endoplasmic reticulum

4.1 Summary

Disulphide bonds are introduced into nascent proteins within the ER by thiol-disulphide exchange reactions with the PDI family of enzymes. In order for this reaction to be maintained the catalytic disulphides of the PDI family members need to be regenerated by enzymes capable of *de novo* synthesis of disulphides. Ero1 and PrxIV are two enzymes that have been established as important generators of ER disulphides. Genetic studies have however found that mammalian cells are still viable and capable of disulphide generation in the absence of these enzymes. Several enzymes have been identified as potential additional contributors to ER disulphide formation. One of these enzymes, VKOR, has since been shown to contribute to disulphide formation in human hepatoma cells. We aimed to quantify the contribution of these sources of ER disulphide formation. We assessed this by analysing the disulphide formation of nascent PPL translated within a G6P optimised RRL translation system that was supplemented with a source of ER. We showed that DPMs depleted of luminal enzymes were incapable of forming disulphides within PPL indicating the luminal enzymes are essential for this process. We then investigated the effect of selectively inactivating enzymes involved in *de novo* synthesis of disulphides. We found that inhibition of Ero1 in DPMs resulted in substantial impairment of disulphide formation, however, this impairment could only be detected following a reductive challenge. By contrast, inhibition of VKOR in addition to Ero1 caused no further impairment of disulphide formation. These findings reinforce the perception that Ero1 is a key generator of ER disulphides but also suggest that alternative pathways capable of PDI oxidation may compensate for loss of Ero1. These results also suggest that the contribution VKOR makes to disulphide generation may be restricted to particular substrates or cell types.

4.2 Introduction

Disulphide bonds are introduced in nascent substrates within the lumen of the ER by the PDI family. The catalytic disulphides of the PDI family members are regenerated by enzymes capable of synthesising disulphide bonds *de novo*. Ero1 and PrxIV are both well established as *de novo* generators, however, several other enzymes such as VKOR, QSOX and GPx7/8 also have been identified as potential generators of disulphides (Bulleid & Ellgaard, 2011). Key questions remain regarding these *de novo* sources: other than Ero1 and PrxIV, which enzymes may act as *de novo* generators? How large is the contribution that each of these sources make to disulphide formation and does this contribution vary between cell types? Does each of these sources have different target substrates? In order to answer the questions surrounding these disulphide sources it is useful to review studies that have investigated them.

Ero1 was originally discovered in yeast and was found to be essential for disulphide formation within this organism (Frand & Kaiser, 1998; Pollard et al, 1998). However, it was later found that loss of Ero1 in fruit flies, or the loss of both of the mammalian isoforms of the enzyme in mice resulted in a mild phenotype and did not substantially affect disulphide formation (Tien et al, 2008; Zito et al, 2010a). This suggested that other pathways to disulphide formation exist in higher eukaryotes. The search for other sources of disulphide generation led to the identification of PrxIV as an additional enzymatic source of ER disulphide generation (Tavender et al, 2010; Zito et al, 2010b).

Similarly to Ero1, PrxIV knockout mice were found to display only a mild phenotype suggesting that disulphide formation is also possible in the absence of the PrxIV pathway (Iuchi et al, 2009). Following this finding, the effects of removal of both the Ero1 isoforms and PrxIV on disulphide formation pathways were assayed by the knockdown of PrxIV in mouse embryonic fibroblast (MEF) cells with disruptive mutations of the Ero1 genes (Zito et al, 2010b). This resulted in several interesting phenotypic features: the cells displayed severely diminished cell growth, had a more reducing environment and displayed impaired collagen secretion. MEF cells in which PrxIV was knocked down and Ero1 was knocked out were also less resilient to a DTT challenge than MEF cells in which Ero1 was knocked out but retained normal PrxIV expression. These findings suggested that the loss of both Ero1 and PrxIV pathways has a more detrimental effect on cells than loss of either pathway alone, however, the continued viability of such cells suggests that disulphide formation was not completely abolished.

In order to identify other *de novo* sources Rutkevich and Williams investigated the effect of functional loss of known and potential disulphide generating enzymes (Rutkevich & Williams, 2012). This was assessed by knockdown of expression or inhibition of these enzymes in a human hepatoma cell line. The effect upon disulphide formation in albumin was then assayed by pulse-chase methodology. No impairment of disulphide formation was detected under these conditions unless the cells were first subjected to a brief DTT reductive challenge. When this reductive challenge was used it was found that knockdown of Ero1α+β resulted in impaired disulphide formation. Furthermore, knockdown of PrxIV, or inhibition of VKOR (both in addition to Ero1α+β knockdown) resulted in further impairment of disulphide formation. Knockdown of QSOX was not found to have any effect. This study was of importance because it provided strong evidence to support the common perception that Ero1 is a major source of disulphides. In addition, the study provided the first *in vivo* evidence that VKOR contributes to ER disulphide formation. It was also observed that disulphide bond formation although impaired remained functional under all assay conditions tested indicating that some disulphide generators remained functional. It was noted that due to toxicity issues

it was not possible to assess the effect of knocking down Ero1α+β and PrxIV and well as inhibiting VKOR (Rutkevich & Williams, 2012).

In order to further assess these *de novo* sources we investigated their influence in the generation of disulphide bonds within nascent PPL using ER sourced from DPMs and HT1080 SP cells. The ER of these sources was treated to remove their luminal components or to selectively inhibit the enzymes Ero1, PrxIV and VKOR. We then supplemented these manipulated ER sources to our G6P optimised RRL translation and assessed the effect upon the disulphide formation. This approach allowed us to assess the overall influence of the ER lumen to disulphide formation and also the contribution of these specific enzymes.

4.3 Results

4.3.1 Preprolactin is an effective substrate for analysis of disulphide formation

We chose to use bovine PPL as the substrate for this investigation as it is a model protein for studies of protein folding and disulphide formation (Kaderbhai & Austen, 1985). Bovine PPL is also not glycosylated (Wallis, 1974). This is advantageous as it prevents our analysis of disulphide formation from being complicated by effects upon the glycosylation machinery. Initial analysis of the disulphide formation of the substrate was conducted in the presence of SP cells as well as DPMs (Fig 4.1). Under reducing conditions a faster mobility species corresponding to the signal peptide cleaved form was observed if a source of ER was present (Fig 4.1 compare lanes 1 and 2 of A and B). The signal sequence of bovine PPL constitutes the first 30 amino acids of the total 229 amino acids (Wallis, 1974). Consequently, its cleavage caused a large change in molecular weight resulting in a detectable shift in gel mobility of the protein. Under non-reducing conditions disulphide bonds remained intact and as such disulphide bonded prolactin can be observed (Fig 4.1 lanes 3 to 8 of A and B). Only one intrachain disulphide bonded prolactin form was visible despite the fact that PPL forms three intrachain disulphides (Wallis, 1974). This is because only one of these disulphides is long range (Fig 1.19) and results in detectable change in gel mobility. The disulphide bonded prolactin was seen to form rapidly and remained stable throughout the course of the assay.

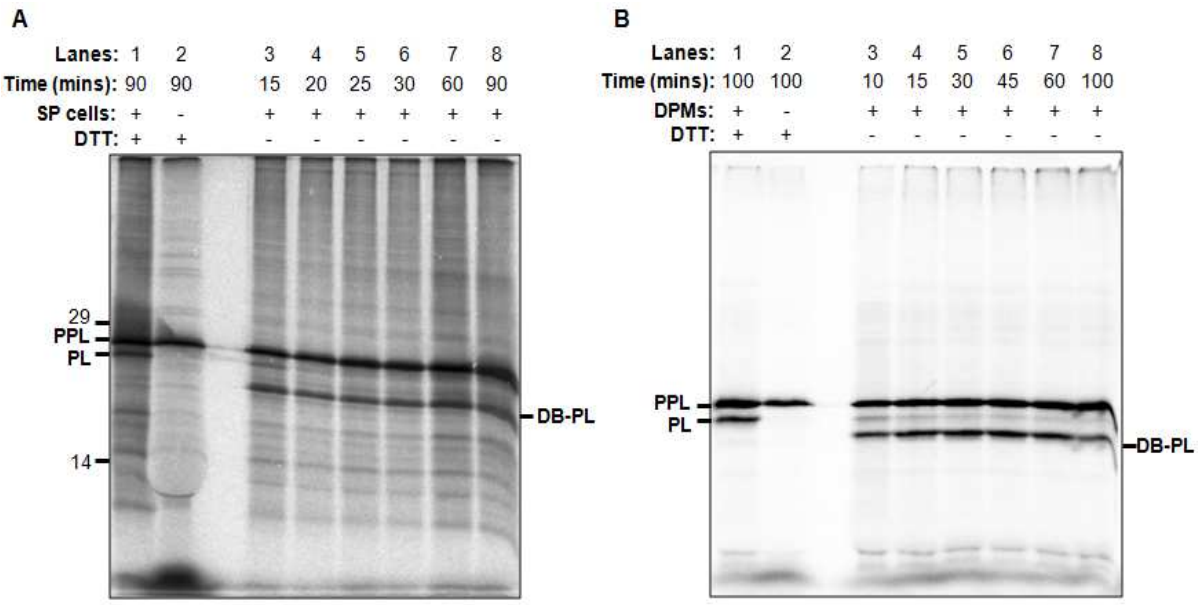

Figure 4.1: Prolactin forms a single detectable disulphide bonded species in the presence of a source of ER. mRNA encoding PPL was translated for the indicated times. ATCA was added to the lysate after 5 min to prevent further translation initiation, thereby allowing a time-synchronised analysis of the prolactin disulphide formation. The translation reactions were supplemented with either SP cells **(A)** or DPMs **(B)** to act as sources of ER. A single intrachain disulphide bonded prolactin species can be observed. PPL: Preprolactin, PL: Prolactin, DB-PL: intrachain disulphide-bonded prolactin.

4.3.2 ER luminal components are essential for disulphide formation of prolactin

The majority of enzymes involved in ER disulphide formation reside in the lumen of the organelle. This includes most of the PDI family members as well as all of the enzymes regarded as known and potential sources of *de novo* disulphide synthesis with the exception of VKOR (Bulleid & Ellgaard, 2011; Kozlov et al, 2010). It has been shown that microsomes that are depleted of their luminal contents are impaired at forming disulphide bonds in nascent proteins translated within a RRL translation system (Bulleid & Freedman, 1988; Marquardt et al, 1993). We sought to verify this finding with our G6P optimised RRL system to confirm whether the luminal enzymes of the microsomes are indeed necessary for disulphide bond formation.

Consistent with previous results (Bulleid & Freedman, 1990) we found that DPMs could be specifically depleted of luminal components by treatment with the detergent saponin (Fig 4.2). Furthermore we showed that ER disulphide bond formation in nascent prolactin was prevented in a RRL supplemented with DPMs that were depleted of their luminal contents (Fig. 4.2C). This result shows that ER luminal enzymes are essential for disulphide bond formation of prolactin.

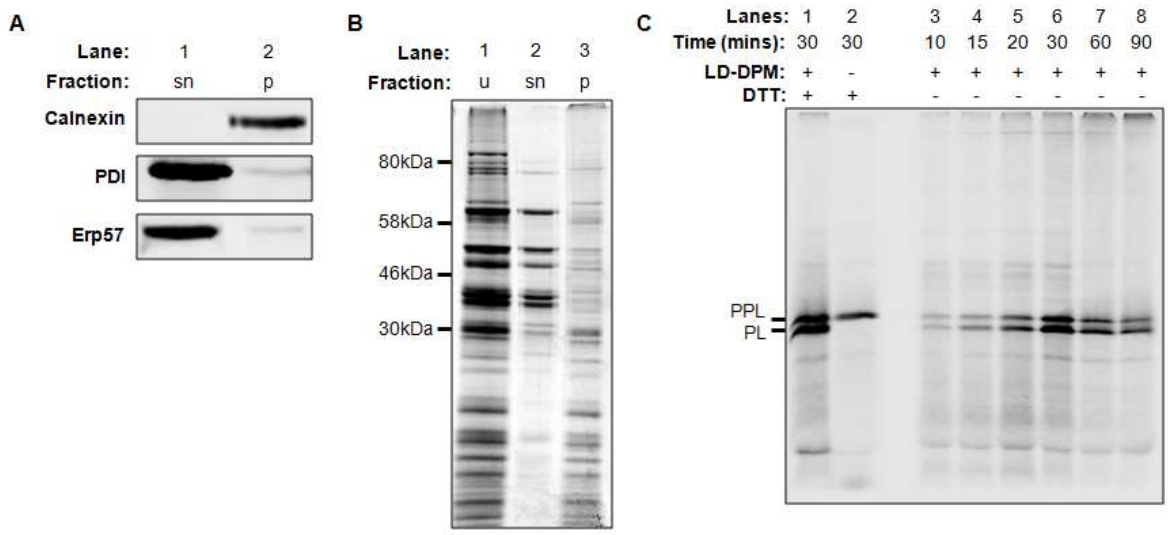

Figure 4.2: Disulphide formation of prolactin within the ER is prevented in the absence of ER luminal proteins. DPMs were treated with 0.5% (v/v) purified saponin to cause the release of their luminal contents. **(A)** Western blotting showed that saponin treatment caused release of luminal proteins such as PDI and ERp57 but not membrane proteins such as calnexin. **(B)** Coomassie blue staining shows the profile of proteins released and retained following saponin treatment. **(C)** It can be seen that when mRNA encoding PPL is translated in RRL supplemented with saponin treated microsomes no disulphide bond formation occurs. sn: supernatant and p: pellet fractions of DPM isolated by centrifugation following saponin treatmen, u: DPM that have not been treated with saponin, LD-DPMs: DPMs depleted of luminal components.

4.3.3 Inhibition of Ero1 following a reductive challenge substantially impairs disulphide bond formation in DPMs

Following the confirmation that the removal of the ER lumen prevents disulphide formation our aim was to determine the enzymes involved in this process and quantify their contribution. Our strategy was to inactivate key components of the ER disulphide bond formation machinery and to assess the effect upon disulphide bond formation of prolactin.

It has been found that disulphide bond formation can be effectively assayed following a brief DTT reductive challenge (Braakman et al, 1992). The DTT treatment used in this technique causes all solvent accessible disulphides to be reduced. Therefore, disulphide bonds have to be generated *de novo* before they are introduced into substrates and cannot come from latent disulphides such as the catalytic disulphides of PDI family members. The method has been used in pulse chase experiments in which DTT is added to cells, removed; and then disulphide formation is analysed at time points following the removed of the reductant. This technique has been used to investigate ER disulphide formation machinery and ER redox dynamics (Appenzeller-Herzog et al, 2010; Cuozzo & Kaiser, 1999; Mezghrani et al, 2001).

Recently, it has been discovered that treatment of HT1080 cells with 10 mM DTT for 10 min causes complete inactivation of PrxIV (Cao et al, 2014). This inactivation occurs because the DTT treatment causes the thiol groups of certain catalytic cysteines of PrxIV to become irreversibly hyperoxidised to sulfinic acid groups (R-SO(OH)) which prevents the catalytic action of PrxIV. This hyperoxidation is thought to occur because Ero1 metabolises DTT (Gross et al, 2006; Tavender & Bulleid, 2010b) which causes hydrogen peroxide to be produced. The high hydrogen peroxide concentration then causes PrxIV hyperoxidaiton (Cao et al, 2014)**.** The inactivation of PrxIV by DTT treatment also occurs with DPMs as well as HT1080 cells (Cao and Bulleid, unpublished data).

The *de novo* disulphide generators Ero1 and VKOR can also be inhibited by the compounds QM295 and warfarin (Blais et al, 2010; Fasco et al, 1983) (Fig 4.3). QM295 has been shown to inhibit 200nM Ero1 *in vitro* with an IC50 of 1.9µM. Ero1 treated with the inhibitor *in vitro* was shown to be predominantly in the reduced state while untreated Ero1 is predominantly in the oxidised form. It was also shown that the Ero1 of mouse embryonic cells treated with 50 µM of QM295 was also predominantly reduced while the enzyme was predominately oxidised in untreated cells. It was concluded that QM295 targets the reduced form of the enzyme and inhibits its activity by preventing re-oxidation (Blais et al, 2010). A concentration of 4 µM warfarin has been shown to inhibit approximately 80% of the VKOR present in rat liver microsomes (Fasco et al, 1983). More recently, it has been shown that addition of warfarin 50 µM to human hepatoma cells in which Ero1 expression was knocked down resulted in impairment of disulphide bond formation in albumin (Rutkevich & Williams, 2012).

Considering this information, we reasoned that through the use of these inhibitors and DTT it would be possible to selectively inactivate Ero1, PrxIV and/or VKOR as well as abolishing solvent accessible disulphides of DPMs. This was achieved by incubating DPMs with (or without) 50 µM QM295 and 50 µM warfarin and incubating the DPMs with 10mM DTT for 10 minutes. QM295 and warfarin inactivate Ero1 and VKOR, while DTT treatment inactivates PrxIV and removes solvent accessible disulphides. The DPMs were resuspended in DTT free buffer before being added to RRL programmed with PPL mRNA. The effect on disulphide bond formation of PPL was then assayed (Fig 4.4).

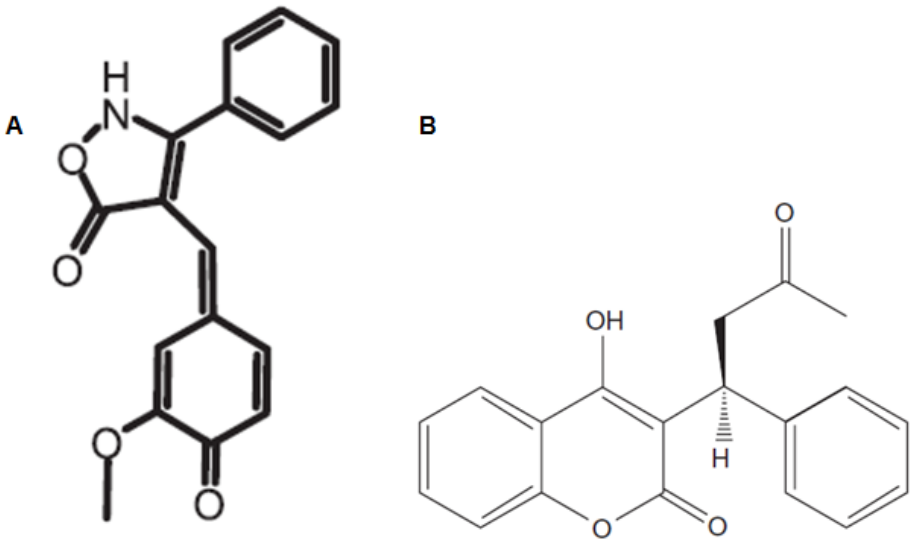

Figure 4.3: Chemical structures of Ero1 and VKOR inhibitors, QM295 and warfarin. (A) QM295 has been shown to be a potent inhibitor of Ero1 *in vitro* and *in vivo*. The name of the inhibitor is derived from the fact it contains a quinone methide group and has a molecular mass of 295 (Blais et al, 2010). **(B)** Warfarin is a drug commonly used as an anticoagulant. It targets the enzyme VKOR which is involved in the vitamin K cycle. Inhibition of VKOR by warfarin prevents regeneration of reduced vitamin K which is an essential cofactor for the blood clotting cascade (Rettie & Tai, 2006). The redox activities of VKOR are also implicated in supporting disulphide formation (Rutkevich & Williams, 2012). Structures of QM295 and warfarin are as originally shown by Blais *et al* and Rettie and Tai respectively (Blais et al, 2010; Rettie & Tai, 2006)

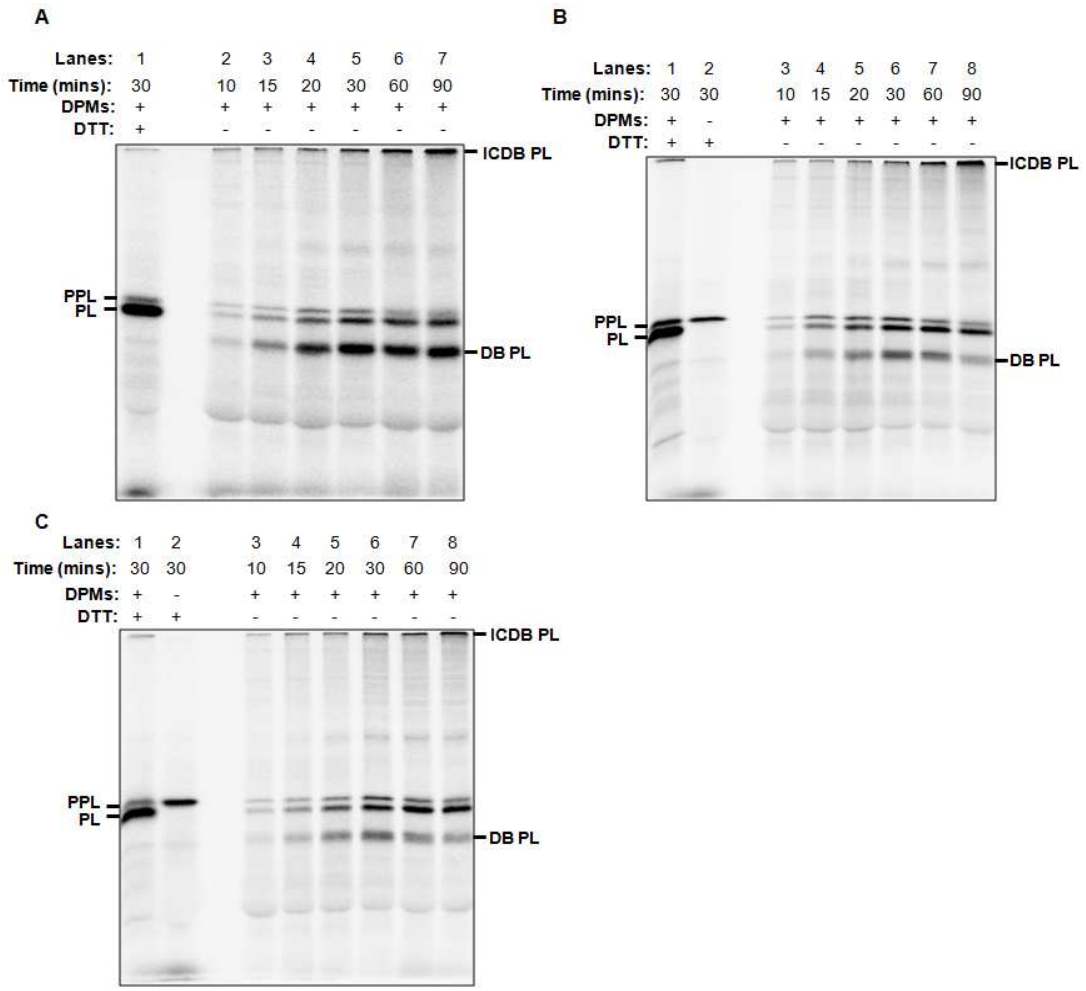

Figure 4.4: Ero1 inhibition following a DTT reductive challenge causes severe impairment of disulphide formation of prolactin in DPMs. DPMs were either not treated with inhibitors **(A)**, treated with QM295 **(B)**, or treated with QM295 and warfarin **(C)**. All DPMs were then exposed to 10 mM DTT for 10 mins before being isolated by centrifugation and resuspended in fresh buffer. The microsomes were then added to RRL programmed with PPL mRNA for translation. Translation reactions proceeded for the indicated times before being treated with NEM. DTT added to the loading buffer of the indicated samples to allow DTT sensitive bands to be identified. It can be seen that when treated with the Ero1 inhibitor QM295 prolactin disulphide bond formation was substantially impaired. Each experimental condition was carried out 3 times and results similar to those shown were obtained with the repeats of each condition. PPL: Preprolactin, PL: non-disulphide bonded Prolactin, DB PL: Intrachain disulphide bonded prolactin ICDB PL: interchain disulphide bonded prolactin/prolactin aggregates.

When treated with only DTT (and hence with PrxIV inactivation) the majority of the translocated prolactin became disulphide bonded and only a minority of the prolactin persisted in the non-disulphide bonded form. Addition of QM295 caused substantial impairment of disulphide bond formation with the majority of nascent prolactin being unable

to form disulphide bonds. Addition of the VKOR inhibitor warfarin, however, did not cause any obvious further reduction of disulphide formation and a minority of the translocated prolactin still remained in the disulphide bonded form (Fig 4.4).

In order to quantify the effects on disulphide formation we repeated each experimental condition twice (3 replicates). We then conducted densitometry of specific gel bands for each of the results and made statistical comparisons between the results for each condition (Fig 4.5). Comparison of the ratios of intrachain disulphides divided by the total translocated protein where made between each condition for each time point. The mean ratio of disulphide bonded/total translocated prolactin was substantially lower in DPMs treated with QM295 then DPMs that had not been treated with this inhibitor. Furthermore, it was found that difference in the ratio values between these two groups was statistically significant for all the time points assayed (t-test p values <0.05).There was however no statistical difference (t-test p values >0.05) for any time point between DPMs that had been treated with DTT and QM295, and the DPMs that had been treated with DTT, QM295 and warfarin. This result shows that Ero1, but not VKOR made a quantitatively large and statistically significant contribution to disulphide bond formation of prolactin within DPMs following a DTT challenge.

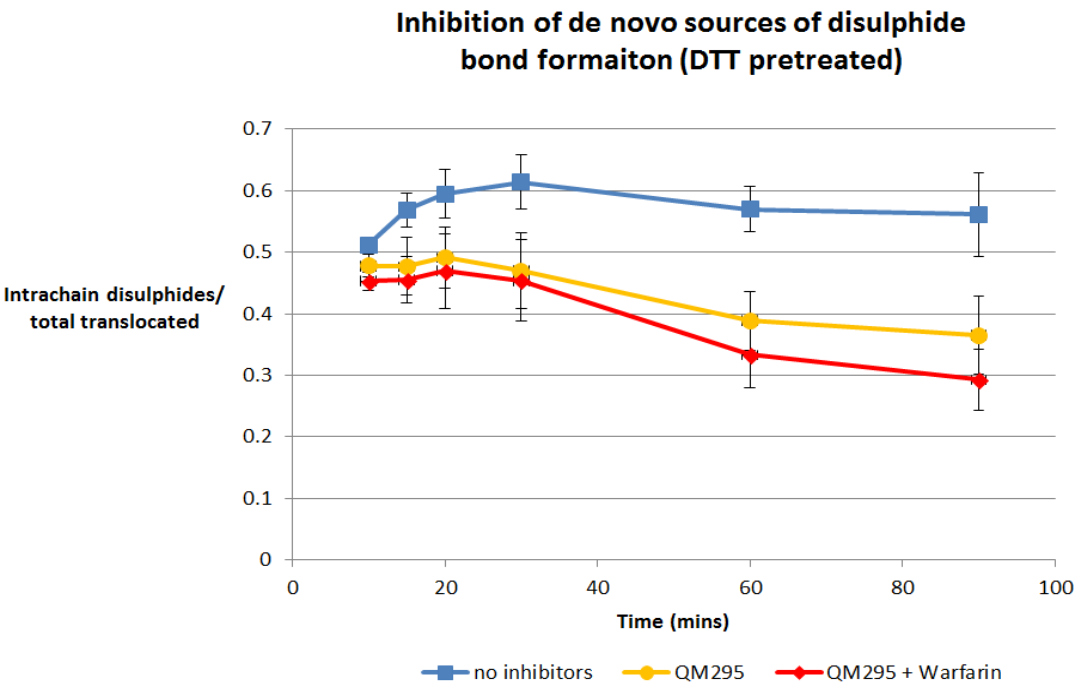

Figure 4.5: Densitometric analysis confirms that inactivation of Ero1 in DPM following a reductive challenge severely impairs prolactin disulphide bond formation. Triplicate results of the experimental data shown in Fig 4.4 were subjected to densitometric analysis. For every time point under non-reducing conditions the desitometric value of the intrachain disulphide prolactin band (DB PL) was divided by the value of the non-disulphide bonded prolactin band (PL) plus the value of DB PL. This gave a ratio of intrachain disulphides/total translocated prolactin. Data point and line colours represent the inhibitor treatment the DPMs were subjected to. DPMs not treated with inhibitors are shown in blue, those treated with QM295 are shown in yellow, while the DPMs treated with QM295 and warfarin are shown in red. Each data point shows the mean ratio of intrachain disulphides/total translocated prolactin at a particular time point. The mean value was calculated from the triplicates of each condition. The error bars shown are +/- one standard deviation. Statistical comparisons were made between the treatment conditions using the student's t-test. Comparison between each time point of the DPMs treated with no inhibitors (blue) and those treated with QM295 (yellow) resulted in p=values ranging from 0.012 and 0.034 indicating statistical significant ($p<0.05$) between the two conditions. Comparison between the DPMs treated with QM295 (yellow) and those treated with QM295 and warfarin (red) resulted in p=values ranging from 0.18 to 0.51 indicating no statistical significance ($p>0.05$). These findings indicate that inhibition of Ero1 following a DTT challenge resulted in a statistically significant impairment of disulphide bond formation. Additional inhibition of VKOR by warfarin resulted in lower mean ratios of intrachain disulphide bond formation as a proportion of total translocated protein, however, these values were not statistically significant.

It should be noted that under the assay conditions we also detected prolactin aggregates at the top of resolving gel that accumulated over the course of the reaction (Fig 4.4). Comparison of the 30 min time points under reducing and non-reducing conditions suggested that these aggregates were at least partially composed of interchain disulphide bonded (ICDB) prolactin. This ICDB/aggregate band was not included in analysis of the disulphide bonded/total translocated ratios. However, it can be seen that this is only a minor species over all time points except the 60 and 90 min time points. Therefore any skewing of our analysis by this product(s) is likely to be limited to these two time points only and do not affect the overall interpretations of our results.

4.3.4 In the absence of a DTT treatment, inhibition of enzymatic sources involved in *de novo* synthesis of disulphide bonds in DPMs does not prevent disulphide formation

We next investigated the effect of inhibition of sources of de novo disulphide formation in the absence a prior treatment of the DPMs with DTT (Fig 4.6). Under these conditions inhibition of Ero1 caused no detectable impairment of disulphide bond formation in nascent prolactin. Combined inhibition of VKOR as well as Ero1 only appeared to impair disulphide bond formation, at the later 60 and 90 min time points (Fig 4.7). It can be seen, however, that less disulphide bonded aggregates were observed at these time points when warfarin was added, which may have resulted in an apparent lower ratio of disulphide bonded/ total translocated prolactin. As a difference in disulphide bonded/total translocated ratios was only observed at these two later time points in which aggregation interferes with the analysis we concluded that the warfarin treatment had not caused additional impairment of disulphide formation.

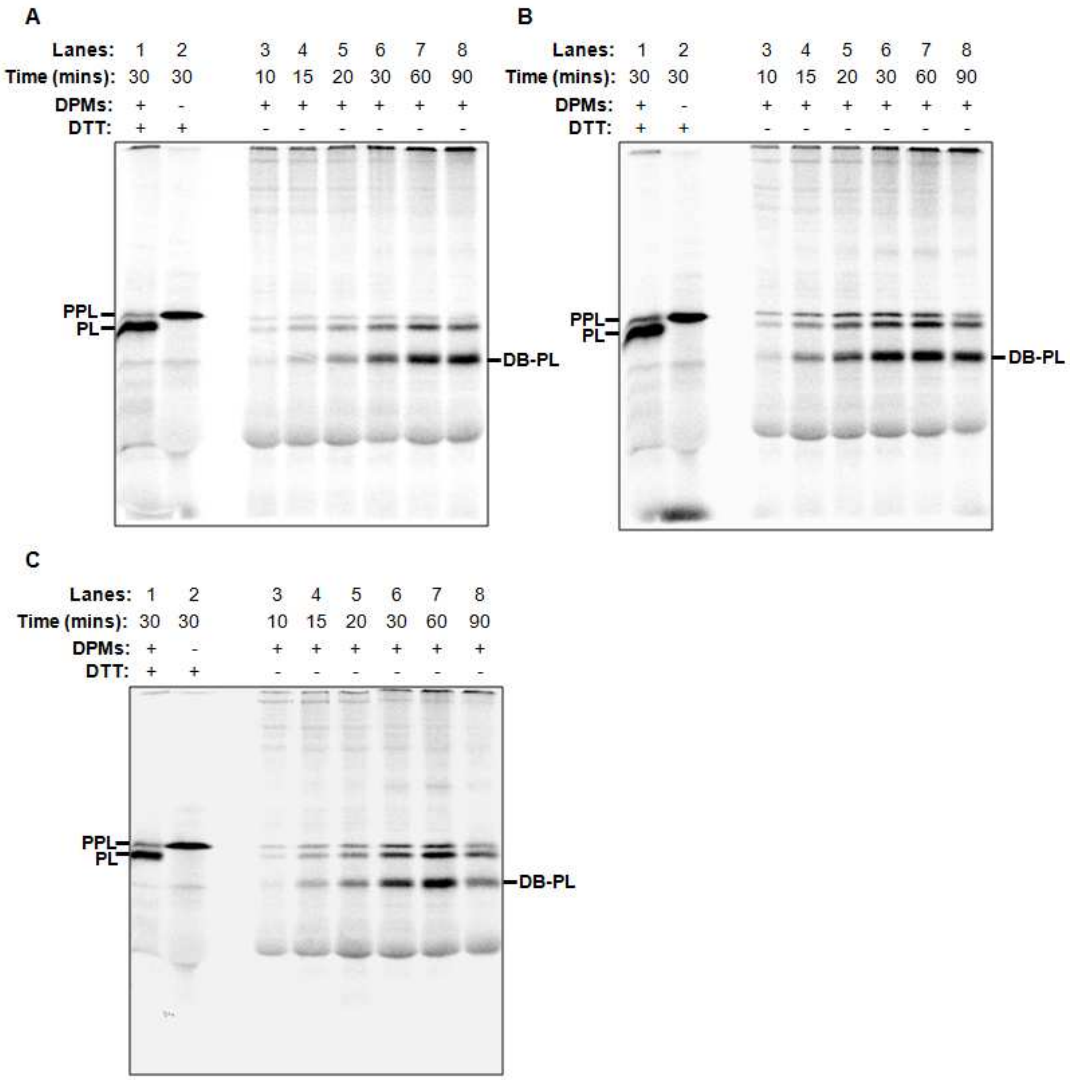

Figure 4.6: In the absence of a DTT reductive challenge inactivation of Ero1 and VKOR in DPMs does not impair disulphide formation in prolactin. DPMs were isolated by centrifugation before being resuspended in the absence of inhibitors **(A)**, in the presence the QM295 **(B)**, or in the presence of QM295 and Warfarin **(C)**. DPMs were then pelleted by centrifugation and resuspended in the absence of inhibitors before being added to RRL programmed withPPL mRNA for translation. 50µM QM295 was also present in the lysate of the reactions shown in (B) and (C). 50µM warfarin was also present in the lysate of the reactions shown in (C). Translation reactions proceeded for the indicated times before being treated with NEM. DTT added to the loading buffer of the indicated samples to allow DTT sensitive bands to be identified. Inactivation of Ero1, or Ero1 and VKOR caused no observable effect on disulphide bond formation. PPL: Preprolactin, PL: non-disulphide bonded Prolactin, DB-PL: Intrachain disulphide bonded prolactin, ICDB PL: interchain disulphide bonded prolactin.

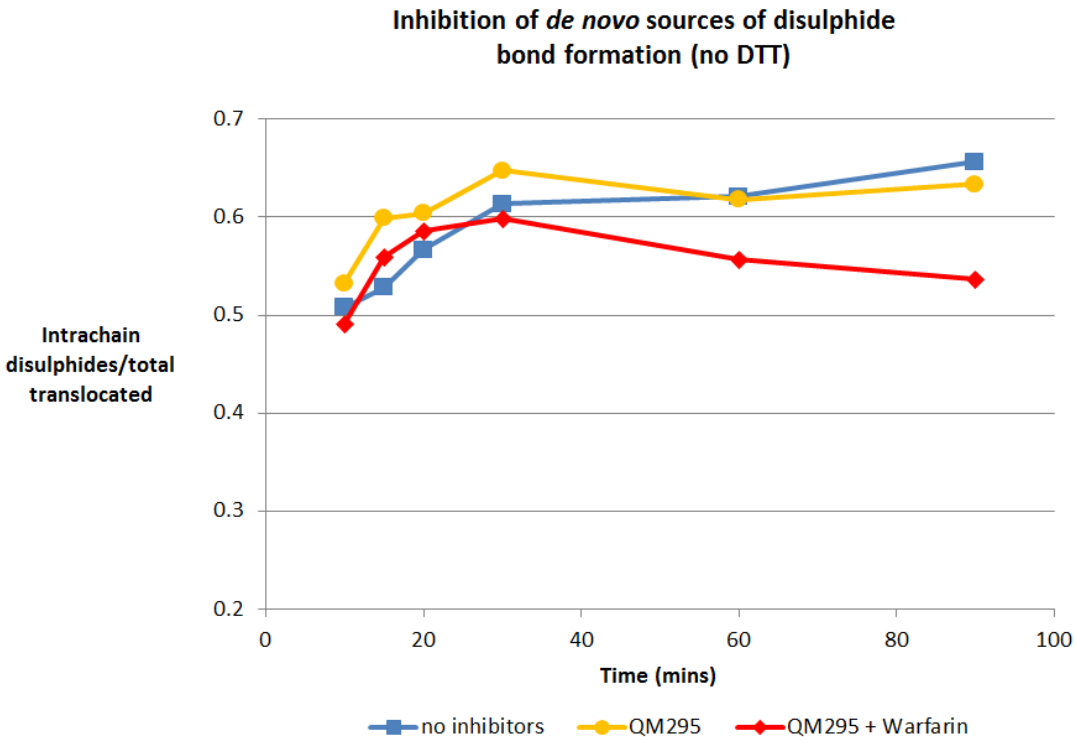

Figure 4.7: Densitometric analysis of inhibition of the enzymatic sources of *de novo* disulphide synthesis in DPMs in the absence of a reductive challenge. The experimental data shown in Fig 4.6 was subjected to densitometric analysis to quantify the effect on disulphide bond formation caused by inactivation of *de novo* enzymatic sources of disulphide generation in DPMs in the absence of a reductive challenge. For each time point of each condition under non-reducing conditions the desitometric value of the intrachain disulphide prolactin band (DB-PL) was divided by the value of the non-disulphide bonded prolactin band (PL) plus the value of DB-PL. This gave a ratio of intrachain disulphides/total translocated prolactin. Data point and line colours represent the inhibitor treatment the DPMs were subjected to. DPMs not treated with inhibitors are shown in blue, those treated with QM295 are shown in yellow, while the DPMs treated with QM295 and warfarin are shown in red. The data shown is from analysis of one experiment for each experimental condition.

Considering these results in conjunction with the results obtained with DTT treatment it can be seen that the two variables tested that had the greatest influence on disulphide formation were the inhibition of Ero1, and treatment with DTT. Disulphide formation can be seen to only be substantially impaired under conditions in which the DPMs had both been DTT treated and Ero1 had been inhibited by QM295. Warfarin treatment did not appear to cause any clear impairment of disulphide formation under any assay conditions. Interestingly,

disulphide bond formation although impaired was at least partially functionally under conditions in which Ero1, PrxIV and VKOR would be expected to be inactive. This suggests that inactivation of these enzymes was not complete or other luminal enzymes were generating disulphides.

4.3.5 SP cells maintain the capacity to form disulphides even when *de novo* enzymatic generators are inactivated

Following on from our findings with DPMs we next experimented with inhibition of enzymatic disulphide sources in SP cells. There were two purposes for using SP cells for this investigation. Firstly, we sought to determine if the SP cells would behave similarly to DPMs under the same assay conditions. Secondly, we aimed to utilise the SP cells to examine prolactin disulphide formation under conditions in which the expression of ER proteins had been altered. As SP cells are derived from tissue culture cells any manipulations that are made to ER protein expression of these cells in tissue culture remain once the cells have become permeabilised for use in the RRL translation system.

Similarly to the DPMs, the SP cells were subjected to conditions designed to impair the disulphide formation apparatus of the ER (Fig 4.8). The most severe condition involved treatment with QM295, warfarin and DTT. To our surprise the SP cells showed no impairment of disulphide formation under any of the treatment conditions. We repeated the experiment with minor changes in assay conditions however the lack of impairment of disulphide bond formation in the SP cells was consistently observed (data not shown). This indicates a clear difference in behaviour between the DPMs and SP cells and indicates that the disulphide formation machinery of SP cells is more robust than that of DPMs.

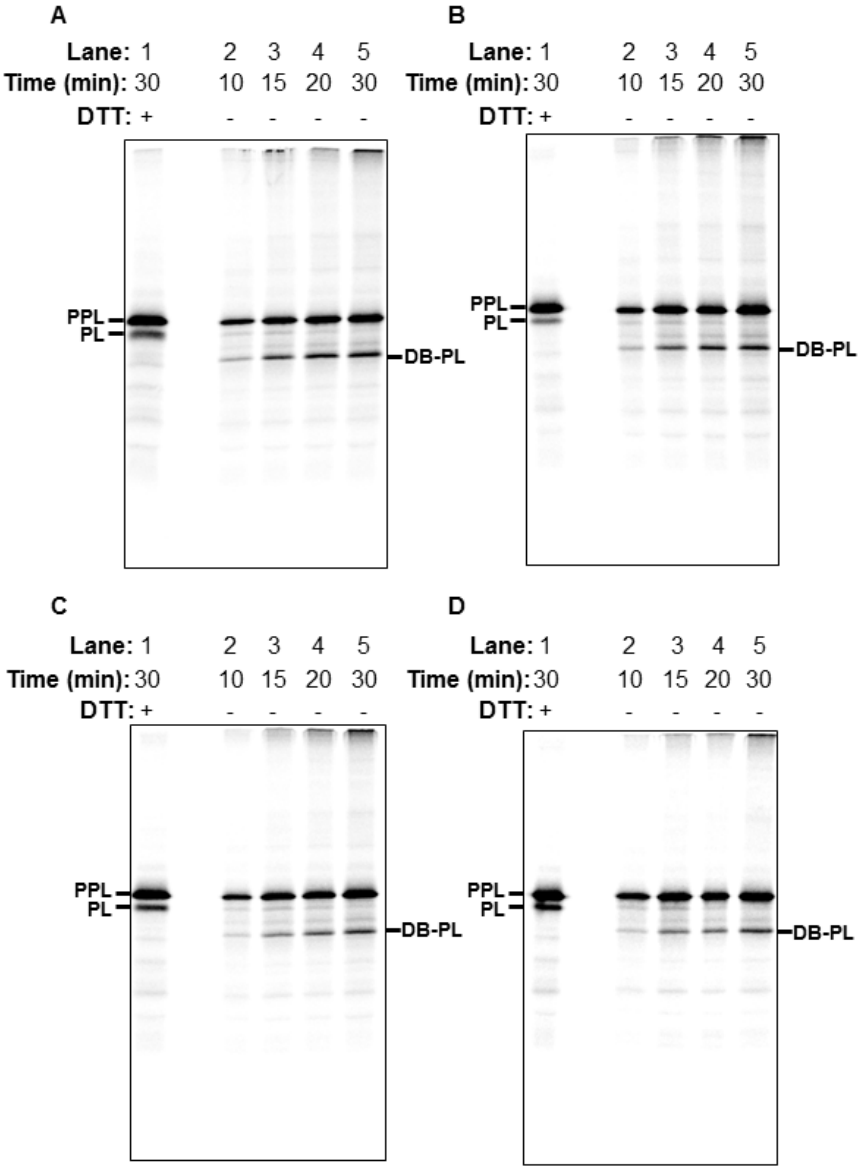

Figure 4.8: Inhibition of enzymatic sources *de novo* sources of disulphide bond formation following a reductive challenge does not impair disulphide bond formation in SP cells. Translation of PPL mRNA was conducted in the presence of SP cells pretreated with or without the inhibitors QM295 and warfarin and the reductant DTT. SP cells were incubated with 50µM of inhibitors for 5 min and then incubated with 10mM DTT for a further 10 min. The cells were then resupsended in fresh KHM buffer and added to RRL for translation reactions. The SP cells were either treated without any inhibitors or DTT **(A)**, with DTT only **(B)**, with QM295 and DTT **(C)**, or with QM295, warfarin and DTT **(D)**. 50µM QM295 was also present in the lysate of the reactions shown in (C) and (D). 50µM warfarin was also present in the lysate of the reactions shown in (D). It can be seen that unlike DPMs there was no impaired disulphide formation of PPL in SP cells in which the Ero1 and VKOR were inhibited and which had been subjected to a DTT reductive challenge.

4.4 Discussion

4.4.1 ER luminal proteins are required for disulphide formation of prolactin

In this study we showed that the luminal enzymes of the ER are required for disulphide bond formation of prolactin. We anticipated that removal of the of the luminal enzymes would either abolish or severely impair disulphide formation as the majority of the enzymes known to be involved in ER disulphide formation reside within the ER lumen. This includes the established *de novo* disulphide generators Ero1 and PrxIV, as well as most of the PDI family members (Bulleid & Ellgaard, 2011; Kozlov et al, 2010). Some of the less well characterised enzymes involved in disulphide generation do however reside in the ER membrane. This includes the five TMX proteins of the PDI family as well as the *de novo* disulphide generator VKOR (Kozlov et al, 2010; Rutkevich & Williams, 2012). Furthermore, it has been shown that VKOR forms mixed disulphides with TMX and TMX4 (Schulman et al, 2010). This thus suggested that a possible VKOR-TMX(4)-prolactin disulphide relay pathway may have still been functional. However, the lack of any prolactin disulphide formation under these conditions suggests that such a pathway does not function within the ER. There are two possible explanations for this. Firstly, VKOR may not be an effective generator of disulphides within these ER sources, or secondly, the TMX and TMX4 PDI family members may not be capable of introducing disulphides into prolactin.

Two previous studies have investigated the contribution of ER luminal factors to disulphides bond formation of nascent proteins using DPMs depleted of their luminal contents within a RRL translation system (Bulleid & Freedman, 1988; Marquardt et al, 1993). A study by Bulleid and Freedman provided early evidence that PDI was involved in disulphide formation. DPMs depleted of luminal components by high pH, and hence lacking PDI, were shown to have reduced, but not complete absence, of disulphide formation of the substrate γ-gliadinn (a wheat storage protein) (Bulleid & Freedman, 1988). A subsequent study by Marquardt *et al* showed that DPMs depleted by treatment with the non-ionic detergent Nikkol were incapable of introducing disulphides into HA. (Marquardt et al, 1993). The complete lack of disulphide formation of HA in depleted DPMs suggests that HA, like PPL is probably not a downstream target of VKOR. As some disulphide bond formation still occurred in γ-gliadin it is possible that this protein is a downstream substrate of VKOR. These studies and our own suggests that a VKOR-TMX(4)-substrate pathway does not operate with the substrates PPL and HA however γ-gliadin may be a substrate of such a pathway.

4.4.2 A VKOR disulphide generation pathway may be specific to particular substrates and/or cell types

The luminal depletion experiments suggested that VKOR pathway could not introduce disulphide bonds into prolactin via the TMX or TMX4 enzymes within ER derived from dog pancreas tissue. VKOR has however also been shown to formed mixed disulphides with the luminal PDI family member ERp18 (Schulman et al, 2010). The findings therefore did not negate the possibility that VKOR could introduce disulphide to prolactin via ERp18. Furthermore, recent evidence from the Bulleid lab has also indicated that PDI family members may shuttle disulphide bonds amongst each other (Oka et al, 2015). Therefore, it also remained a possibility that VKOR could support the introduction of disulphides into its target PDI family members which could in turn transfer disulphide to PDI family members capable of introducing disulphide to prolactin. Our inhibitions studies provide further information regarding these possibilities. Inhibition of VKOR in DPMs (following a reductive challenge and inactivation of Ero1 and PrxIV) resulted in a lesser amount of disulphide formation of prolactin relative to the same conditions without the inhibition of VKOR. The decrease in disulphide formation was only slight and was not statistically significant which suggested that VKOR made either a small or no contribution to disulphide formation in prolactin within DPMs. This assay should have been sensitive to the detection of disulphide generation of VKOR as both Ero1 and PrxIV had been inactivated and therefore a large amount of the functional redundancy within the disulphide formation machinery should have been removed. Therefore, if VKOR was capable of supporting disulphide formation of prolactin, it would have been expected to be contributing a large proportion of the remaining disulphide generating capacity of the microsomes. As such, the lack of a clear effect upon VKOR inhibition shows it does not make a substantial contribution to prolactin disulphide formation within the ER of dog pancreas tissue.

The lack of observable VKOR derived disulphide formation in DPMs is in contrast to a previous findings from Rutkevich *et al* that VKOR makes a contribution to the disulphide formation of albumin (Rutkevich & Williams, 2012). The study by Rutkevich and Williams was carried out using pulse chase methodology with a human hepatoma cell line. Functionally the experimental approaches used by ourselves and Rutkevich and Williams to detect VKOR disulphide bond formation activity are highly similar. Both involve assaying the disulphide formation of a substrate following a reductive challenge in an ER system in which Ero1 was inactivated and comparing the effect with inactivation of VKOR by warfarin. Aside from the methodological difference of using a pulse chase or *in vitro* system, the two primary biological differences are between the substrates used to assay the oxidative folding and the cellular source of the ER used.

This suggests two possible causes for the difference in the VKOR disulphide generation activity between our study and that of Rutkevich and Williams. Firstly, VKOR may only introduce disulphide bonds into a specific subset of downstream protein targets. These targets are likely to be substrates which the PDI family members TMX, TMX4 and ERp18 interact with. It would be inferred from this hypothesis that albumin, but not preprolactin is a target of these PDI family members. A second possibility is that VKOR may be an important contributor to disulphide formation within particular tissues. It has been previously shown that VKOR mRNA is highly expressed in liver cells compared to other tissues and also that VKOR is up regulated in cancer tissues (Wang et al, 2005). It may be expected that VKOR disulphide generation activity is particularly high in human hepatoma cells in which the study by Rutkevich and Williams was conducted. In any case, the findings of the study reported in this chapter imply that the contribution that VKOR makes as a *de novo* source of disulphide is not universal but is specific to particular substrates or cell types.

4.4.3 Ero1 disulphide generation activity can only be detected following a reductive challenge

Ero1 is commonly regarded as the primary generator of disulphides within the ER (Tavender & Bulleid, 2010a). However, in this investigation the disulphide generation activities of Ero1 could only be detected in DPMs following a reductive challenge. This finding is consistent with those reported by Rutkevich and Williams in which it was found that the knockdown of Ero1 in human hepatoma cells only resulted in an impairment of disulphide formation of albumin following a reductive challenge (Rutkevich & Williams, 2012). Furthermore Zito *et al* detected a modest delay in the oxidative folding of IgM in cells derived from Ero1 knock out mice following a reductive challenge (Zito et al, 2010a). These findings consistently indicate that the ER maintains an effective capacity to introduce disulphides in the absence of Ero1. It has been considered that the alternative disulphide generators including PrxIV and VKOR may compensate for loss of Ero1 (Bulleid & Ellgaard, 2011). In this investigation however, prolactin disulphide formation in DPM still occurred under conditions in which Ero1, PrxIV and VKOR would be expected to be inactivated. However, ER luminal depletion did abolish the disulphide formation of prolactin. These findings indicate that the ER disulphide bond formation machinery is remarkably robust and suggest that alternative routes of disulphide generation from ER luminal sources may persist even in the absence of these three enzymes.

4.4.4 Inactivation of Ero1, PrxIV and VKOR does not impair disulphide formation in SP cells

Curiously, we found that when using SP cells, impairment of disulphide bond formation of prolactin could not be detected even when the SP cells had been DTT treated and the enzymes Ero1, VKOR and PrxIV were inactivated. This contrasts the clear impairment of disulphide formation that we observed with DPMs under the same conditions. It also contrasts the findings of Rutkevich and Williams who detected impair disulphide formation of albumin in human hepatoma cells following a reductive challenge if Ero1 was knocked down (Rutkevich & Williams, 2012). However, the finding is somewhat consistent with the discovery that immunoglobulin secretion and disulphide formation proceeded normally in mice in which Ero1 was knocked out (Zito et al, 2010a).

The findings of this chapter and other studies suggest disulphide bond formation machinery of the ER is fairly robust and resilient to loss of function of particular enzymes. Therefore the findings that inactivation of several *de novo* disulphide sources results in disulphide impairment in DPMs but not SP cells could be an important clue in elucidating the biochemical and molecular mechanisms that enable the disulphide formation to function in such a robust manner. There are several key differences between DPMs and SP cells that may be responsible for these different observations: they are derived from different cell types; the ER of SP cells remains as a single reticular membrane while that of DPM is in the form of vesicles, also, the ER of SP cells remains in contact with other cellular organelles including the mitochondria (Kornmann et al, 2009) while DPMs are isolated from other organelles. One possible hypothesis is that oxidising equivalents such as hydrogen peroxide could be supplied to the ER by the mitochondria and may also support ER oxidative folding. This hydrogen peroxide could perhaps support direct oxidation of PDI or protein substrates as has been shown to occur *in vitro* (Karala et al, 2009). Alternatively, the hydrogen peroxide may be supporting peroxidase enzymes that will still be active such as the glutathione peroxidases 7 and 8 (Nguyen et al, 2011). Such a suggestion is highly speculative at this stage but may warrant further investigation.

Chapter 5: Results 3: Analysis of mixed disulphide interactions between PDI and prolactin during and following translocation to the ER

5.1 Summary

PDI is an ER luminal enzyme that plays a key role in the oxidative folding of nascent secretory proteins. We sought to investigate the interactions between PDI and nascent proteins during ER translocation. For this study we used a RRL system to translate mRNA encoding PPL or mRNA encoding PPL that stalled on the ribosome. Translation was carried out in the presence of HT1080 SP cells over expressing a substrate trapping PDI mutant. The translationally stalled PPL only partially translocated across the ER membrane while the non-stalled PPL was unhindered in its translocation to the ER. We immunoisolated translation product and showed that mixed disulphide species formed with PDI and translationally stalled prolactin as well prolactin that had translocated to the ER lumen. These results indicate that prolactin is a substrate for PDI and that PDI may interact with prolactin during translocation across the ER membrane. We also detected a change in the prolactin-PDI mixed disulphide species between stalled and luminal prolactin. This indicates that PDI may form additional interactions with prolactin following completion of translocation to the ER lumen.

5.2 Introduction

The folding of nascent secretory protein within the ER occurs in conjunction with the formation of disulphide bonds in a process known as oxidative folding. The PDI family of oxidoreductases play a key role in this process by catalysing the oxidation of thiols as well as the rearrangement and reduction of disulphides. In addition, the PDI family also acts as chaperones that bind unfolded and misfolded proteins to prevent their aggregation (Ali Khan & Mutus, 2014). Due to these roles the PDI family is of importance to our understanding of the maturation of secretory proteins and the molecular processes of protein folding diseases (Wilkinson & Gilbert, 2004).

The enzyme PDI is the most extensively studied member of the PDI family. PDI has been analysed by structural, biophysical and cellular approaches. These studies have yielded significant insight into how the enzyme interacts with its substrates and supports the oxidative folding process. It is now understood that PDI is one of several enzymes that interact with and catalyses oxidative folding in secretory proteins. A current research goal is to understand the initial sequence of events of the interaction between PDI and its nascent substrates (Hatahet & Ruddock, 2009). In this study we analysed the mixed disulphide species that form between PDI and the substrate prolactin upon entry to the ER. In order to understand the current stage of PDI research it is useful to review some of the preceding studies.

The structure of PDI has been solved in yeast and humans (Tian et al, 2008; Tian et al, 2006; Wang et al, 2013). The structure is made up of four domains that adopt a “horseshoe” or “U” shaped conformation. The outermost thioredoxin domains (designated a and a’) contain the CXXC catalytic motifs involved in thiol-disulphide exchange reactions. These two domains are bound to the internal domains (designated b and b’) which form a large hydrophobic cleft (Fig 5.1). Crystallisation of yeast PDI at high temperature (22°C) have shown that the a and a’ domains are highly flexible while the b and b’ domains are both rigid (Tian et al, 2008). Furthermore, it has been shown that the enzyme undergoes redox regulated conformational changes in which it adopts an open conformation while oxidised but a closed conformation when reduced (Wang et al, 2013).

These findings have lead to a model for oxidation of substrates: It is proposed that the open conformation of the oxidised form facilitates interaction of PDI with reduced and unfolded substrates which bind to the hydrophobic cleft of the b and b’ domains. PDI then catalyses oxidative folding of the substrate and becomes reduced in the process. This in turn causes the PDI to adopt a more closed conformation and induces release of the substrate (Tian et al, 2008; Tian et al, 2006; Wang et al, 2013).

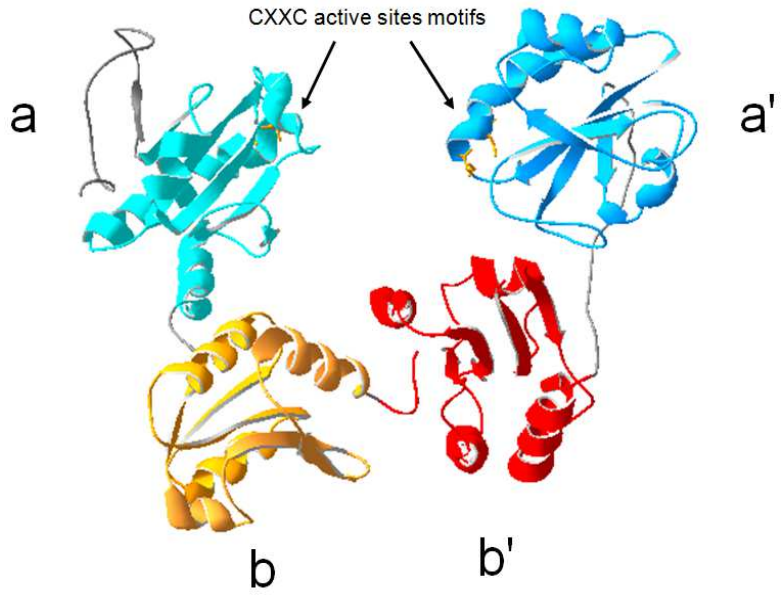

Figure 5.1: Structure of PDI: The image shown was produced using DeepView and represents the structure of human PDI (4EKZ). Domains a, a’, b and b’ are shown in aqua, blue, orange and red respectively. The CXXC active sites in the a and a’ domains are indicated. The structure shown was solved by Wang et al by x-ray crysallography (Wang et al, 2013).

The interaction between PDI and a folded, partly folded and unfolded protein has been investigated by NMR spectroscopy (Irvine et al, 2014). This study used bovine pancreatic trypsin inhibitor (BPTI) as a substrate. Natively disulphide-bonded BPTI represented the folded form; BPTI in which all the cysteines were alkylated (and hence incapable of forming disulphides) represented the unfolded form; and a BPTI mutant capable of only forming two disulphides (rather than three) represented a partly folded form. Analysis of the interaction between PDI and these BPTI forms showed that PDI bound all three forms, however, the binding to the native BPTI was weaker than the binding to the unfolded or partly folded forms. These findings suggest that PDI preferentially interacts with substrates that have not completed folding (Irvine et al, 2014). This implies that PDI may interact with substrates in the early stages of translocation to the ER when protein substrates would still be in an unfolded state.

The effect of PDI on both protein folding and disulphide formation has also been specifically analysed using a biophysical approach (Kosuri et al, 2012). This study involved unravelling a single molecule substrate by force generated by atomic force microscopy. The substrate was then allowed to interact with the catalytic a domain of PDI (PDIa) to form a mixed disulphide species. Following this the folding and disulphide bond formation of the substrate was analysed by unravelling the substrate with a further force pulse. The length of extensions of the substrate that were unravelled with each force pulse indicated if the substrate had folded or if it had folded and also formed a disulphide bonded. It was found that protein folding was not impeded by PDIa attachment but protein folding did appear to be the rate limiting step of disulphide formation. It was therefore concluded that protein folding drives PDI catalysed disulphide formation (Kosuri et al, 2012).

The biological substrates of the PDI family members have been investigated by isolating the covalently bound mixed disulphide species that form during thiol-disulphide exchange reactions (Sevier & Kaiser, 2002) (Fig 5.2). These species are normally transient reaction intermediates, however it has been found that these species can be stabilised by mutating the CXXC catalytic motif of the thioredoxin domains. The N-terminal cysteine of this motif is responsible for targeting substrates while the C-terminal cysteine is primarily responsible for resolution of the mixed disulphide species that forms with a substrate (Walker et al, 1996). As such, mutation of the C-terminal residue to either an alanine or serine residue prevents resolution of the species by this mechanism and thus stabilises the species. This method has been used to capture and isolate mixed disulphide species between PDI family members and substrates. A specific procedure that has been used is to over express a PDI family member containing CXXA/S active site mutations and an epitope tag. The PDI and any substrate it is bound to can then be immumoisolated using an antibody that targets the

tag (Jessop et al, 2009b). This technique has allowed the identification of a number of substrate proteins of the PDI family and has also shown that certain substrates are the target of multiple PDI family members (Jessop et al, 2007; Jessop et al, 2009b; Oka et al, 2013).

It has been reported that disulphide bond formation can occur both co-translationally and post-translationally with the substrates HA, and glycoproteins expressed in Semliki Forest virus (SFV) (Chen et al, 1995; Molinari & Helenius, 1999). The SFV proteins were also found to co-precipitate with PDI and ERp57. This suggests that PDI family members may interact with substrates co-translationally by thiol-disulphide exchange reactions (Molinari & Helenius, 1999).

These studies collective suggest a model in which PDI interacts with nascent unfolded substrates, both co-translational and post-translationally. This interaction catalyses the disulphide formation step of oxidative folding which leads to natively folded proteins with a low affinity for subsequent reactions with PDI.

This model implies that PDI will interact with and form mixed disulphide species with its substrates during translocation to the ER lumen. To investigate this premise we analysed the interaction between PDI and nascent prolactin during translocation of prolactin across the ER membrane. Our approach was to use a RRL *in vitro* translation system in which we either expressed PPL or translationally stalled PPL in the presence of SP-cells expressing a substrate trapping PDI mutant. We then immunoisolated mixed disulphide species between PDI and prolactin. Translationally stalled PPL is only partially exposed to the ER lumen while the non-stalled prolactin is fully imported into the ER lumen. This approach has allowed us to analyse PDI interactions with prolactin during and following translocation into the ER.

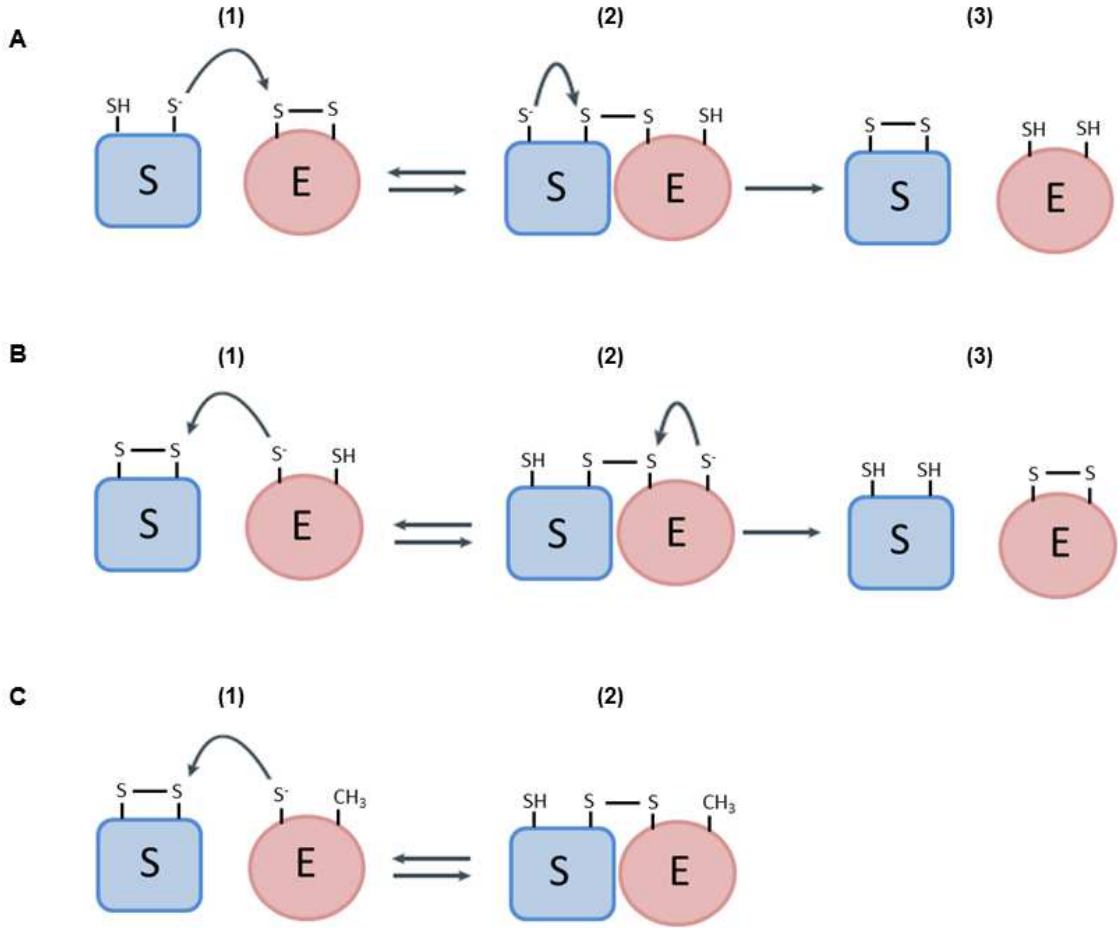

Figure 5.2: Transfer of disulphide bonds by thiol-disulphide exchange reactions occurs via a transient mixed disulphide species. (A) Cysteine residues can be oxidised to form a disulphide bond within a protein substrate: (1) The thiol group of the protein substrate in the form of a thiolate anion carries out a nucleophilic attack on the disulphide bond of a CXXC catalytic motif of an oxidoreductase enzyme. (2) This leads to the formation of a transient mixed disulphide species. (3) The mixed disulphide species is resolved by a nucleophilic attack on the mixed disulphide conducted by the second cysteine of the CXXC motif of the oxidoreductase enzyme which, in the form of thiolate anion, attacks the mixed disulphide. This leads to the resolution of the mixed disulphide and the completion of the thiol-disulphide exchange reaction. **(B)** The reverse reaction can also occur in which disulphide bonds in a protein substrate are reduced to thiol groups. The reaction scheme proceeds by the opposite pathway to the reaction depicted in (A). **(C)** Transient mixed disulphide species can be stabilised using a mutant oxidoreductase enzyme in which the second cysteine of the CXXC catalytic motif has been mutated to a CXXA motif: The reaction proceeds as shown in (B) except that the mixed disulphide species cannot be resolved as the resolving cysteine of the CXXC catalytic motif is not present. S: Substrate in which disulphide formation or reduction is catalysed. E: Oxidoreductase enzyme catalysing formation or reduction of disulphide bonds. The figure shown is based upon a figure originally shown by Sevier and Kaiser (Sevier & Kaiser, 2002).

5.3 Results

5.3.1 Optimised immunoisolation of prolactin-PDI mixed disulphide species

Our first objective was to determine whether PDI and nascent prolactin interact by thiol-disulphide exchange reactions. These reactions require a mixed disulphide species to be formed; therefore, we sought to capture and isolate this species. To achieve this we utilised a HT1080 cell line over expressing a substrate trapping PDI mutant (stPDI) in which the CXXC motif of both active sites had been mutated to CXXA (Jessop et al, 2009b). This CXXA mutation stabilises the normally transient mixed disulphide species that wild type PDI (wtPDI) forms with its substrates (Walker et al, 1996). This makes the species easier to capture. In addition to the CXXA mutations, stPDI is also V5 tagged; this thus enables immunoisolation of stPDI in conjunction to any prolactin that it is bound to (Fig 5.3).

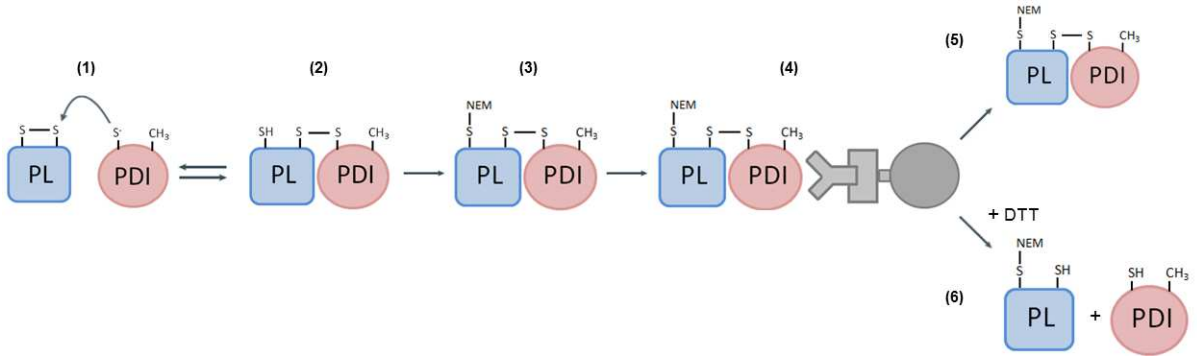

Figure 5.3: Method of detection of isolated prolactin-stPDI mixed disulphide species. Nascent prolactin is synthesised in a RRL system in the presence of SP cells generated from HT1080 cells transfected with stPDI. **(1,2)** stPDI may act as a reductase towards disulphides within prolactin causing a mixed disulphide species to form. As the CXXA motif of stPDI lacks a C-terminal cysteine this mixed disulphide species cannot be resolved efficiently. **(3)** The mixed disulphide species is further stabilised by treatment with the alkylating agent NEM which reacts with free thiols and prevents further reactions of these groups. **(4)** stPDI is specifically immunoisolated via its V5 tag, allowing isolation of stPDI bound to prolactin. **(5,6)** The isolated prolactin-stPDI species is maintained under non-reducing conditions, or reduced by the addition of DTT which caused the mixed disulphide between prolactin and stPDI to be broken. Samples may then be processed by SDS-PAGE gel electrophoresis and exposed to a phosphor image plate as described in the materials and methods. PL: prolactin, PDI: stPDI.

An initial obstacle we had with the immunoisolation of prolactin-stPDI mixed disulphide species was that both PPL and PL bound non-specifically to the Sepharose beads used for the immunoisolation. This was problematic because it resulted in contamination from PPL that was not interacting with stPDI. The PPL remained bound to the beads even following the washing steps of the immunoisolation procedure (Fig 5.4A, lane 2). We discovered, however, that pre-incubating the Sepharose beads with 1% BSA prevented almost all non-specific binding of the protein (Fig 5.4A, lane 3). This was thought to be due to BSA blocking the available non-specific binding sites on the Sepharose beads. This method allowed for very effective and specific detection of stPDI boud to prolactin in a mixed disulphide species. The specificity of the detection of this secies is clearly shown by the observation that prolactin could be isolated in samples reduced in loading buffer only if the V5 antibody was used in the immunoislation procedure (Fig 5.4B, compare lane 2 and 4). As the V5 antibody will specifically react with the V5 tag present on stPDI this result demonstrates that only prolactin bound to the stPDI was specifically detected.

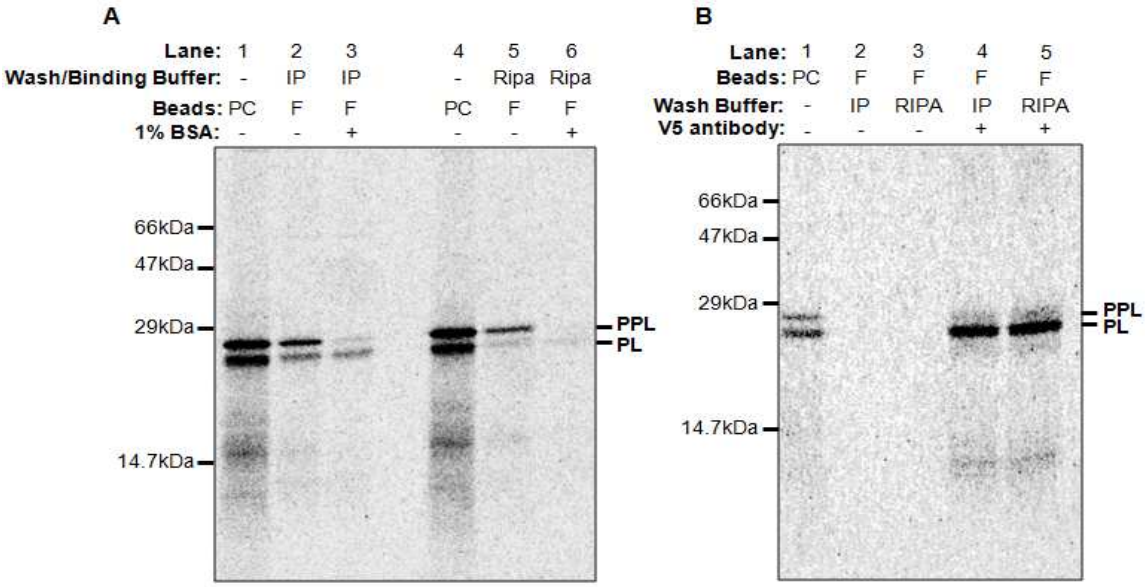

Figure 5.4: Optimisation of immunoisolation of prolactin bound to stPDI: Optimisation of the conditions used for immunoisolation of prolactin bound to stPDI was required to prevent contamination of the immunoisolate from PPL binding to the Sepharose beads. Immmunoisolation using a V5 antibody was carried out on RRL samples in which mRNA encoding PPL had been translated for 30 min in the presence of SP cells generated from cell cultures of HT1080 cells transfected with stPDI. DTT was added to all samples following the immunisolation procedure. The DTT abolished any mixed disulphides between PL and stPDI allowing isolated prolactin to be examined. The effect that varying specific factors of the immunoisolation procedure had upon the binding of PPL contaminants to the beads was examined. **(A)** Pre-treating the protein A conjugated Sepharose beads with 1% BSA prevented the vast majority of PPL binding to the beads. Use of the stringent RIPA buffer for washing and binding steps was less effective than the IP buffer as it did not reduce PPL binding to the beads but instead reduced the desired immunisolation of PL bound to stPDI. **(B)** The immunoisolation procedure was conducted with and without V5 antibody to confirm that that the PL isolated by this procedure was from PL bound to stPDI. PL was only immunoisolated when V5 antibody was present. The effect of using RIPA buffer instead of IP buffer on the washing but not binding steps was examined. There was no observable difference between using these different buffers on the immunoisolation. PPL: preprolactin, PL: prolactin, BSA: bovine serum albumin, PC: preclear protein A Sepharose beads, F: final protein A Sepharose beads.

5.3.2 Analysis of prolactin disulphide formation in the presence of stPDI

Having optimised the immunoisolation procedure our next objective was to assess how the presence of stPDI affects disulphide formation of prolactin and to specifically analyse the prolactin-stPDI mixed disulphide species that forms.

In order to assess the effect of stPDI on prolactin disulphide formation we compared the output of PPL translation in the presence SP cell expressing stPDI with our previous findings with untransfected SP cells (compare Fig 5.5A with Fig 4.1A). With both cell types, the reduced samples of the translation products showed the expected bands of full length PPL and signal cleaved PL. Under non-reducing conditions a faster mobility band corresponding to native disulphide bonded prolactin (DB-PL) was also observed with both sample sets. However, in the presence of SP cells expressing stPDI, only a fraction of the prolactin molecules were in the DB-PL form, while in untransfected cells all of the prolactin was in this form. This suggested that in the presence of stPDI only a minority of the prolactin molecules had completed the oxidative folding process and achieved a native disulphide configuration. Several additional bands were also observed in the presence of stPDI. These comprised one band of the same mobility as reduced prolactin and several bands of higher molecular weight species. The presence of a subset of the prolactin molecules in the apparent reduced form indicated that the long range disulphide between cysteines 88 and 204 (signal sequence included in numbering) had failed to form suggesting that these molecules may have misfolded. The observation of several higher molecular weight DTT sensitive bands indicated that a large fraction of the prolactin was interchain disulphide bonded to other molecules. As these bands were only observed in the presence of stPDI we expected a large fraction of these mixed disulphide species to be composed of prolactin bound to stPDI.

V5 immunoisolation indicated that a substantial amount of the PL was indeed in a mixed disulphide species with stPDI (Fig 5.5B). Surprisingly, rather than one prolactin-stPDI species we detected multiple forms with a large range in SDS-PAGE gel mobility. The range of prolactin-stPDI forms observed corresponded to the mobility of many of the prolactin interchain disulphide species observed in the translation output (compare lane 2 of Fig 5.5A with Lane 2 of Fig 5.5B). This indicated that the prolactin-stPDI species accounted for a large proportion of the observed interchain disulphide bonded prolactin forms. We had anticipated that there would be one prolactin-stPDI form with the approximate gel mobility of 80kDa species as this is the sum of the individual molecular weights of PL (22.7kDa) and PDI (57.1kDa). Indeed the V5 immunoisolation shows a single strong band of approximately this size. However, the presence of other forms indicates that the prolactin-stPDI species isolated are not all homogenous and most likely include species that are also interacting with other proteins.

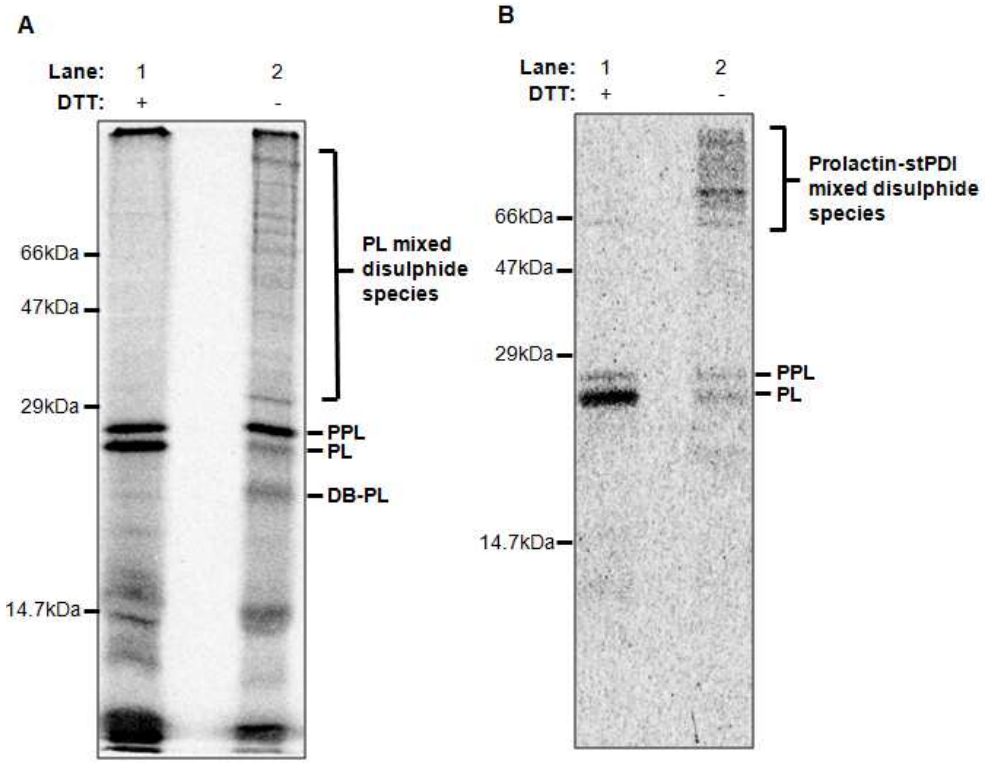

Figure 5.5: Prolactin forms a mixed disulphide with PDI. (A) mRNA encoding PPL was translated for 30 min in the presence of SP cells generated from cell cultures of HT1080 cells over expressing stPDI. **(B)** Immunoisolation using a V5 antibody was carried out using RRL translation samples produced under the conditions described in (A). PPL: preprolactin, PL: prolactin, DB-PL: Intramolecular disulphide bonded prolactin.

5.3.3 Mixed disulphide species between prolactin and PDI can be detected with wtPDI as well as stPDI

We next investigated if it was possible to isolate trapped prolactin-PDI mixed disulphide species using SP cells generated from untransfected HT1080 cells and HT1080 cells over expressing wtPDI. We found that mixed disulphide species could be trapped using SP cells derived from wtPDI over expression cells but not untransfected cells (Fig 5.6). This suggested that a high concentration of PDI is required for these mixed disulphide species to be detected. This is perhaps not surprising as it may be expected that at any one time only a fraction of the prolactin molecules are in a mixed disulphide interaction with PDI. Indeed, a previous study in yeast cells found that only a fraction of the substrate carboxypeptidase Y was in a mixed disulphide species with wtPDI at any one time (Frand & Kaiser, 1999). Therefore, if a higher concentration of PDI is present, it may be anticipated that the proportion of prolactin molecules interacting with the enzyme may be higher and thus a mixed disulphide interaction may be more readily detected. A comparison of PDI expression between untransfected HT080 cell and HT1080 cells over expressing stPDI confirmed the

PDI expression is substantially higher in cell lines over expressing PDI (Fig 5.7). This may explain why the interaction with prolactin could not be detected when only endogenous levels of PDI are present during the reaction.

Interestingly, it can be seen that one prolactin-PDI species is prominent when stPDI over expressing SP cells are used; however, there are no prominent species when wtPDI over expressing SP cells are used (Fig 5.6, compare lanes 5 and 6). This indicates that this particular prolactin-PDI mixed disulphide species is stabilised when CXXA mutant PDI is involved in the interaction.

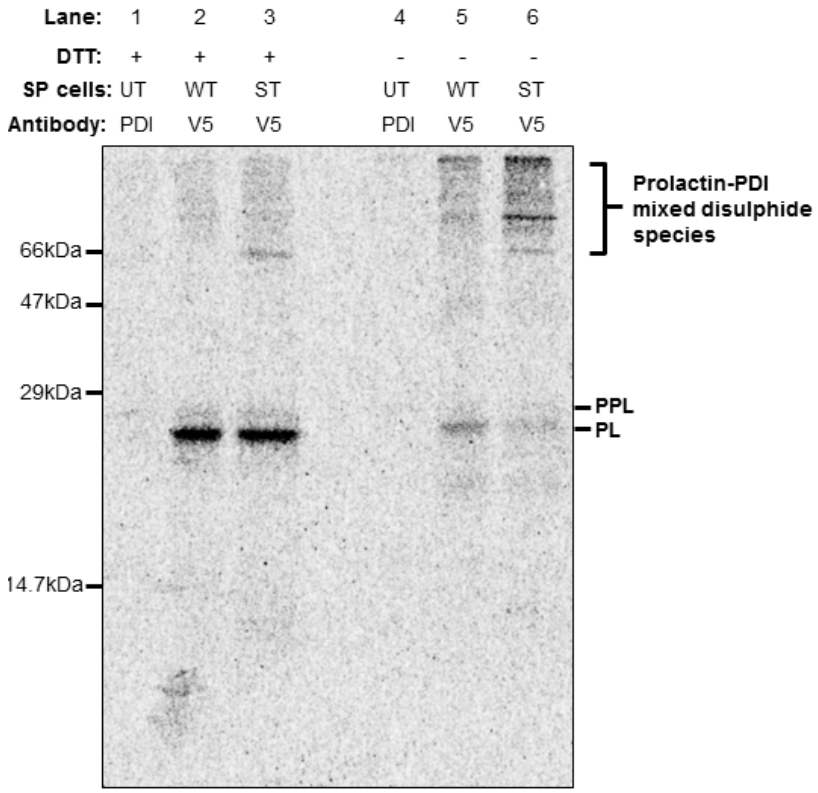

Figure 5.6: Prolactin-PDI mixed disulphide species can be detected in HT1080 cells over expressing wtPDI or stPDI but not in untransfected HT1080 cells expressing endogenous PDI only. mRNA encoding PPL was translated for 30 min in the presence of SP cells generated from cell cultures of either untransfected HT1080 (UT), HT1080 over expressing wtPDI (WT) or HT1080 cells over expressing stPDI (ST). V5 immunoisolation was then carried out on all samples.

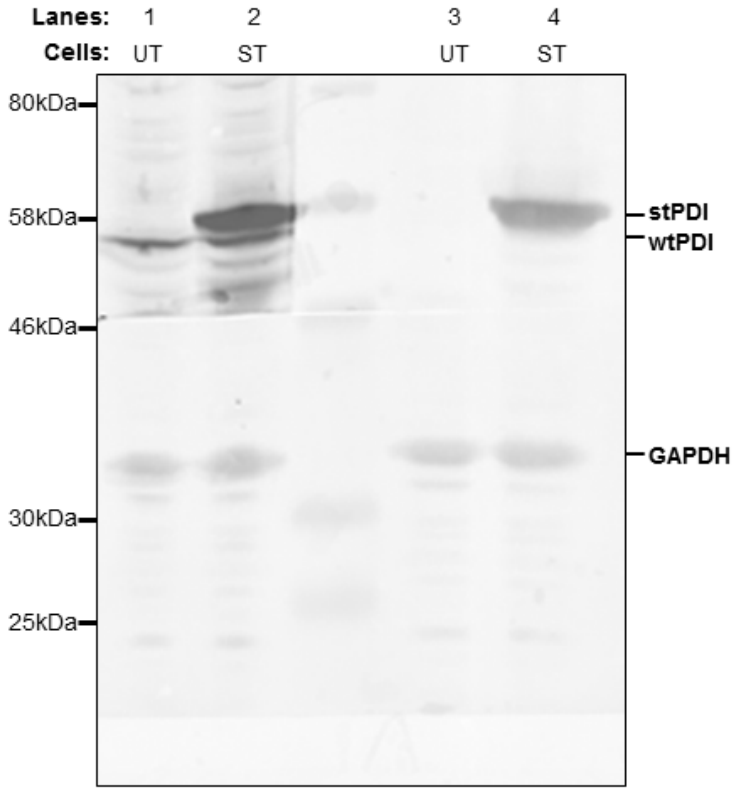

Figure 5.7: PDI expression in untransfected HT1080 cells and HT1080 cells over expressing stPDI. Western blotting was carried out on samples of 50,000 cells from either untransfected HT1080 (UT) or HT1080 cells over expressing stPDI (ST). The nitrocellulose membrane shown was cut into 3 sections; one horizontal cut was made just above the 46kDa marker shown and one vertical cut was made to the immediate right of lane 2. This allowed the membrane to be probed with 3 different primary antibodies without any issues from cross-reactivity between the antibodies. Lanes 1 and 2 were probed with an anti-PDI antibody; lanes 3 and 4 were probed with an anti-V5 antibody. The lower half of all lanes was probed with an anti-GAPDH antibody. It can be seen that stPDI is over expressed in transfected cells relative to the endogenous wtPDI. Equal loading is shown by the GADPH loading control. N.B. The molecular weight of stPDI is slighter larger than that of wtPDI due to the V5 tag present on stPDI.

5.3.4 Translationally stalled prolactin is only partially exposed to the ER lumen

Our next objective was to determine if PDI interacts with prolactin during translocation to the ER lumen. In order to investigate this interaction we generated a mRNA sequence encoding PPL in which the stop codon of the sequence had been removed. The stop codon of a mRNA sequence is required to allow the protein synthesised to be released from the ribosome. In the absence of a stop sequence the protein remains bound to the ribosome via the terminal tRNA. Photocrosslinking experiments with PPL have shown that approximately 30 amino acids of the sequence occupies the ribosome exit tunnel while approximately 50 amino acids of the sequence transverse the Sec61 translocation (Mothes et al, 1998). We reasoned that our translationally stalled preprolactin (tsPPL) would have the three N-terminal

cysteine residues exposed to the ER lumen, however the three C-terminal cysteines of the sequence would be shielded from the lumen by the channel of the ribosome (Fig 5.8). Crucially, the prolactin would be unable to form the long range disulphide between cysteines 88 and 204 that forms in natively folded prolactin (Wallis, 1974) and which is thought to be responsible for the gel-shifted disulphide bonded prolactin (Fig 4.1).

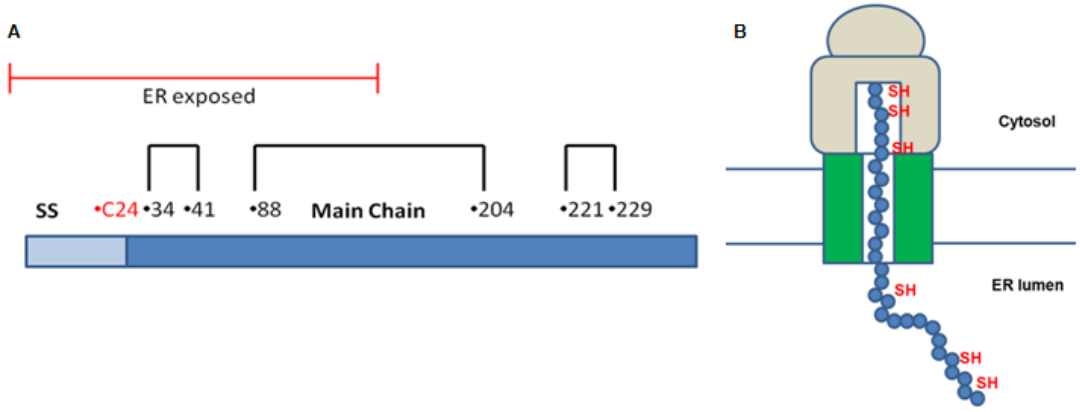

Figure 5.8: Exposure of cysteine residues to the ER lumen of translationally stalled preprolactin. **(A)** Diagram showing all 7 cysteine residues of the bovine preprolactin sequence and the cysteines exposed to the ER lumen when mRNA encoding tsPPL is expressed. The native disulphide pairing of cysteine residues from PPL that is not stalled is also shown. (**B)** Diagram illustrating the positioning of cysteine residues relative to the ribosome and Sec61 translocon in tsPPL. N.B. The PPL signal sequence (SS) contains one cysteine residue a position 24 however the SS is removed during entry to the ER.

5.4.5 Translationally stalled prolactin is unable to form a long range intramolecular disulphide bond

We examined the disulphide bond formation of tsPPL (Fig 5.9). As predicted we found that the stalled sequence was indeed incapable of forming a long range intrachain disulphide bond. This was apparent as no fast mobility DTT-sensitive prolactin band was visible indicative of such a long range disulphide. The translationally stalled prolactin appeared to be reasonably stable as the majority of the prolactin had the same mobility as the reduced form over the course of the 90 min experiment. This suggested that the translated sequence remained bound to the ribosome throughout the reaction.

A fraction of the stalled prolactin appeared to accumulate at the interface of the resolving gel over the course of the reaction. Comparison of reducing and non-reducing lanes indicated that this material was DTT sensitive suggesting it contained disulphides (Fig 5.9 compare lanes 1 and 2). The lack of gel mobility suggested it was aggregated material and therefore it was designated as interchain disulphide bonded prolactin aggregates. The gradual

accumulation of this material may indicate the misfolding and aggregation of neighbouring stalled prolactin chains. This is likely to be a consequence of prolonged stalling of the prolactin oxidative folding process.

A faint higher molecular weight doublet was visible throughout the reaction. This doublet was visible when tsPPL but not PPL mRNA was translated. We reasoned that this doublet was caused by tsPPL still bound to tRNA. The ester bond that connects peptides and tRNA is known to be easily hydrolysed, particularly at basic pH such as the basic pH of SDS-PAGE gels (Bresler et al, 1968; Kirchdoerfer et al, 2007). We therefore reasoned that the that the ester bond of the majority of stPPL had become hydrolysed following the translation reaction while a minority of the stPPL had remained bound throughout the analysis procedure.

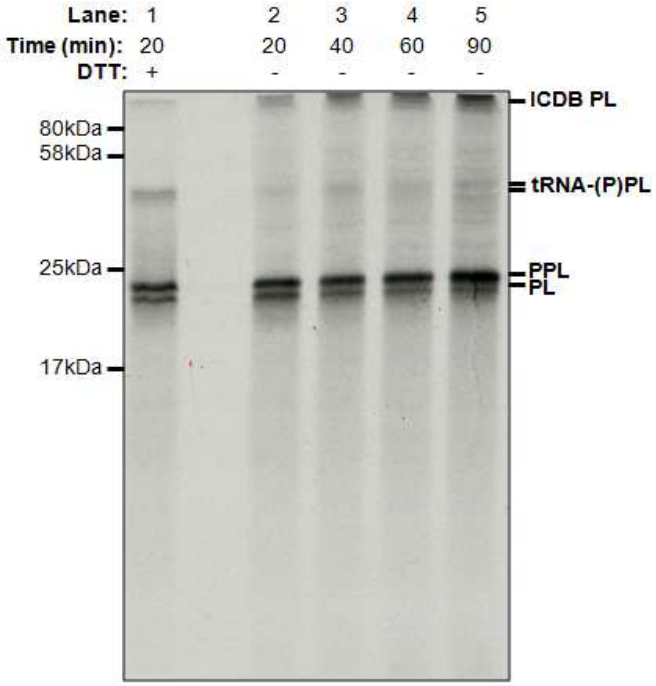

Figure 5.9: Translationally stalled preprolactin is prevented from forming a long range intrachain disulphide bonds. mRNA encoding tsPPL was translated in the presence of untransfected HT1080 SP cells for the indicated times. ATCA was added to a concentration of 75 µM 5 min after incubating the translation mixture at 30°C to prevent further translation initiation. This allows the disulphide bond formation of time synchronised set of nascent translationally stalled prolactin to be analysed. PPL: Preprolactin, PL: non-disulphide bonded prolactin, tRNA-(P)/PL: tRNA bound preprolactin/prolactin, ICDB PL: interchain disulphide bonded prolactin aggregates.

We found that translationally stalled prolactin could be effectively released from the ribosome by treatment with the ribonuclease enzyme RNase A (Fig 5.10). The release of stalled prolactin was evident for two reasons. Firstly, no tRNA bound PPL was visible in treated samples, and secondly a fraction of the prolactin formed a long range intrachain

disulphides following the treatment indicating that C-terminal cysteines had become accessible to the ER lumen.

While we observed that in RNase A treated samples about half of the prolactin had formed a long range intrachain disulphide we also noted that half of the prolactin remained at the same gel mobility as reduced prolactin. This suggested the this prolactin had either not formed disulphide bonds or had only formed short ranged disulphide bonds that had no detectable effect on gel mobility. This indicates that the disruption of the protein translocation and the oxidative folding process caused by the stalling and release of the prolactin may have made incorrect intrachain disulphide pairing and/or misfolding more likely. One possible explanation for this is that the prolonged exposure of only the three N-terminal cysteines to the ER lumen increased the likelihood of non-native disulphide pairings between these cysteines. Possible non-native pairings could have occurred between cysteines 31 or 41 with cysteine 88 (refer to Fig 5.8). This in turn may have prevented the formation of the long range disulphide between 88 and 204 and as such prevented the formation of a detectable long-ranged disulphide bonded.

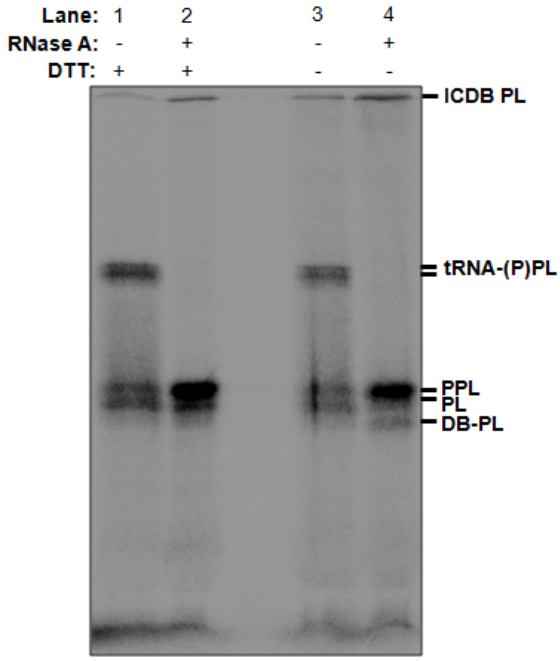

Figure 5.10: RNase A treatment causes release of translationally stalled prolactin from the ribosome. mRNA encoding tsPPL was translated in the presence of DPMs for 20 min. RNase A was then added to the indicated samples and these samples were incubated at 30°C for a further 20 min. PPL: preprolactin, PL: prolactin, DB-PL: intrachain disulphide bonded prolactin, tRNA-(P)/PL: tRNA bound preprolactin/prolactin, ICDB PL: interchain disulphide bonded prolactin aggregates.

5.4.6 PDI interacts with luminal exposed cysteines of prolactin during translocation of the protein to the ER

Mixed disulphide species were successfully isolated when tsPPL was translated in the presence of SP cell expressing stPDI (Fig 5.11). This shows that stPDI was capable of interacting with the three N-terminal cysteines of prolactin during translocation of the molecule to the ER lumen. Two mixed disulphide species were observed when prolactin was stalled; a faint slow mobility form, and a prominent fast mobility form. Following release of the stalled prolactin both of these forms were observed as major bands. These two mixed disulphide species were also both observed as major bands if non-stalling prolactin was translated.

The increase in intensity of the slow mobility form following release of stalled prolactin may indicate that a second form of prolactin-PDI interaction gains in stability as the C-terminal proportion of the molecule enters the ER lumen. Alternatively, this second slower mobility form may only be capable of forming at later stages of translocation or following entry to the ER lumen. If this were the case, the observation of this form as a faint band when stalled prolactin was translated may be due to a minority of the prolactin protein becoming released to the lumen without the addition of RNase A.

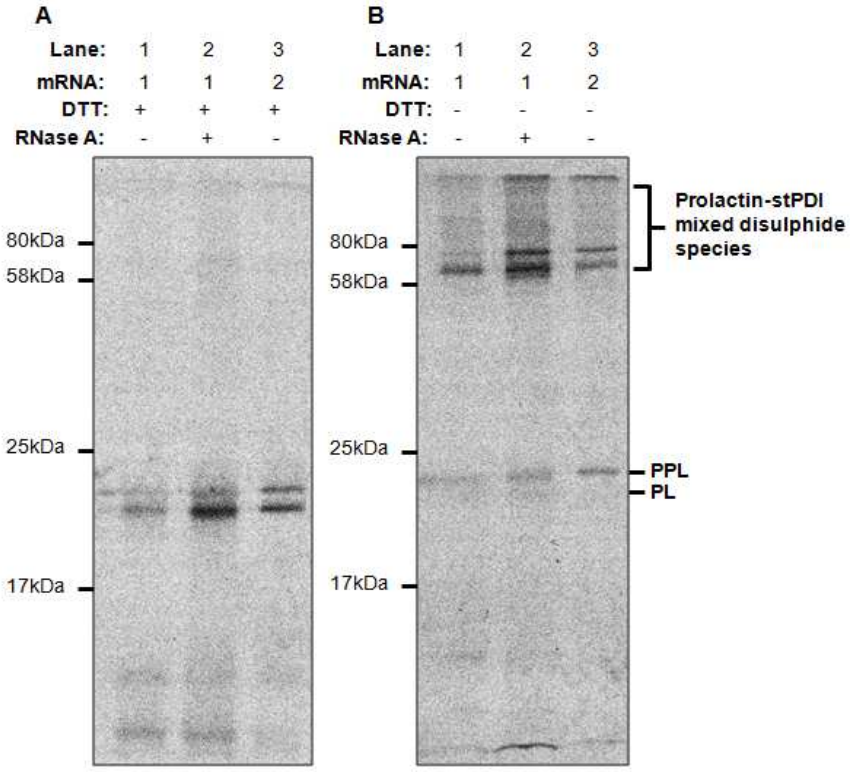

Figure 5.11: stPDI forms mixed disulphide species with prolactin during and following translocation to the ER lumen. (A,B) mRNA encoding either tsPPL (1) or PPL (2) was translated for 20 min in the presence of HT1080 SP cells over expressing stPDI. RNase A was added to the indicated samples to cause release of the tsPPL. Prolactin-stPDI mixed disulphide species were captured following the translation reaction by V5 immunoisolation.

Surprisingly the multiple stPDI-prolactin observed in earlier experiments were not observed under the same conditions with this experiment (Compare Fig 5.5B lane 2 and Fig 5.6 lane 6 with Fig 5.11B lane 3). One possible cause for this experimental variation was the use of a new batch of RRL for this experiment which was not used for previous experiments. Each batch of RRL is produced from different rabbits and as such there is some small variation in the composition of each lysate. A consistent feature of each of these results was a major band of approximately 80kDa in size. As the other multiple bands observed in the earlier experiments are relatively weak compared to this major band it may be that these different stPDI-prolactin forms are sensitive to the subtle variations in biochemical and molecular composition between the lysates. Further experimentation with this approach will give a clearer indication of the different species of this interaction.

5.5 Discussion

5.5.1 PDI acts as a reductase of prolactin during translocation to the ER

In this study we identified that prolactin is a substrate of PDI. We detected this interaction by using a substrate-trapping PDI mutant which allowed the isolation of mixed disulphide species between prolactin and PDI. Interactions between PDI and PPL have been detected using a cross-linking approach in a study by Klappa *et al* (1995) (Klappa et al, 1995). While the cross-linking approach of Klappa *et al* (1995) was indicative of a general interaction between PDI and PPL the isolation of mixed disulphide species in this study indicates that the two molecules engage in thiol-disulphide exchange reactions.

The stPDI mutant used in this investigation targets disulphide bonded substrates with which it forms mixed disulphide species. The reaction steps involved in forming these mixed disulphide species matches the reaction steps of substrate recognition and interaction of endogenous PDI family members acting in a reductase functional role (Sevier & Kaiser, 2002). This therefore suggests that PPL is a substrate of reductase enzymatic activity of PDI.

A previous investigation made a comparison of the mixed disulphide species isolated from the lysates of several cells lines over expressing PDI family members with CXXA substrate trapping mutations (Jessop et al, 2009b). It was found that each PDI family member formed mixed disulphide species with a distinct set of substrates. Only a few mixed disulphide interactions were detected for PDI which implied it may only have a few substrates in its role as a reductase (Jessop et al, 2009b). The results of this chapter indicate that prolactin is part of the small subset of secretory proteins which are reductase targets of PDI.

Prolactin also formed mixed disulphide in the ER of SP cells over expressing wtPDI. The transfected wtPDI is not biased towards oxidase or reductase reactions and acts in the same manner as endogenous PDI. Therefore, the observation of mixed disulphides with wtPDI indicates the interaction of stPDI with prolactin was not merely an aberration caused by the active site mutations within the enzyme.

Prolactin-stPDI interactions were observed with translationally stalled prolactin and an additional prominent mixed disulphide species was detected following translocation to the ER. This indicates that reduction of disulphides by stPDI can occur during the ER translocation process but suggests that complete translocation of a substrate may allow additional interactions between the enzyme and substrate to occur.

5.5.2 Native disulphide bonded prolactin may still form in the presence of stPDI

The CXXA mutations of the stPDI used in this investigation prevent resolution of mixed disulphide species by a C-terminal cysteine (Sevier & Kaiser, 2002). Despite this substrate trapping, analysis of prolactin disulphide formation in the presence of stPDI indicated that a large fraction of the nascent prolactin was capable of forming native intrachain disulphide bonds. This shows that not all of the nascent prolactin was locked in a mixed disulphide with stPDI when the reaction samples were analysed.

There are two possible pathways that may have allowed native disulphide formation in the presence of stPDI. Firstly, endogenous wtPDI may have catalysed disulphide formation in the correctly folded fraction of prolactin. The prolactin that achieved correct folding may then have had a low affinity for subsequent interactions with stPDI (Irvine et al, 2014), allowing it to remain as a monomeric species. Secondly, it is likely that the prolactin-stPDI species that form are resolved at a slow rate by reaction with glutathione. Resolution of substrate trapping species by such a reaction has been suggested previously (Dick & Cresswell, 2002; Walker & Gilbert, 1997). This model of reaction resolution is supported by the finding that a glutathione redox buffer increases the catalytic rate of PDI mutants containing only a single active site cysteine by 20 to 40 fold (Walker & Gilbert, 1997). Therefore, the prolactin that was observed in a monomeric natively disulphide bonded form may have been freed from prior interaction with stPDI by this mechanism of reaction resolution.

5.5.3 PDI may interact with its substrates in conjunction with other enzymes

In two experiments we observed a multiple prolactin-stPDI species with a large range of gel mobility. These species remain intact following heating in SDS-PAGE buffer but were sensitive to DTT indicating that these species contain interchain disulphides.

There are several explanations as to why multiple prolactin-PDI mixed disulphide bands may be detected. First, PDI has two active sites and could be in mixed disulphide species with one or two molecules at any one time. Potentially, this could allow PDI to form mixed disulphide with one or two prolactin moleceuls, or one prolactin molecule and another protein within the ER such as Ero1. Secondly, the prolactin and PDI molecules may have varying amounts of internal disulphide bonding which would affect their hydrodynamic volume and thus affect their mobility on the gel. And thirdly, the PDI enzyme may be acting in an enzyme complex containing other oxidoreductases such as Ero1, ERp57 or GPx7. Other studies have also indicated that such complexes may form (Di Jeso et al, 2005; Frand & Kaiser, 1999; Molinari & Helenius, 1999; Wang et al, 2014).

In summary, the results of this chapter show that PDI interacts with prolactin during and following translocation to the ER. This finding suggests that the interactions between PDI

family members and their substrates during oxidative folding begin as the substrates translocate to the ER lumen. The relative intensities of different prolactin-PDI species were shown to change following release of a stalled prolactin to the ER lumen. Evidence suggestive of multiple higher molecular weight mixed disulphide species comprising prolactin and PDI was also observed. This suggests a complex sequence of interactions between prolactin, PDI and possible other components. Further investigation with this approach may allow greater elucidation of the nature of these interactions.

Chapter 6: Discussion

6.1 General discussion

6.1.1 Cell free protein synthesis systems capable of disulphide bond formation

In this thesis we have described a modified RRL translation system in which the G6P concentration has been optimised to allow specific detection of ER disulphide bond formation and mixed disulphide species. This system was designed to be used as tool for investigation of the biological processes involved in disulphide formation within the ER. This system is an improvement to the DTT/GSSG RRL systems that have commonly been used for these types of investigations (Kaderbhai & Austen, 1985; Marquardt et al, 1993).

Cell-free protein synthesis (CFPS) systems remains a common experimental tool which continues to be developed for use in a range of applications (Chong, 2014). The modifications that we have made to our CFPS system bears some relation to other modified systems, albeit for different purposes. We found that the addition of G6P to our RRL was a useful modification as it biochemically supported redox control of thiol groups within the lysate. In a separate development G6P (along with other metabolites of the glycolytic pathway) has also been identified as an effective energy source to add to *E.coli* lysate systems (Calhoun & Swartz, 2005; Kim et al, 2007). The addition of G6P to this *E.coli* system is one of many modifications that have been made with the goal of optimising the *E.coli* lysate for use as a low cost and versatile system to produce protein for commercial applications (Carlson et al, 2012). With this goal in mind, G6P is used as a energy source for *E.coli* lysate as it is substantially cheaper than traditional energy sources such as phosphoenolpyruvate or creatine phosphate (Calhoun & Swartz, 2005).

Our RRL system is optimised to assay the formation of disulphide bonds within supplemented sources of ER. CFPS have also been generated in which the conditions are optimised for disulphide bond formation in the absence of ER (Goerke & Swartz, 2008; Ryabova et al, 1997). Such systems have been developed for a range of purposes including, the preparation of disulphide bonded proteins for structural and functional analysis (Matsuda et al, 2013), and also more specific purposes such as the testing of the effects of protein sequence changes on the binding or recombinant antibodies (Ryabova et al, 1997). Another focus of such systems is to optimise the production of disulphide bonded proteins for commercial goals such as the production of proteins for therapeutic or vaccination purposes (Goerke & Swartz, 2008; Kim & Swartz, 2004). These non-ER disulphide production CFPS achieve disulphide bond formation by the addition of a mixture of oxidised and reduced glutathione as well as the addition of oxidoreductase enzymes such as PDI or Dsb family members (Kim & Swartz, 2004; Ryabova et al, 1997; Yin & Swartz, 2004).

While there are parallels between our RRL systems and these other CFPS there are also important distinctions between each system. A central focus of the commercial CFPS that produced disulphide bonded proteins is to optimise low cost protein production (Carlson et al, 2012). On the other hand the central focus of our system is to resemble as closely as possible the cellular processes of maturation of secretory protein within the ER in order to study these processes in a physiologically relevant context. Due to these differing goals each of these CFPS has separate developmental paths.

6.1.2 The redox state of PDI family members in intact cells and in SP cells

The redox status of the PDI family members is of importance as it relates to the readiness of these enzymes to carry out their dual roles as either oxidase or reductase enzymes (Jessop & Bulleid, 2004). ERp57, ERp72, P5 and PDI-R have been found to reside in a predominantly reduced state within HeLa and HT1080 cells while PDI is in a partially reduced/oxidised state in these cells (Jessop & Bulleid, 2004; Mezghrani et al, 2001). Molteni *et al* conducted a direct comparison of the redox state of PDI in intact and semi-permeabilised (SP) HeLa cells. It was shown that PDI was in a predominately oxidised state in the SP cells compared to the partial reduced/oxidised state of the intact cells (Molteni et al, 2004). However, exposure of the SP cells to 10mM GSH caused the redox state of these enzymes to become more reduced. It was, therefore, reasoned that the influence of cytosolic GSH upon the ER causes PDI to be maintained in a more reduced state in intact cells (Molteni et al, 2004).

The most common experimental application of SP cells is their use in CFPS systems. It is tempting to make inferences from the findings of Molteni *et al* regarding the redox state of PDI in SP cells when used in an *in vitro* translation. Based upon their evaluation it would be expected that the redox state of PDI when used in a CFPS system would match the redox state in an intact cell if used in a system in which redox homeostasis is biochemically supported, such as with our G6P optimised RRL system. The reasoning behind this is as that the lysate of such a system would support NAPDH production and therefore contain reduced glutathione. The influence of the reduced glutathione from the lysate would cause the PDI of the SP cells to remain in a reduced state. This reasoning is supported by the finding by Jackson *et al* that addition of G6P to gel-filtered RRL supports generation of GSH (Jackson et al, 1983a).

6.1.3 Functional overlap between the enzymatic pathways of PDI oxidation

It has been shown that there are multiple enzymes within the ER that are capable of oxidising PDI or other PDI family members (Bulleid & Ellgaard, 2011). While Ero1 is generally regarded as the primary ER oxidase it has been shown that its deletion from fruit

flies or mice has a mild phenotype and does not substantially effect disulphide formation (Tien et al, 2008; Zito et al, 2010a). Indeed, in our experiments we only detected impairment of the disulphide bond formation of PPL in DPMs if both Ero1 and PrxIV had been inactivated and the microsomes had been subjected to DTT treatment immediately prior to the reaction. This is consistent with cellular experiments that have indicated that both a brief DTT treatment and functional loss of Ero1 or PrxIV are required to detect impaired disulphide formation (Rutkevich & Williams, 2012). These findings suggests a remarkable level of resilience and plasticity within the oxidative pathways of the ER and also suggests that the multiple disulphide bond formation pathways may confer a level of functional overlap allowing for loss of individual pathways to be compensated for.

The fact that this resilience of the oxidative pathways was observed in our DPM supplemented RRL assay is interesting as it is not possible for loss of single pathways to be compensated for by up regulation of other compensatory pathways as might occur in genetic studies in cell culture or animal models. This suggests that in the DPM there is sufficient functional overlap in the pathways of oxidising PDI family members to allow no functional impairment to be detected in the absence of a DTT challenge when Ero1, PrxIV and VKOR activity are lost. It could be argued that this may be a specific feature of cells with a high secretory output. Indeed the pancreatic cells from which DPMs are derived have a particularly high secretory load and may be better equipped to handle functional loss of individual oxidase pathways. However, no impairment was observed with HT1080 SP cells which are not professional secreting cells arguing against this explanation.

6.1.4 Nascent secretory proteins may begin oxidative folding upon translocation to the ER

We showed in chapter 5 that PDI interacts and forms a mixed disulphide species with nascent prolactin during and following translocation of the protein across the ER membrane. It has also been reported that PDI assists in the oxidative folding by acting as a “place holder” of nascent proteins that allows proteins to fold into conformations that facilitate catalysis of disulphide bond formation at the active sites of PDI (Kosuri et al, 2012). A recent NMR study indicated that PDI interacts strongly with unfolded proteins substrates and weakly with folded substrates (Irvine et al, 2014).

Considering these findings and our own, it would appear that PDI and possibly other PDI family members interact with nascent unfolded proteins upon entry to the ER. These interactions catalyse oxidative folding leading to natively folded proteins. Weaker interactions between PDI and possibly other family members may continue following completion of the protein folding.

6.2 Future experiments

6.2.1 Comparison between ER disulphide formation in cells and in G6P optimised RRL

We have shown that our G6P-optimised RRL translation system is a useful experimental tool for investigations with a focus on disulphide bond formation. However, in order to further evaluate how effective this system is as a tool for investigating ER maturation processes it is important to determine how closely the system resembles these processes within cells. This should be achievable by comparison of the disulphide bond formation within our RRL system with those formed in cells under equivalent conditions.

We noted that disulphide bond formation of HA within our RRL system closely resembles the disulphide bond formation of HA that has been identified in a pulse chase assay by Braakman et al (Braakman et al, 1991). Comparison of our results with those of Braakman et al however shows there to be a difference in the number of disulphide bonded HA intermediates detected by these two methods. These experiments did differ in several variables; most crucially, different cell types were used between the experiments. In addition the assays were conducted under different temperatures, and also the HA from the pulse chase assay was isolated with a HA antibody which may preferentially bind to particularly disulphide bonded forms. A comparison should be made between these two assays under the same conditions in order to definitively determine how similar the disulphide formation observed in our assay is to that which occurs in cells.

6.2.2 Identify the cellular or substrate specificity of the VKOR disulphide generation pathway

A current research goal is to characterise the various enzymatic pathways that contribute to disulphide formation within the ER (Bulleid & Ellgaard, 2011). A study by Rutkevich and Williams identified for the first time that VKOR (in addition to Ero1 and PrxIV), is capable of generating disulphides in cells (Rutkevich & Williams, 2012). However, contrary to these findings, our investigation of the *de novo* sources found that VKOR made no detectable contribution to disulphide formation.

As the VKOR disulphide formation pathway is still relatively new to the field, the circumstances in which it contributes to disulphide formation remains to be fully characterised. The differences between our findings and that of Rutkevich and Williams could be explained by a possible specificity of the VKOR pathway towards particular substrates. Alternatively, the extent of the contribution that VKOR makes to disulphide generation may differ widely amongst cells of different tissues.

In our study, we analysed the disulphide formation of prolactin within microsomes generated from dog pancreas tissue. The Rutkevich and Williams study analysed disulphide formation of albumin in a human hepatoma cell line. The expression of VKOR mRNA varies widely amongst different tissues. It is especially highly expressed in the liver and is also unregulated in cancer tissues (Wang et al, 2005). If the extent of VKOR disulphide generation activity is strongly related to tissue expression then it would be expect that VKOR activity would be particularly high in the hepatoma cell line use by Rutkevich and Williams. A direct comparison of the disulphide activity amongst different sources would be needed to evaluate this.

The findings of Rutkevich and Williams showed a circumstance in which the VKOR pathway is active (i.e. with an albumin substrate in hepatoma cells). Therefore, a comparison of VKOR activity amongst different sources of ER and with different protein substrates would be necessary to determine how wide spread VKOR activity is. Such a comparison could be made using the assays systems we described in chapter 4, programmed with mRNA of different substrates (including albumin) and containing microsomes derived from particular cell types (including hepatoma cells).

6.2.3 Identify factors supporting a robust ER disulphide formation pathway in cells

Our results in chapter 4 showed that disulphide formation was substantially impaired in DPMs in which the enzymes Ero1, and PrxIV had been inactivated if the DPMs had also been subjected to a reductive challenge. By contrast, no impairment of disulphide formation in HT1080 SP cells was detected under the same conditions. This indicates that the disulphide formation machinery of SP cells is more robust than that of the DPMs. This is of interest as our findings and that of other studies now indicate that the disulphide formation within the ER of cells is a robust process that remains functional even if key enzymes involved in the process are absent or inactive (Bulleid & Ellgaard, 2011; Rutkevich & Williams, 2012). Therefore, elucidating the factors that are responsible for impairment of disulphide formation in DPMs but not SP cells could allow us to understand the factors that allow disulphide formation to function in a robust manner within cells.

We noted several key factors that may account for the observed difference between HT1080 SP cells and DPMs.

1. DPMs and HT1080 SP cells are derived from different tissues.
2. The ER of SP cells is a single reticular membrane while the ER of DPMs is in vesicles.
3. The ER of SP cells is in contact with other organelles (including the mitochondria) while the DPMs are isolated from organelles.

As hydrogen peroxide (H_2O_2) has been shown to support PDI oxidation *in vitro* (Karala et al, 2009) we also hypothesised that H_2O_2 derived from the mitochondria may support ER disulphide generation .

The particular factor that is responsible for the observed differences could be determined by supplementing our disulphide impairment assay with two different sources of ER: Namely microsomes, or microsomes associated with mitochondria; both of which would be derived from an HT1080 cell line. If disulphide impairment failed to occur with HT1080 microsomes it would indicate that tissue specific ER differences were responsible for the observed differences between DPMs and SP cells. If disulphide impairment did occur it would indicate that either being part of a reticular membrane and/or being associated with other organelles supports disulphide generation.

Microsomes associated with mitochondria are also known as mitochondrial associated membranes (MAMs). It has been shown that MAMs can be isolated from tissue cultures cells through a series of centrifugation steps (Wieckowski et al, 2009). Supplementing our assay with MAMs would indicate if the disulphide formation of the ER is supported by mitochondrial activity. If the MAMs are shown to support disulphide formation it could subsequently be determined if an active electron transport chain (ETC) plays a role in this process. This could be achieved by treatment of the MAMs with cyanide which is known to inhibit the ETC (Berg et al, 2007).

6.2.4 Further investigation of the interactions between PDI and nascent prolactin

In chapter 5 we showed that PDI interacts with nascent PPL as a reductase during and following translocation of the protein to the ER lumen. We have identified the following objectives for further investigation of the interaction of PDI family members with nascent prolactin.

Objectives

1. Analyse the interactions between nascent chain prolactin and other PDI family members.
2. Compare the interactions between PDI and stalled prolactin containing different amounts of cysteines exposed to the ER lumen.
3. Analyse the kinetics of interactions between PDI and nascent chain during and following folding.

The assay we used for the identification mixed disulphide species between prolactin and PDI is applicable for use with all PDI family members or with other secretory protein substrates. Cell lines expressing substrate-trapping mutants of different PDI family members could be

used for such an assay. Such cell lines have been generated previously within the Bulleid lab and used in an investigation by Jessop *et al* to evaluate the substrate targets of PDI family members (Jessop et al, 2009b). These cell lines could be used to generate SP cells and identify interactions with other PDI family members and prolactin during translocation to the ER lumen.

We detected mixed disulphide species between PDI and prolactin that depended upon whether the prolactin was translationally stalled or whether the prolactin had been released to the ER lumen. We noted that the translationally stalled prolactin had only three cysteine residues exposed to the ER lumen while the released prolactin had all six of its cysteine residues exposed. It would therefore be interesting to determine if the change in PDI interactions we observed following release of the prolactin were due to greater exposure of cysteines to the ER lumen or due to a change in the possible interactions with PDI following completion of translocation of prolactin to the lumen. This could be determined by creating a longer translational stalled prolactin construct which contains a C-terminal region containing random coil. The additional C-terminal region could occupy the ribosome and Sec61 translocon tunnels thus allowing the entire prolactin region of the molecule to be exposed to the ER lumen. The interactions between PDI and the C-terminal extended translationally stalled prolactin could be compared when the protein is in the stalled and released forms. If a change in interaction was still observed upon release of this construct it would indicate that PDI interacts different with prolactin following completion of translocation. On the other hand if the stalled and released C-terminal extended prolactin have the same interactions with PDI it would indicate the interaction of PDI with the nascent prolactin is related to the number of cysteine residues exposed.

A study using NMR to analyse the interactions between PDI and its substrates reported that PDI interacts strongly with unfolded substrates and weakly with substrates that had completed folding (Irvine et al, 2014). As such, it would be predicted that a high proportion of nascent prolactin molecules would be in a mixed disulphide interaction with PDI during the early stages of a RRL translation reaction programmed when the protein had not yet achieved native folding. It would then be expected that the proportion of prolactin molecules interacting with PDI would become lower following completion of folding. This interaction could be assayed by carrying out an ATCA time synchronised prolactin translation in the presence of SP cells expressing substrate trapping PDI. The prolactin-PDI mixed disulphide species formed during early time points of this reaction could then be analysed and the relative intensities of the isolated species compared. This would allow the intensity of the PDI interactions with nascent prolactin to be followed from the unfolded to folded state.

There are also several interesting subsequent experiments that could be conducted with this approach. For example, it could be examined if there is a difference in the time-dependent mixed disulphide species of prolactin-PDI interactions detected with SP cells over expressing wtPDI or stPDI. A difference between these PDI forms may be expected as mixed disulphide species with stPDI are stabilised, also stPDI favours a reductase function compared to wtPDI which has no bias to acting as an oxidase or reductase. This interaction could also be assayed at higher incubation temperatures which would promote misfolding and aggregation of the nascent prolactin. As such it may be expected that under such conditions prolactin would interact more strongly with PDI as the enzyme would target misfolded proteins as a chaperone and may be required for reduction of aberrant disulphides.

6.2.5 Evaluate the influence of cytoplasmic redox factors on oxidative folding within the ER

It occurs to us that the G6P optimised RRL translation system that we have described here is potentially an effective system for investigating the influence of external redox factors on oxidative folding within the ER. There a two main reasons for this. Firstly, we have shown that within this system, disulphide bond formation occurs specifically within sources of ER (DPMs or SP cells) but not within the lysate. This indicates that within the lysate the ER of these sources remains a distinct environment for oxidative folding. Secondly, the lysate itself is quasi cellular in nature and contains constituents and factors of the cytosol which can be manipulated by inhibition of specific enzymes. We have shown that inhibition of particular enzymes of this lysate that biochemically support the redox systems can lead to loss of thiol redox control. Therefore, this system provides a means to investigate oxidative folding within a source of ER that is exposed to an external redox environment that can be specifically manipulated.

Several important studies have analysed the influence of the redox buffer glutathione upon oxidative folding (Chakravarthi & Bulleid, 2004; Molteni et al, 2004; Tsunoda et al, 2014). It is useful to review the findings of these studies to understand how best to further the research on this topic.

Molteni *et al* investigated the influence of external glutathione on disulphide formation within the ER (Molteni et al, 2004). In this study, oxidative folding of immunoglobulin J chain (JcM) was assayed within the ER of intact or SP cells suspended in DMEM. It was found that the rate of disulphide bond formation of JcM following a DTT reductive challenge was faster within SP cells than intact cells. However, if 10mM GSH was added to the DMEM disulphide

bond formation within the SP cells resembled that of intact cells. This suggested that cytosolic GSH can influence the oxidative folding within the ER (Molteni et al, 2004).

In a related study, Chakravathi and Bulleid investigated the effect of depleting intracellular glutathione levels on oxidative folding (Chakravarthi & Bulleid, 2004). The method they used was to culture cells in the presence of buthionine sulfoxide (BSO), which is an inhibitor of γ-glutamylcysteine synthetase. The cells were subjected to a brief DTT reductive challenge after which oxidative folding of tissue plasminogen activator (tPA) was assayed by pulse chase methodology. The BSO-treated cells were substantially depleted of glutathione with less than 1mM GSH being present in the treated cells compared to the ~13mM GSH present in non-depleted cells. It was found that the glutathione depleted cells had a faster rate of tPA disulphide bond formation. However this did not result in an increased rate of formation of native disulphide bonded tPA. This suggested that the reductive but not the oxidative pathway was impaired by glutathione depletion. No difference in the oxidative folding of tPA between BSO treated and non-treated cells was detected if the cells were not first treated with DTT before the pulse chase assay (Chakravarthi & Bulleid, 2004).

Recently, a study by Tsunoda *et al* has investigated the role of glutathione on disulphide formation using a different approach (Tsunoda et al, 2014). In this study, the glutathione degrading enzyme ChaC1 was specifically expressed within the ER lumen of HeLa cells to depleted the organelle of this chemical. The oxidative folding of low density lipoprotein (LDL) receptor was then assayed by pulse-chase methodology. The LDL receptor was chosen for analysis in this study because it contains multiple disulphides, many of which have been shown to require rearrangement (Jansens et al, 2002). Hence reduction reactions would be required for native disulphide configuration to be achieved. It was found that there was no detectable difference in oxidative folding of LDL receptor between cells in which the ER was depleted of reduced glutathione or those in which glutathione levels were not altered (Tsunoda et al, 2014). This indicates that glutathione is not essential for the ER reductive pathway.

It should be noted that in the study by Tsunoda *et al* the cells were not treated with DTT before the chase analysis was conducted. Therefore, it can be concluded that in absence of a reductive challenge the depletion of ER luminal glutathione had no detectable on the oxidative folding of the LDLR. These results are therefore consistent with the experiments of Chakravarthi and Bulleid, which only detected a phenotype of glutathione depletion on oxidative folding of tPA when the cells were first briefly treated with DTT before chase analysis was conducted (Chakravarthi & Bulleid, 2004).

Analyses of these reports suggest that glutathione may act in the ER reductive pathway but alternative electron donors may also facilitate this reaction and compensate for loss of the chemical. It has also been hypothesized that the thioredoxin system may also influence ER redox processes; this prospect remains to fully explored (Bulleid & van Lith, 2014). Several possible mechanisms for glutathione or thioredoxin mediated effects on ER oxidative folding have been suggested (Fig 6.1) (Bulleid & van Lith, 2014). These proposed pathways suggest that glutathione or thioredoxin is involved in supporting a disulphide reduction pathway by supporting the reduction of PDI family members. Further investigation of the influence of glutathione and particularly thioredoxin upon ER oxidative folding will indicate which of these potential pathways operate in cells.

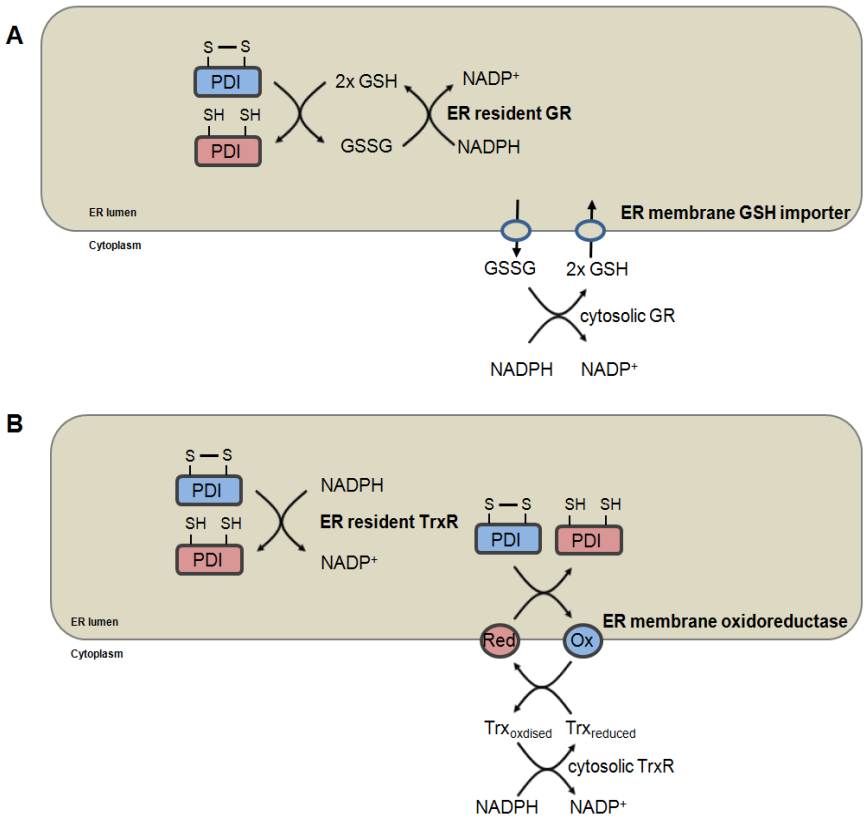

Figure 6.1: Hypothesised mechanisms of PDI reduction. (A) Glutathione mediated reduction: PDI may be reduced by GSH either generated by an ER resident glutathione reductase or imported into the ER lumen by a protein channel. (**B)** Thioredoxin mediated reduction: PDI may be reduced by an ER resident thioredoxin reductase or by the transfer or reducing equivalents from cytosolic thioredoxin, via an ER membrane oxidoreductase. It should be noted that a critical components of each of these possible models remain to be identified. These components are the ER resident glutathione reductase, the ER localised GSH transporter, the ER resident thioredoxin reductase or the suggested ER membrane oxidoreductase. The above figure was adapated from a figure shown by Bulleid and Van Lith (Bulleid & van Lith, 2014).

Our RRL system could be used to answer to two important questions on this topic.

1) Does external reduced thioredoxin influence ER oxidative folding and if so how does its influence compare with that of glutathione?
2) Can external oxidising equivalents influence disulphide bond formation within the ER lumen in the absence of ER luminal enzymes?

It may be possible to achieve the first objective using the translation substrate HA. We found that when we analysed the disulphide bond formation of HA that formation of the native disulphide form was a lengthy process that involves multiple intermediates. Clearly the oxidative folding of a multi-disulphide bonded protein such as HA is a complex and lengthy molecular process that most likely involves disulphide reduction steps as well as thiol oxidation. If external redox factors strongly influence ER oxidative folding then it would be expected that perturbing these external redox factors during the oxidative folding of HA may have a detectable effect.

The disulphide bond formation of an ATCA time synchronised cohort of HA molecules could be analysed in RRL in which the redox pathways of the lysate have been manipulated. A preliminary experiment could study oxidative folding in a lysate devoid of G6P and as such lacking NADPH generation and hence support of the glutathione and thioredoxin redox pathways. Subsequent experiments could specifically inhibit glutathione or thioredoxin reductase by treatment of a G6P supported lysate with carmustine or auranofin. This would thus investigate the specific contribution of external reduced glutathione or thioredoxin to the HA oxidative folding. In each case the specific disulphide bond formation events could be analysed by NEM treatment of samples and processing by SDS-PAGE electrophoresis and autoradiography.

Considering the previous findings of Molteni *et al* and Charkravarthi and Bulleid (Chakravarthi & Bulleid, 2004; Molteni et al, 2004) it is likely that an effect on oxidative folding will be detected when the GSH concentration is reduced, but only if the SP cells are first subjected to a DTT reductive challenge. The effect of reduction of reduced thioredoxin on ER oxidative folding remains untested and therefore it cannot be predicted if there will be an effect. However, we showed in chapter 3 a clear affect on disulphide formation of HA in RRL lacking a source of ER if the thioredoxin pathway was impaired. The lysate is biochemically similar to cellular cytosol. Therefore, an effect that would or wouldn't be detected within the ER could be clearly contrasted to this clear effect that occurs externally of the organelle.

We have shown in this thesis that disulphide bond formation is prevented from occurring within luminal depleted DPMs. The experiment we carried out was conducted in the

presence of G6P optimised RRL. Therefore, thiol redox control would have been maintained within the external lysate during the reaction. It would be interesting to determine if disulphide bonds would form within luminal depleted DPMs in the presence of RRL in which G6P is not added. As we have shown thiol redox control would be lost in such lysate. It remains to be determined if disulphide bond formation would still be prevented within luminal depleted DPMs under such conditions. If disulphide bond formation were to occur it would suggest that the transfer of external oxidising equivalents to the DPM lumen allows the formation of ER disulphide bonds even in the absence of ER luminal enzymes. If disulphide bond formation was still prevented within the luminally depleted DPMs it would indicate that the DPM luminal environment remained distinct from that of the lysate. This would imply that oxidising equivalents could not travel freely from the lysate to the DPM lumen or that a DPM membrane protein catalyses reactions to maintain distinct redox conditions within the ER.

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