Glutathione is required to regulate the formation of native disulphide bonds within proteins entering the secretory pathway.

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Running title: role of glutathione in disulphide bond formation
Summary

The formation of native disulphide bonds is an essential event in the folding and maturation of proteins entering the secretory pathway. For native disulphides to form efficiently an oxidative pathway is required for disulphide bond formation and a reductive pathway is required to ensure isomerisation of non-native disulphide bonds.

The oxidative pathway involves the oxidation of substrate proteins by PDI, which in turn is oxidised by endoplasmic reticulum oxidase (Ero1). Here we demonstrate that overexpression of Ero1 results in the acceleration of disulphide bond formation and correct protein folding. In contrast, lowering the levels of glutathione within the cell resulted in acceleration of disulphide bond formation but did not lead to correct protein folding. These results demonstrate that lowering the level of glutathione in the cell compromises the reductive pathway and prevents disulphide bond isomerisation from occurring efficiently, highlighting the crucial role played by glutathione in native disulphide bond formation within the mammalian endoplasmic reticulum.

Keywords: protein disulphide isomerase/endoplasmic reticulum oxidase/glutathione/protein folding/glycosylation
Introduction

The endoplasmic reticulum (ER) provides an environment that allows the oxidative folding and post-translational modification of proteins entering the secretory pathway. The compartmentalisation of the ER away from the cytosol allows the correct redox conditions to be established (1), which in turn enables a distinct set of folding catalysts to facilitate the formation of native disulphide bonds. A growing family of ER oxidoreductases is thought to be responsible for catalysing the formation, isomerisation and reduction of disulphide bonds (2). These oxidoreductases contain active sites homologous to the active site found in the cytosolic reductase thioredoxin, characterised by a pair of cysteine residues (CxxC) that shuttle between the disulphide and dithiol form (3). The reactions that these enzymes catalyse require the individual active sites to be maintained in either the oxidised disulphide form, for disulphide bond formation, or the reduced dithiol form, for isomerisation or reduction of disulphide bonds (4). How the active sites are maintained in either their reduced or oxidised state and how the ER maintains an environment conducive to disulphide bond formation, isomerisation and reduction has been the subject of intense speculation over the past 40 years (5,6). Recently, components of the oxidative pathway have been identified, however we know very little about the reductive pathway even though it is clear that disulphide bond isomerisation and reduction are essential for cell viability (7).

It is now firmly established that the oxidoreductase PDI catalyses the formation of
disulphide bonds within the eukaryotic ER (8,9). During disulphide bond formation
an intra-chain disulphide bond between the cysteine residues within the active site is
able to accept two electrons from the polypeptide chain substrate resulting in the
reduction of the PDI active site. Ero1p has been shown to be responsible for the
oxidation of PDI in yeast, a defect in Ero1p leads to a lack of disulphide bond
formation demonstrating the crucial role played by Ero1p in the oxidative folding
pathway (5,6). There is growing evidence to suggest that Ero1p is an FAD-
dependent oxidase that is able to pass electrons from PDI to the ultimate electron
acceptor oxygen (10). However, Ero1p can also catalyse the oxidation of PDI under
anaerobic conditions suggesting the possibility that alternative electron acceptors
could substitute for oxygen under these conditions (11).

Although a clear mechanism exists to oxidise PDI, in mammalian cells most of the
ER oxidoreductases, including PDI appear to be in a predominantly reduced form at
steady state (12). This would suggest that a pathway exists to maintain these proteins
in a reduced state within the cell. That PDI is predominantly reduced in mammalian
cells contrasts with the situation in yeast where PDI is clearly predominantly oxidised
(9). The yeast ER maybe more oxidising than the mammalian ER explaining this
discrepancy, however, despite the differences it is clear that PDI in mammalian cells
can be oxidised by Ero1 and catalyses disulphide bond formation (12). At least one
other ER oxidoreductases, ERp57, does not appear to be a substrate for Ero1 as
judged by a lack of oxidation in cells overexpressing Ero1 (12). ERp57 has also been
shown to act as a reductase, at least in vitro, to allow the breaking of non-native disulphide bonds within MHC-class I heavy chain (13). Hence there is a requirement for both an oxidative pathway for disulphide bond formation and a reductive pathway to allow reduction and isomerisation of non-native disulphides and for these pathways to co-exist in the same intra-cellular compartment.

The mechanism for maintaining ER oxidoreductases in a reduced state could involve a protein-mediated process such as exists in the E.coli periplasm where DsbD maintains the main enzyme catalysing disulphide isomerisation DsbC in a reduced state (14). Alternatively a glutathione buffer may be involved, eliminating the requirement for a separate protein-mediated pathway for their reduction. Maintaining a pool of GSH within the ER could be brought about by continuous transport from the cytosol where glutathione reductase maintains a high concentration of GSH. Indeed some evidence exists to suggest the presence of a transport system that allows the selective passage of GSH rather than GSSG (15). Also, elimination of glutathione from yeast cells by removal of the enzyme involved in the first step of synthesis, γglutamylcysteine synthetase, did not prevent disulphide bond formation but did render the cells more prone to hyperoxidation, suggesting a direct or indirect role for glutathione in maintaining a redox balance in the yeast ER (16). Hence the possibility remains that reduction of ER oxidoreductases can be bought about either directly by glutathione or by a separate enzyme catalysed pathway.

To address this point we have chosen to study the folding and disulphide bond
formation of human tissue type plasminogen (tPA). This protein contains 17 disulphide bonds and is secreted from recombinant cell lines as a mixture of two glycoforms that differ in their extent of core N-linked glycosylation. We have previously shown that conditions preventing disulphide bond formation, such as addition of the reducing agent DTT to culture medium of living cells, lead to complete glycosylation of a sequon that would otherwise undergo variable glycosylation in untreated cells (17). The extent of glycosylation of tPA is therefore intimately linked to the rate of protein folding and disulphide bond formation. The close temporal relationship between glycosylation and folding of tPA allows us to evaluate the affect of altering the redox conditions on both the rate of disulphide bond formation and also the rate of protein folding. Here we present evidence establishing that a rate-limiting step in the oxidative pathway and protein folding is the oxidation of PDI. We also show that a high concentration of intra-cellular glutathione is required to ensure the formation of native disulphide bonds.
Experimental Procedures:

Cell lines and treatment

tPA expressing CHO cells (ATCC CRL-9606) were cultured in HAM F-12 medium supplemented with 2 mM L-glutamine and 10% fetal bovine serum (tissue culture media and supplements obtained from Invitrogen) plus appropriate antibiotics at 37°C. In the buthionine sulfoximine (BSO, Sigma) experiments, 0.5 mM BSO was added to the media 6 h after seeding. Cells were cultured in the presence of BSO for 16 h. During the cell labelling experiments with cells cultured in the presence of BSO, 0.5 mM BSO was added to the starve, pulse and chase media.

Generation of stable transfected cell lines

pcDNA3.1-ERO1-L±myc vector, a kind gift from Prof. Roberto Sitia (San Raffaele University, Milano, Italy), was linearized with SmaI and transfected into tPA expressing CHO cells using FuGENE-6 transfection reagent (Roche Diagnostics). Briefly, 2µg of plasmid DNA was mixed together with various amounts of FuGENE-6, incubated at room temperature for 20 minutes and added in a dropwise manner to 6 × 10⁴ CHO cells maintained in 6 well dishes. 48 h after transfection, cells from each well were seeded into 150 mm dishes in the selection medium, which consisted of growth medium containing 0.4 mg/ml G418 (Sigma). The cells were grown in the selection medium for 2-3 weeks and the neomycin resistant clones were selected. The
stably transfected cells were maintained in the selection growth medium and overexpression of Ero1-L±myc was examined by Western blot analysis.

**Immunofluorescence**

Cells were grown on coverslips to 50-70% confluency, washed twice in PBS and fixed in methanol for 10 minutes at -20°C followed by a brief rehydration with PBS. Cells were stained with primary antibodies, mouse anti-c-myc hybridoma supernatant, clone 9E10 (Sigma) and rabbit anti-calnexin polyclonal antibody (Stressgen Bioreagents), diluted in PBS, for 20 minutes. After three 5 minutes washes in PBS, cells were incubated with the secondary antibodies, Alexa Fluor 594 Donkey anti-mouse antibody and Alexa Fluor 448 Donkey anti-rabbit antibody (Molecular Probes Inc.), diluted in PBS, for 20 minutes, followed by four 5 minutes washes in PBS. DNA was stained with DAPI (Sigma). All the incubations were carried out at room temperature. The coverslips were mounted onto slides with Mowiol (Calbiochem) containing 25mg/ml DABCO (Sigma) as an anti-fade agent. Slides were viewed on a Leica confocal microscope and images captured using a charge-coupled camera.

**Western Blotting**

The proteins separated by SDS-PAGE were transferred electrophoretically from the gels on to a nitrocellulose membrane. Blots were blocked in TTBS (100mM Tris-HCl pH 7.5, 0.9% w/v NaCl, 0.1% v/v Tween 80) containing 3% (w/v) milk followed
by incubation for 1 h. in the primary antibody, rabbit anti-\textit{myc} polyclonal antibody (Santa Cruz Biotechnology) or anti-PDI antibody (31), diluted to 1:500 in TTBS buffer. Blots were then washed three times for 10 minutes each in TTBS and incubated with 1:1000 dilution of goat anti-rabbit IgG-HRP (DAKO), in TTBS, for 1 h. After washing three times for 10 minutes each in TTBS, the membranes were treated with Super Signal West Pico chemiluminescent substrate (Pierce) for 5 minutes. All incubations were carried out at room temperature and the proteins were visualized by exposure to Fuji Medical X-ray film.

**Radiolabelling and Immunoisolation.**

For pulse chase experiments, $2 \times 10^6$ cells per 6 cm dish were washed twice with pre-warmed methionine- and cysteine- free DMEM (Sigma), and then pre-incubated in the same medium for 20 minutes at $37^\circ$C. Each monolayer was pulse-labelled with $50 \mu$Ci of $^{35}$S-EXPRESS (Amersham) for 10 minutes at $37^\circ$C and then chased with excess of methionine and cysteine. Dithiothreitol (DTT) was added in the pulse chase medium, where indicated. 0.5 mM cycloheximide (Sigma) was included in the chase medium to block completion of labelled nascent chains. At the end of the labelling period or chase times, the cells were transferred to ice and washed twice with ice cold phosphate-buffered saline (PBS) containing 20mM N-ethylmaleimide (NEM, Sigma) to minimise disulphide bond rearrangements. The cells were lysed in 1 ml of lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl,
1% Triton X-100, 0.1% SDS, 1 mM PMSF, 20 mM NEM) for 10 minutes on ice. Lysates were centrifuged at 12,000g for 20 minutes to remove insoluble material, and incubated overnight with goat anti-human tPA polyclonal antibody (Cambio) or mouse anti-human tPA monoclonal antibody (American Diagnostica Inc.) and protein G-Sepharose (Zymed).

To monitor secretion of tPA, chase media from the radiolabelled cells were collected at the indicated times and immunoisolated with monoclonal or polyclonal tPA antibodies.

Immune complexes were washed in IP buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM PMSF) and solubilised in 50 ¼l of sample buffer (0.25mM Tris-HCl pH 6.8, 2% w/v SDS, 20% v/v glycerol, 0.004% w/v bromophenol blue). DTT (50mM) was added to reduced samples where indicated. All samples, reduced and non-reduced, were boiled for 5 mins prior to electrophoresis. Samples were resolved by SDS-PAGE and visualized by either autoradiography using Kodak Biomax MR film or phosphorimage analysis using a Fuji Film Bas 1800 phosphorimager.

**Glutathione assays**

Total glutathione levels were measured using the DTNB [5, 5’-dithiobis(2-nitrobenzoic acid), Sigma]- GSSG reductase recycling assay (32). CHO cells (2 × 10^6) were harvested by trypsinisation and cell pellet was lysed in 100 μl of ice cold 8 mM
HCl / 1.3 % 5-sulfosalicylic acid (SSA, Sigma) by agitating with glass beads. The lysate was incubated on ice for 30 minutes. Glass beads, precipitated protein and cell debris was removed by centrifugation at 12,000g for 5 minutes. Total glutathione was measured by adding 10 µl of the lysate to 1 ml assay mixture pre-warmed to 30°C in a spectrophotometer cuvette. GSSG reductase was then added with mixing to initiate the assay and change in absorbance at 405 nm was measured over 4 minutes. Standard curves were generated using 0.5 - 4 nmol GSH solubilised in 8 mM HCl / 1.3 % SSA.

**PDI redox state**

The in vivo redox state of PDI was assayed by modification with the thiol – reactive reagent acetamido-maleimidylstilbene-disulphonic acid AMS (Molecular Probes). Cells were incubated for 10 min at 37°C with or without DTT (5 mM) or 4,4′dithiodipyridine (0.5 mM). At the end of the incubation period, the cells were transferred to ice and washed twice with ice cold phosphate-buffered saline (PBS) containing 20mM NEM to minimise disulphide bond rearrangements. The cells were lysed in 1 ml of lysis buffer (without NEM) for 10 minutes on ice. Cell lysates were centrifuged at 12,000g for 20 minutes to remove insoluble material. The supernatant was transferred into a fresh tube, 1% SDS and 10mM TCEP was added to all the samples. Samples were boiled for 2 min, cooled and treated with 25 mM AMS for 60 min at room temperature, in the dark.
Results

Overexpression of Ero1-L± leads to enhanced oxidative protein folding

Our initial studies focused on the consequence of overexpressing Ero1 on the ability of tPA to form disulphide bonds, fold correctly and be secreted. Our approach was to generate stable cell-lines expressing both t-PA and wild type Ero1 or Ero1 containing single cysteine point mutations known to compromise function (18,19). A CHO cell-line expressing t-PA (CHO-tPA) was transfected with myc-tagged Ero1-L± and stable cell-lines selected. Isolated cell-lines were screened for expression of Ero1 by Western blot analysis (Fig. 1A, lanes 1-4). To assess the oxidation state of the transfected Ero1-L±, cells were treated with the membrane permeable alkylating agent N-ethylmaleimide (NEM), to block free thiol groups and prevent rearrangement of disulphide bonds. Analysis of the cell-lysate by electrophoresis carried out under non-reducing conditions (Fig. 1A, lanes 5-8), revealed that the myc-tagged exogenously expressed Ero1-L± migrated with a greater mobility than when reduced prior to electrophoresis (Fig 1A, lane 1 and 5). The increased mobility indicates the formation of intra-chain disulphide bonds as previously described for the endogenous protein (19). Some higher molecular weight bands can also be seen when the cell lysate was separated under non-reducing conditions. These higher molecular weight bands are likely to be mixed disulphides between exogenously expressed Ero1-L± and endogenous PDI, as characterised previously (12). No such high molecular weight bands were seen with either cysteine mutants demonstrating
their inability to form functional mixed disulphides with PDI (Fig. 1A, lanes 6 and 7). These results demonstrate that the wild type Ero1-L± expressed in the CHO cells has folded correctly and is functional as indicated by its ability to form intrachain disulphides and mixed disulphides with other known ER oxidoreductases.

The intracellular location of exogenously expressed Ero1-L± protein in the stably transfected CHO-tPA cells was examined by indirect immunofluorescence. Cells were stained with a mixture of anti-myc antibody and antibody to an ER resident protein, calnexin. Secondary antibodies were conjugated to Alexa Fluor 594 (red) or Alexa Fluor 488 (green) dyes, to enable double labelling and to visualise Ero1-L± and calnexin in the same cells. A characteristic ER reticular appearance for calnexin was visualized in cells. Overlay images of myc with calnexin immunofluorescence showed co-localisation of the protein with calnexin indicating that transfected Ero1-L± is localised in the ER (Fig. 1B).

In order to investigate the affect of overexpression of Ero1-L± on the extent of glycosylation of tPA, untransfected (UT) and Ero1-L± transfected (Ero1) stable CHO-tPA cells were radiolabelled for 10 minutes in the absence or presence of varying concentrations of DTT in the labelling medium. The cells were treated with NEM, the cells lysed and the tPA immunoisolated with a goat polyclonal antibody specific for tPA. The immunoisolated proteins were subjected to both reducing and non-reducing SDS-PAGE (Fig. 2A and 2B). In the untransfected CHO-tPA cells, the tPA synthesized during the 10 minutes labelling period in the absence of DTT
migrated as a doublet when reduced prior to electrophoresis (Fig. 2A upper panel UT, lane 1) indicating that variable glycosylation had occurred. When subjected to non-reducing SDS-PAGE (Fig 2B, upper panel UT, lane 1) tPA migrated with a greater mobility and with a more diffuse pattern than on reducing gels, indicating that during the 10 minutes labelling period in the absence of DTT, the tPA had formed intrachain disulphide bonds. As the concentration of DTT in the labelling medium was increased from 0 to 5mM, the doublet progressively disappeared until only a single band of the over glycosylated tPA was present at concentrations of DTT above 0.75mM (Fig. 2A, lanes 2-10). This change in migration was accompanied by a progressive reduction in the formation of disulphide bonds as indicated by the slower migrating sharper bands under non-reducing conditions (Fig 2B, lanes 2-10). Thus, at 1mM DTT no disulphide bonds were formed as no faster migrating protein was seen when the sample was run under non-reducing conditions. In addition the reduction of proteins within the cell with higher concentrations of DTT also caused a slight decrease in mobility of the protein when run under reducing conditions (Fig. 2A lanes 8-10). This is due to full alkylation by NEM of the 34-cysteine residues in tPA when the protein is reduced and is a further indication of the lack of disulphide bond formation at these concentrations of DTT.

In the cell-line overexpressing Ero1-L± the tPA synthesised was more resistant to the effects of reduction with DTT. This resistance is apparent both by the presence of variably glycosylated tPA at DTT concentrations as high as 1mM and a lack of full
reduction of disulphide bonds at 2mM DTT (Fig. 2B, middle panel). Hence overexpression of Ero1 leads to formation of disulphide bonds at higher DTT concentrations indicating that Ero1 can act as an oxidase and can function in the presence of reducing agents. Additionally the ability to form disulphide bonds corresponds to an ability to also fold into a conformation that restricts glycosylation at higher concentrations of DTT, again highlighting the intimate link between disulphide bond formation and protein folding.

The ability of cysteine mutants of Ero1 to bring about a similar effect was also investigated. Mammalian Ero1 contains 14 cysteine residues of which three (Cys 391, 394 and 397) have been shown, by single point mutation, to be involved in either stabilisation of structure or enzymatic function (12,19). An analysis of single cysteine mutations has also been carried out with yeast Ero1 and four cysteine residues (Cys100, Cys105, Cys352 and Cys 355) were found to be absolutely required for function (18). These correspond to Cys99, Cys104, Cys394 and Cys397 in the human protein. We created two Ero1 cysteine mutants with mutation in cys99 or both cys99 and cys394 and prepared separate stable cell-lines expressing these proteins (Fig. 1A). Both the cell-line expressing Ero1-cys99 (Fig. 2A and B, lower panel) and the cell line expressing Ero1-cys99/394 (data not shown) decreased the ability of the cells to resist the effect of DTT on disulphide bond formation. At 0.1mM DTT there was no disulphide bond formation and the synthesised tPA was fully glycosylated (Fig. 2A and B, lane 2). This result demonstrates that the effect on disulphide bond
formation by reducing agent was a direct consequence of overexpression of Ero1 and not due to other pleiotropic effects such as the induction of another protein involved in disulphide bond formation.

**Ero1-L± accelerates oxidative folding of correctly folded tPA in the mammalian ER.**

The previous experiments highlighted the role of Ero1 in facilitating disulphide bond formation in the presence of reducing agents. However these results do not address whether the overexpression of Ero1 actually accelerates the oxidative folding pathway. To address this point we studied the post-translational folding of tPA in cells following labelling in the presence of a concentration of DTT known to prevent disulphide bond formation. All previous studies using mild concentrations of DTT to inhibit co-translational disulphide bond formation in newly synthesised secretory and membrane proteins have shown that the effects of DTT are reversible, and that the proteins undergo post-translational disulphide bond formation when DTT was removed from the culture medium of the cells (20-23). These results demonstrated that oxidising conditions within the ER necessary for disulphide bond formation could be rapidly restored when DTT was removed from the cells.

To determine the time required to re-establish oxidising conditions within the ER and to form the native complement of disulphide bonds in reduced tPA, CHO cells were pulsed in the presence of DTT and then chased with an excess of non-radiolabelled cysteine and methionine in the absence of DTT. Cycloheximide was added to the
chase medium to inhibit protein synthesis. Cells were chased for up to 90 minutes, treated with NEM, lysed and tPA immunoisolated using the goat anti-human tPA polyclonal antibody. Time courses for intracellular disulphide bond formation in the untransfected (UT) and Ero1-L± transfected (Ero1) CHO-tPA cells were constructed by separating the immunoisolated proteins by SDS-PAGE under reducing and non-reducing conditions (Fig. 3). When untransfected CHO-tPA cells were labelled for 10 minutes in the presence of 5mM DTT, non-reducing SDS-PAGE (Fig. 3B, UT) revealed a sharp band at 0 minutes into the chase, indicating that no disulphide bond formation had occurred. After 5 minutes of the chase the banding pattern became more diffuse and protein migrated with greater mobility indicating partial disulphide bond formation. By 10 minutes of the chase there was a further increase in mobility, which was also observed when the samples were reduced prior to electrophoresis (Fig. 3A, UT). This was due to differential alkylation of partially oxidised tPA. No further increase in mobility was observed after 45 minutes of chase indicating that in the untransfected CHO-tPA cells disulphide bond formation was complete between 30-45 minutes into the chase.

In the Ero1-L± overexpressing cells, tPA migrated as a diffuse band of disulphide bonded protein within 5 minutes of the chase (Fig 3B, Ero1) and there was no further increase in mobility after 20 minutes of chase, indicating that in these cells disulphide bond formation was complete between 10-20 minutes into the chase. Thus overexpression of Ero1-L± accelerates the recovery from DTT treatment, allowing
more rapid oxidative folding within mammalian cells. The acceleration of the oxidative pathway would suggest that the rate-limiting step in the pathway is the oxidation of PDI by Ero1 as any increase in Ero1 causes acceleration in the rate of oxidation of the substrate.

An increase in the intensity of tPA immunoisolated during the chase period after labelling in the presence of DTT was observed at early time points in both cell lines (Fig. 3A and B). This could reflect a lack of protein synthesis inhibition by cycloheximide. However, this is unlikely to be the case as only fully glycosylated tPA was detected during the chase and any protein synthesised during the chase would be variably glycosylated as the redox conditions within the ER are restored rapidly (24). Affinity of the polyclonal antibody for reduced and non-reduced tPA was determined (results not shown) and it was found that only 35% of the tPA immunoisolated from the non-reducing sample was precipitated from the reducing sample, demonstrating that the antibody has a reduced affinity for non-disulphide bonded tPA. Thus, the increase in the intensity of immunoisolated tPA at early time points during the chase period after DTT treatment indicates post-translational folding and disulphide formation of tPA during this period.

The ability to accelerate the oxidation of tPA after treatment with DTT could lead to the formation of native or non-native disulphide bonds. If non-native disulphide bonds were formed then the acceleration in rate of disulphide bond formation would not necessarily lead to an increase in the rate of formation of correctly folded tPA.
To investigate this point we determined whether the tPA formed after recovery from DTT treatment had undergone the conformational maturation required for its exit from the ER and secretion from the cell.

For this purpose we used the pulse chase protocol as described above, with the chase times extended to 240 minutes. After each chase time, tPA was immunoisolated from both the cell lysates and the chase medium. The immunoisolated tPA was separated under reducing conditions, to construct time courses for secretion of tPA synthesised in the presence of DTT, by the untransfected (Fig. 4A) and Ero1-L± overexpressing (Fig. 4B) CHO-tPA cells. When the untransfected CHO-tPA cells were labelled for 10 minutes in the presence of 5mM DTT a sharp band was observed immediately after the pulse (Fig. 4A) indicating the presence of only fully glycosylated tPA. The protein formed disulphide bonds within 30 minutes as evidenced by an increase in band intensity and a slightly faster mobility due to lack of alkylation by NEM upon disulphide bond formation. Secreted tPA was observed initially in the chase media after 75 minutes and increased in intensity at subsequent time points. Therefore, after synthesis in the presence of DTT, tPA took between 60-75 minutes to reach the cell surface. If it took 30-45 minutes for the tPA to fold (Fig. 3) then the rate of secretion in this cell-line is approximately 30 minutes.

The same experiment was carried out with the Ero1-L± overexpressing CHO-tPA cells. A clear difference in the secretion pattern of tPA was seen following labelling of these cells in the presence of 5mM DTT for 10 minutes (Fig.4B). As compared to
the 60-75 minutes time period taken by the first tPA molecules to reach the cell surface in the untransfected CHO-tPA cells, in the Ero1-L± overexpressing cells, the first tPA molecules reached the cell surface within 45 minutes. As the tPA in this cell-line took between 10-20 minutes to form disulphide bonds the rate of secretion in this cell-line is also approximately 30 minutes. Hence overexpression of Ero1 does not effect the rate of secretion but does accelerate the formation of native disulphide bonds giving rise to tPA that is secreted from the cell. The fact that the tPA is secreted from the cell and that it has escaped the ER quality control system for glycoproteins suggests that it has folded correctly.

**Glutathione is required for isomerisation of non-native disulphide bonds**

Genetic evidence in yeast has demonstrated that glutathione is dispensable for formation and rearrangement of disulphide bonds (Frand & Kaiser 1998, Cuozzo and Kaiser 1999) and instead potentially functions to protect the cell from hyper-oxidising conditions. In order to investigate the role of glutathione during oxidative protein folding in mammalian cells, we reduced the intracellular glutathione levels by culturing cells in the presence of buthionine sulfoximine (BSO), which is an inhibitor of ³-glutamylcysteine synthetase. Cells cultured in the presence of 0.5 mM BSO for 16 h were found to contain limited amounts of GSH (decrease from approximately 13mM to < 1mM).

To determine whether the loss of glutathione had any effect on the posttranslational folding of tPA, the pulse chase approach was used to follow the folding of tPA in
cells pre-treated with BSO. The results for the CHO-tPA cells, show that disulphide bond formation occurs within 15 minutes as evidenced both by an increased mobility of the immunoisolated tPA separated under reducing conditions and the appearance of a diffuse, faster migrating product when separated under non-reducing conditions (Fig. 5). A further slight increase in mobility is also observed between 60-75 minutes. It would appear from these results that there is a more rapid initial formation of disulphide bonds in cells treated with BSO but that there is slower process of maturation. On comparing these results with the posttranslational oxidative folding of tPA in BSO untreated CHO-tPA cells (Fig. 3), it is clear that there is a profound acceleration in the rate of disulphide bond formation in the glutathione depleted cells.

The slight increase in mobility of tPA molecules after 60 minutes of chase led us to postulate that the tPA molecules might have formed a non-native structure within this time period. The increase in mobility may be a consequence of rearrangement of such non-native disulphides. In order to test this hypothesis we checked for appearance of tPA molecules in the chase media. If the tPA molecules formed at the end of 15 minutes are correctly folded then they should appear into the media within 45-60 minutes as seen with the tPA molecules synthesised by the Ero1-L± transfected cells (Fig. 4). When the chase media from the BSO treated CHO-tPA cells was immunoisolated with the polyclonal anti-tPA antibody, tPA could be detected only after 75 minutes. Thus, although the depletion of glutathione accelerates the rate of
disulphide bond formation, this does not lead to more rapid secretion. These results suggest that the faster migrating tPA molecules observed at the early time points are malfolded and contain non-native disulphide bonds. Such non-native disulphides would need to undergo isomerisation to attain their native structure before they can exit the ER and be secreted from the cells providing a potential explanation for the time delay observed between folding and appearance of tPA in the media.

When CHO-tPA cells, pre-treated with BSO were pulsed in the absence of DTT, no difference in the folding and secretion of tPA was observed, as compared to the BSO untreated cells (results not shown). Thus in the absence of any reductive stress, glutathione seems to be dispensable for the oxidative folding of tPA at least as far as our experimental approach can detect. This experiment shows that the rate of secretion of tPA is not adversely affected by the presence of BSO ruling out this particular explanation for the lack of secretion of tPA at earlier time points in BSO treated cells.

To investigate the possibility that malfolded protein was formed during the more rapid oxidation of tPA in BSO-treated cells we made use of a conformational-specific antibody to tPA. This antibody (PAM 1) is used in the selective purification of native human one-chain tPA, recognising an epitope in the native protein (25). Untransfected CHO-tPA cells were pulsed with radiolabelled methionine in the presence of 5mM DTT followed by chase in the absence of DTT for up to 180 minutes. Cells were pre-treated with 0.5mM BSO for 16 hours where indicated. The
correctly folded tPA was immunoisolated from both cell lysates and media with the PAM 1 antibody. The immunoisolated protein was separated under reducing conditions to construct time courses for secretion of tPA in the BSO treated and untreated CHO-tPA cells (Fig. 6 A/B).

In the BSO untreated cells, the first correctly folded tPA molecules were observed after 45-60 minutes of chase (Fig. 6A). This result confirms our earlier observation that in the untransfected CHO-tPA cells, disulphide bond formation was complete between 30-45 minutes into the chase (Fig. 3). In the cells pre-treated with BSO, no native tPA molecules could be seen until 45-60 minutes into the chase as judged by immunoisolation with the PAM 1 antibody. This result indicates that the disulphide-bonded tPA molecules seen at the early time points in BSO-treated cells (Fig. 5) are non-native forms of tPA. Immunoisolation of tPA from the media in either the BSO treated or untreated cells with the monoclonal antibody revealed that the first molecules of tPA were secreted into the media 75-90 minutes into the chase. Even in the BSO-treated cells tPA ultimately was folded correctly and was secreted at the same rate as in untreated cells. Therefore the consequence of lowering the concentration of glutathione in the cell is that when the cell recovers from a reductive stress, proteins form non-native disulphide bonds that require a period of time to be resolved to yield correct native disulphide bonds.

One explanation for the effect of lowering the level of glutathione on the formation of disulphide bonds is that there is a general effect on the redox state of ER
oxidoreductases. Hence in the presence of decreased concentrations of glutathione these enzymes may become oxidised and less able to carry of disulphide isomerisation. To test this possibility we measured the redox state of PDI either in untreated cells, Ero1 transfected cells or cells incubated in the presence of BSO for 16 hours. We treated cells with NEM to prevent disulphide interchange prior to solubilising the cell membranes with a detergent-containing buffer. Using this approach any free thiols are alkylated by NEM and any cysteine residues that have formed disulphide bonds are protected from alkylation. The resulting cell lysate was suspended in a denaturing buffer in the presence of the reducing agent TCEP to break disulphide bonds followed by AMS modification. TCEP does not prevent maleimide-based alkylation and therefore does not inhibit AMS. Proteins were separated by SDS-PAGE, any increase in mobility is due to AMS modification of cysteine residues that are oxidised within cells. Prior to the addition of NEM, cells were either untreated or treated with the membrane permeable oxidising agent 4,4’-dithiodipyridine (4-DPS) (0.5mM) or with DTT (5mM). AMS did not modify PDI after treatment with DTT, indicating that PDI was reduced (figure 6C, lane 1). If the cells were treated with 4-DPS PDI became oxidised as judged by alkylation with AMS (Fig. 3, lane 2). At steady state PDI was mainly reduced though there was some oxidised material present (Fig 6C, lane 3). After treatment with BSO there was not a noticeable difference in the redox state of PDI (Fig. 6C, lane 4) though in the Ero1 overexpressing cell-line there was clearly more oxidised than reduced PDI (Fig. 6C,
 lane 5). Hence, decreasing the concentration of glutathione in the cells does not dramatically affect the redox state of PDI.
Discussion

The role of Ero1 in the oxidative pathway

The ability of Ero1 to facilitate the oxidation of proteins within the ER in both yeast and mammalian cells is now well established (2). Evidence for a role for Ero1 in oxidation of PDI and formation of disulphide bonds in secreted proteins, came from both genetic and biochemical approaches in yeast, which demonstrated that Ero1 accepts electrons from an ER oxidoreductase such as PDI which in turn accepts electrons from the substrate protein (9,10,26). The role of Ero1 in mammalian cells has been investigated previously by studying the affect of overexpression on oxidation of PDI and secreted proteins (12). These studies and the results presented here show clearly that PDI is a substrate for Ero1 and that there is a marked positive effect of overexpression of Ero1 on the ability of mammalian cells to allow disulphide bond formation. In addition, we demonstrate here that a consequence of this effect on the oxidative pathway is acceleration in the rate of protein folding and the acquisition of a native structure. Hence, for tPA at least, the rate of protein folding within the cell is potentially limited by the capacity of the cell to facilitate disulphide bond formation. These results prove that the ability of proteins to adopt the correct three-dimensional structure within cells can be accelerated by manipulating the level of the enzymes responsible for disulphide bond formation within the ER.

That overexpression of Ero1, a protein involved exclusively in the oxidative pathway,
leads to an increase in the rate of protein folding argues that the rate limiting step in folding is the oxidation of disulphide bonds. However, this interpretation does need to be viewed with a certain degree of caution, as both in our studies and those published previously (12) the affect of overexpression of Ero1 is only manifest when the cells are challenged with a reducing agent. Hence, overexpression of Ero1 accelerates recovery after treatment with a reducing agent and allows disulphide bond formation to occur in the presence of elevated levels of DTT. One could argue that under steady state conditions the rate of folding of proteins such as tPA is already maximal and any effect on this rate can only be observed when the pathway is compromised. However, what we can conclude from these studies is that Ero1 is able to function and catalyse the formation of disulphide bonds under reducing conditions, a conclusion borne out by the ability of purified yeast Ero1 to catalyse the formation of disulphide bonds in the presence of 2mM GSH (10). This conclusion challenges the idea that an oxidising environment within the ER lumen is required to allow disulphide bonds to form and emphasises the importance of a specific oxidative pathway for catalysis.

The fact that PDI was more oxidised in the Ero1 overexpressing cell-line and that PDI forms mixed disulphides with Ero1 strongly suggests that it is a substrate for Ero1. It has previously been shown that the oxidation state of ERp57 is not altered in cells overexpressing Ero1 and that no mixed disulphides are formed between ERp57 and Ero1, leading to the conclusion that ERp57 is not a substrate for Ero1 (12).
ERp57 is an abundant ER oxidoreductase that is a member of the PDI family of proteins, but is distinct in that it associates with calnexin and calreticulin and has been postulated specifically to be involved in disulphide bond formation in glycoproteins substrates (27). We also looked at the redox state of ERp57 and found that it is reduced both in the untransfected and Ero1 overexpressing cell-line (SC and NJB unpublished results). The protein we used in this study is a glycoprotein and interacts with calnexin (28), however the fact that Ero1 overexpression effects disulphide bond formation would suggest that PDI rather than ERp57 is involved in at least the oxidation of disulphide bonds in glycoprotein substrates. Clearly this does not rule out a role for ERp57 in the isomerisation of non-native disulphides a role that has been demonstrated in vitro with purified glycoproteins (13). The observation that in cells the active site disulphide in ERp57 is reduced and that this protein is not a substrate for Ero1 suggests a kinetic segregation of an oxidative pathway involving PDI and a reductive pathway involving ERp57 or one of the other ER oxidoreductases. If this is the case then clearly a pathway must exist to maintain a level of reducing equivalents in the ER lumen to facilitate reduction of ERp57.

**The role of glutathione in maintaining the reductive pathway**

The ability to eliminate glutathione biosynthesis in yeast cells has established that glutathione is not required for the oxidation of disulphide bonds (16). However, the depletion of glutathione from yeast cells causes oxidation of proteins within the ER and suppresses a temperature sensitive Ero1 mutant suggesting a role in the reduction
of disulphide bonds. Our results in mammalian cells demonstrate that a normal level of glutathione is required within the cell to prevent the formation of aberrant disulphides during the posttranslational folding of tPA. The formation of native disulphide within the ER lumen is therefore dependent upon both an oxidative pathway and a reductive pathway to prevent the formation of non-native disulphides, the reductive pathway being compromised when the level of glutathione within the cell is decreased. The fact that glutathione can cross the ER membrane either through a specific transporter (15) or simply through pores in the membrane (29) suggests that this low molecular weight thiol could provide the necessary reducing equivalents to facilitate the reduction of folding proteins directly or via reduction of ER oxidoreductases such as ERp57. As glutathione is a poor substrate of Ero1 in vitro (26), it is unlikely to be directly oxidised by the Ero1 in vivo therefore providing segregation of the pathways of oxidation and reduction within the ER.

In general terms the ability to catalyse two thermodynamically opposed reactions within the same cellular compartment requires that the two reaction pathways be kinetically partitioned. In the prokaryotic periplasm this partitioning is solved by the presence of two separated enzyme-catalysed pathways. These pathways ensure a flow of electrons either from the substrate protein to a terminal electron acceptor such as oxygen via DsbA, DsbB and ubiquinone (30), or from thioredoxin in the cytosol via the membrane protein DsbB to DsbC in the periplasmic space (14). Our results and those of others lead us to conclude that in the mammalian ER this problem has
been solved by the evolution of an oxidative pathway that specifically catalyses the oxidation of PDI and that can function in the presence of high concentrations of reduced glutathione. These concentrations of glutathione are sufficient to maintain other ER oxidoreductases such as ERp57 in a reduced form and therefore able to catalyse the reduction of non-native disulphides or facilitate the reduction of polypeptides destined for retrotranslocation from the ER to the cytosol for degradation.

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Figure legends.

Figure 1: Expression of wild-type and cysteine mutants of Ero1-L± in HEK 293 cells

Stable cell-lines expressing wild type or cysteine-to-alanine mutants of Ero1L± were isolated and tested for expression of exogenous Ero1. (A) Cell lysates were separated by SDS-PAGE under either reducing (lanes 1-4) or non-reducing conditions (lanes 5-8). Separated proteins were transferred to nitrocellulose and probed with anti-myc antibody. Clear bands corresponding to exogenously expressed and myc-tagged Ero1 were seen in all cell-lines apart from the untransfected cells (ut) (lane 5 and 8). (B) A stable cell line expressing wild-type Ero1 was analysed by indirect immunofluorescence. Cells were stained with a mixture of anti-myc antibody and antibody to an ER resident protein, calnexin. Secondary antibodies were conjugated to Alexa Fluor 594 (red) or Alexa Fluor 488 (green) dyes, to enable double labelling and to visualise Ero1 and calnexin in the same cells. The co-localisation of exogenously expressed Ero1 with calnexin (merged image, bottom right) indicates an ER localisation of Ero1 in this cell-line.

Figure 2: Overexpression of wild type Ero1-L± allows disulphide bond formation to occur at higher concentrations of DTT

Either untransfected cells or stable cell-lines expressing Ero1-L± or Ero1-C99A were pulsed with radiolabeled amino acids in the absence (lane 1) or presence of increasing concentration of DTT (lanes 2-10). Cells were lysed and newly
synthesised tPA immunisolated prior to separation by SDS-PAGE carried out under reducing (A) or non-reducing (B) conditions. Radiolabelled protein was visualised by autoradiography.

**Figure 3: Overexpression of Ero1-L± increases the rate of disulphide bond formation after treatment with DTT.**

Untransfected cells or cells stably expressing Ero1-L± were radiolabelled in the presence of 5mM DTT, conditions which prevent the formation of disulphide bonds in newly synthesised proteins. Cells were then allowed to recover in the absence of DTT and samples taken at various time points as indicated. Cells were lysed and newly synthesised tPA immunisolated prior to separation by SDS-PAGE, which was carried out under reducing (A) or non-reducing (B) conditions. Radiolabelled protein was visualised by autoradiography.

**Figure 4: Overexpression of Ero1-L± increases the rate of protein folding and native disulphide bond formation following treatment with DTT**

Untransfected (A) or cells stably expressing Ero1-L± (B) were radiolabelled in the presence of 5mM DTT. Cells were then allowed to recover in the absence of DTT and samples taken at various time points as indicated. Cells were lysed and media was collected. Newly synthesised tPA was immunisolated from both the cell lysate and the media at each time point prior to separation by SDS-PAGE carried out under reducing conditions. Radiolabelled protein was visualised by autoradiography.
Figure 5: Cells with lowered cellular glutathione show an increased rate of recovery from reduction with DTT

Non-transfected cells were treated with BSO for 16hr and then pulse labelled with radiolabelled amino acids in the presence of 5mM DTT. Cells were lysed at various time points after removal of DTT and disulphide bond formation followed by separating the immunoisolated tPA by reducing and non-reducing SDS-PAGE. Media was also collected at each time point and secreted tPA immunoisolated and separated by SDS-PAGE run under reducing conditions. Radiolabelled protein was visualised by autoradiography.

Figure 6: Acceleration of recovery of disulphide bond formation by lowering glutathione levels does not result in the formation of native disulphide bonds.

Non-transfected cells were incubated in the absence (A) or presence (B) of BSO for 16hr to reduce the levels of cellular glutathione. Cells were pulse labelled in the presence of 5mM DTT to prevent disulphide bond formation and then allowed to recover in the absence of DTT for various time periods as indicated. Media and cell-lysates from each time point were prepared and tPA immunoisolated using a monoclonal antibody that recognises an epitope present in the final native structure. Samples were separated by SDS-PAGE carried out under reducing conditions and radiolabelled protein visualised by autoradiography. (C) The redox state of PDI was evaluated in non-transfected cells reduced with DTT (lane 1) oxidised with 4,4’dithiodipyridine (DPS) (lane 2) or after incubation in the absence (lane 3) or
presence (lane 4) of BSO to reduce the level of cellular glutathione. The redox state of PDI in Ero1-L± overexpressing cells was also evaluated (lane 5). In each instance cells were treated with NEM prior to cell lysis and cellular proteins were alkylated with AMS in the presence of the reducing agent TCEP. Samples were separated by SDS-PAGE, transferred to nitrocellulose and the resulting blots probed with an antibody to PDI.
### Panel A

| LANE | wt | C99A | C99/334A | Lt |
|------|----|------|----------|----|
| 1    |    |      |          |    |
| 2    |    |      |          |    |
| 3    |    |      |          |    |
| 4    |    |      |          |    |
| 5    |    |      |          |    |
| 6    |    |      |          |    |
| 7    |    |      |          |    |
| 8    |    |      |          |    |

**Labelled Proteins:**
- Mixed disulphides
- Ero1

### Panel B

#### Calnexin
- Green

#### Myc
- Red

**DNA Staining:**
- DAPI
- Merge
### A

| LANE | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|------|---|---|---|---|---|---|---|---|---|----|
| DTT (mM) | 0 | 0.1 | 0.2 | 0.3 | 0.4 | 0.5 | 0.75 | 1 | 2 | 5 |

- **UT**
- **Ero1**
- **Ero1-C99A**

**Reduced**

### B

| LANE | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|------|---|---|---|---|---|---|---|---|---|----|
| DTT (mM) | 0 | 0.1 | 0.2 | 0.3 | 0.4 | 0.5 | 0.75 | 1 | 2 | 5 |

- **UT**
- **Ero1**
- **Ero1-C99A**

**Non-reduced**
A

10 min pulse +5 mM DTT
Chase (min) 0 5 10 20 30 45 60 75 90

UT

Ero1

reduced

B

10 min pulse +5 mM DTT
Chase (min) 0 5 10 20 30 45 60 75 90

UT

Ero1

non-reduced
A) Cell lysate and media from untransfected cells show changes over time with a 10 min pulse and 5 mM DTT chase.

B) Cell lysate and media from Ero-1 overexpressing cells also show changes over time with a 10 min pulse and 5 mM DTT chase.
Glutathione is required to regulate the formation of native disulphide bonds within proteins entering the secretory pathway
Seema Chakravarthi and Neil J. Bulleid

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