The refolding of hen egg white riboflavin-binding protein: effect of protein disulphide isomerase on the reoxidation of the reduced protein

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Hen egg white riboflavin-binding protein (RfBP) contains nine disulphide bonds. Provided these remain intact, the refolding of RfBP after incubation in 6 M guanidinium chloride is highly efficient with at least 95 % of the binding activity regained within 3 min. Kinetic studies indicate that this regain consists of at least two phases. When the disulphide bonds of RfBP are reduced, reoxidation using a mixture of oxidized and reduced glutathione leads to less than 5 % recovery of activity. However, if protein disulphide isomerase (PDI; EC 5.3.4.1) is present during the reoxidation nearly 50 % activity can be regained, suggesting that PDI may play an important role in the maturation of RfBP *in vivo*.

INTRODUCTION

Hen egg white riboflavin-binding protein (RfBP) acts as a source of riboflavin to the developing embryo [1]. It is the most abundant vitamin-binding protein in the egg white. Mutations which give rise to a lack of RfBP lead to embryo death at 13 days. Yolk and serum RfBP are synthesized in the liver, whereas the albumin protein is produced in the oviduct. Although all three proteins are products of the same gene, it is known that yolk and albumin RfBP differ in a number of respects including their glycosylation patterns. RfBP binds riboflavin tightly (K_d = 1.3 nM) in a 1:1 ratio. On formation of the complex, the fluorescence of riboflavin is completely quenched; this quenching is thought to be due to the stacking of aromatic groups within the hydrophobic binding pocket [2]. The quenching provides a convenient assay for the integrity of the riboflavin-binding site of the protein.

RfBP consists of a single polypeptide chain of 219 amino acids of molecular mass 29.2 kDa. It has been reported that crystals diffracting to 0.28 nm have been obtained [3]; however, no further three-dimensional structural information is available. RfBP undergoes a number of post-translational modifications, namely: the formation of nine disulphide bonds [4], extensive glycosylation on Asn-36 and Asn-147 [5], and the phosphorylation of eight serine side chains between Ser-186 and Ser-197 [6]. It also undergoes limited proteolysis with the cleavage of a 17-residue signal peptide from the N-terminus and two arginine residues from the C-terminus [7]. These features, coupled with the relatively small size of the protein, make RfBP an interesting system in which to study the effects of post-translational modifications on protein unfolding and refolding.

In preliminary work on the unfolding and refolding of RfBP, Allen et al. demonstrated that, provided the disulphide bonds remained intact, the protein could be refolded from its denatured form with high efficiency, when dialysis was used to remove the denaturing agent (GdnHCl) [8]. In conditions under which the disulphide bonds in RfBP were kept reduced, the unfolding of RfBP by GdnHCl could not be reversed by subsequent dialysis.

The refolding of many reduced denatured proteins *in vitro* is facilitated by protein disulphide isomerase (PDI), an enzyme which is present in the lumen of the endoplasmic reticulum and which is thought to catalyse the formation and shuffling of disulphide bonds in secreted proteins [9]. The action of PDI has been established mainly through studies on model substrates [10] and on small highly soluble proteins which refold spontaneously with reasonable efficiency; in such cases, PDI catalyses net disulphide formation and isomerization steps associated with protein conformational changes (see references in [9]). However, there has recently been increasing interest in the action of PDI on proteins which refold inefficiently [11–13], and there is some debate as to whether PDI has a distinct chaperone activity [14,15].

In the present paper, we report studies of the kinetics of refolding of RfBP (disulphide bonds intact) after denaturation by GdnHCl. We also report on the reoxidation of reduced RfBP using the GSH/GSSG redox system; there is only a very small recovery of biological activity (< 5 %). (It should be noted that there are over 3.4×10^7 possible combinations of 18 sulphydryl groups to form nine disulphide bonds.) However, in the presence of PDI, the yield of active RfBP can be increased significantly (up to at least 50 %). This suggests that PDI plays an important role in the maturation of RfBP *in vivo*.

MATERIALS AND METHODS

Hen egg white RfBP was purified by the method of Hamazume et al. [5] as modified by Walker et al. [16] scaled up to process 400 ml of egg white. The eggs were obtained from local commercial sources. The holoprotein complex was isolated via ion-exchange chromatography, on two Sephadex A50 DEAE columns (10 cm × 9.6 cm²). The second column gives pure holo-RfBP, from SDS/PAGE studies. The apoprotein was obtained by ion-exchange chromatography of the holoprotein, on an SP

Abbreviations used: RfBP, riboflavin-binding protein; PDI, protein disulphide isomerase; CD, circular dichroism; DTT, dithiothreitol; Fab, antibody fragment (antigen binding); GdnHCl, guanidinium chloride; Nbs_2, 5,5′-dithiobis(2-nitrobenzoate).

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Sephadex C50 column (12 cm × 3.1 cm^2), at pH 3.8 (the riboflavin–RfBP complex dissociates between pH 3.8 and 4 [1]). SDS/PAGE does not give an accurate estimation of the molecular mass of RfBP due to its high degree of phosphorylation and glycosylation [1].

Protein and riboflavin concentrations were determined spectrophotometrically. The molar absorption coefficients of RfBP and riboflavin are 49000 $M^{-1} \cdot cm^{-1}$ (282 nm) and 12500 $M^{-1} \cdot cm^{-1}$ (455 nm) respectively [8]. The activity of the purified RfBP was verified by its ability to quench the fluorescence of riboflavin (excitation and emission wavelengths 370 nm and 520 nm respectively). The titration showed that complete quenching was observed at a 1:1 molar ratio of RfBP to riboflavin, indicating that the protein was fully active. The structural integrity of the RfBP was also assessed by circular dichroism (CD) and intrinsic protein fluorescence measurements.

Fluorescence and CD measurements were made on a Perkin-Elmer LS50 and Jasco J-600 spectropolarimeter respectively at 20 °C unless otherwise indicated.

Apo-RfBP (10 mg/ml) was unfolded by incubation in 6 M GdnHCl (dissolved in 50 mM sodium phosphate buffer, pH 7.0) for 15 min at room temperature [8]. The concentrations of GdnHCl solutions were checked by refractive index measurements [17]. The extent of secondary and tertiary structure loss was measured by CD and tryptophan fluorescence respectively, and the riboflavin-binding ability was also assessed. CD measurements were made from 190 nm to 260 nm. Protein fluorescence was measured from 300 nm to 400 nm, after excitation at 290 nm. Refolding was achieved by lowering the denaturant concentration to 0.1 M by dilution with the sodium phosphate buffer. The degree of refolding was gauged by the extent of secondary and tertiary structure recovery, as measured by far-UV CD and protein fluorescence against time. The refolding was carried out in the presence and absence of equimolar riboflavin. In order to monitor the recovery of binding activity, the fluorescence of riboflavin was measured against time in the experiment in which refolding was performed in the presence of equimolar riboflavin. The dilution (of denatured protein into buffer) and mixing were performed within the cuvette in the fluorimeter to minimize the 'dead' time of the experiment. Protein fluorescence was measured at 350 nm (excitation at 290 nm) and riboflavin fluorescence was measured at 520 nm (after excitation at 370 nm).

Apo-RfBP was reduced by the method of Kozik [18]. RfBP (1 mg) was incubated overnight in 8 M urea and 1 mM dithiothreitol (DTT) at room temperature. All buffers and solutions contained 2.7 mM EDTA and were freshly purged with O_2-free nitrogen for 2 min to minimize random reoxidation by atmospheric oxygen, catalysed by trace metals. The reduced RfBP was separated from excess urea and DTT by gel filtration on Sephadex G-25. The elution buffer (50 mM sodium phosphate, pH 7.8) contained 1 mM GSH, to prevent reoxidation of the reduced RfBP. The sulphydryl content of reduced RfBP was determined spectrophotometrically by reaction with 5,5′-dithiobis(2-nitrobenzoate) (Nbs_2) [19] using a sample which had been gel-filtered in the absence of GSH.

Reoxidation was initiated by the addition of GSSG to a final concentration of 0.1 mM. This ratio of [GSH]/[GSSG] (10:1) was found to be the optimum through reoxidation studies in the absence of PDI (results not shown). When the effect of PDI was being studied, PDI was added to the solution of reduced RfBP in GSH immediately prior to the addition of GSSG [13]. The reoxidation reactions were performed at 4 °C. Control experiments were performed with equivalent molar ratios of BSA in place of PDI. The extent of recovery of the native structure of RfBP was assessed by measuring the quenching of riboflavin fluorescence of samples taken from the reoxidation mixtures. Control experiments showed that the presence of PDI or BSA did not interfere with the measurement of riboflavin binding.

PDI was purified from bovine liver by the method of Freedman et al. [20].

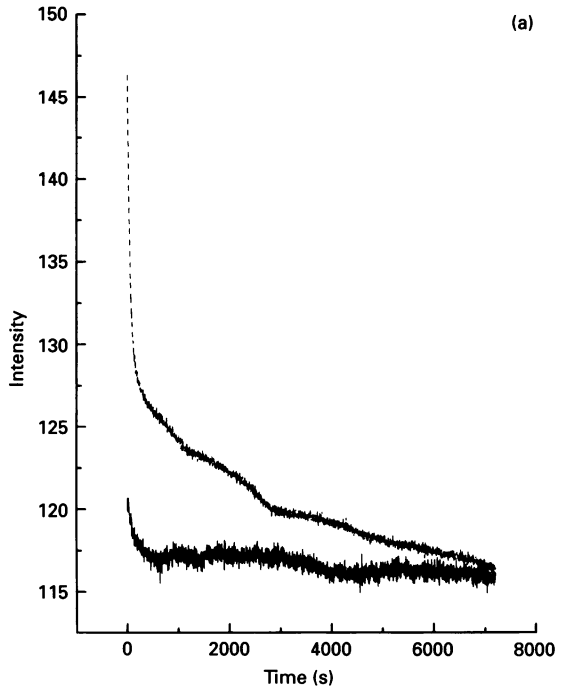
RESULTS

Denaturation and refolding of apo-RfBP

In agreement with the results of Allen et al. [8], apo-RfBP appeared to be fully denatured after incubation in 6 M GdnHCl; there was a total loss of secondary and tertiary structure and the riboflavin-binding activity was lost. In 6 M GdnHCl, the wavelength of maximum fluorescence emission is identical to that of tryptophan model compounds (356 nm), indicating complete exposure of tryptophan residues to the aqueous environment. Similarly, the CD spectrum above 205 nm (a lower limit imposed by the absorbance of GdnHCl) of the protein in 6 M GdnHCl is indicative of a random coil. This unfolding occurred despite the retention of the nine disulphide bonds in the protein. In agreement with the earlier data [8], the fluorescence changes indicated that the unfolding was not a simple two-state process; a first change occurs between 0 M and 2 M GdnHCl and a second between 5 M and 6 M GdnHCl. There may be an intermediate state of RfBP stable between 2 M and 4 M GdnHCl.

As the lowering of the concentration of GdnHCl through dilution is rapid compared with refolding, the kinetics of refolding of the denatured protein can be followed spectroscopically. Most of our studies involved fluorescence measurements of two types: (i) intrinsic protein fluorescence to monitor the regain of the tertiary structure of RfBP, and (ii) riboflavin fluorescence as it is quenched by the protein during refolding to generate the riboflavin-binding activity. The changes in protein fluorescence appear to be at least biphasic. In the absence of riboflavin, 95% of the refolding (i.e. total fluorescence changes) occurred within 50 s (the changes were too rapid to permit detailed analysis of the fast portion of the process; it is possible that a number of rapid steps are involved). This was followed by a slower phase, lasting up to 2 h, in which the final 5% of the fluorescence change occurred (Figure 1). In the presence of equimolar riboflavin, only 90% of the tertiary structure was regained after the initial rapid phase, and a further 5% of the total change expected (i.e. the difference between RfBP in 6 M GdnHCl and RfBP in 0.1 M GdnHCl) occurred over the subsequent 2 h (Figure 1). The slower phase, seen in both the presence and absence of riboflavin, could be indicative of some of the slower steps thought to be involved in protein folding, such as *cis/trans* isomerization of Xaa-Pro peptide bonds [21]. The results also seem to imply that the presence of riboflavin in some way slightly impedes the acquisition of the final native tertiary structure. In the riboflavin fluorescence quenching experiment, the native RfBP quenches the riboflavin fluorescence almost immediately, while the refolding RfBP takes almost 200 s to reach an endpoint, although the main part of the process is complete within 50 s (Figure 2).

The rate of regain of secondary structure of RfBP during refolding was examined by far-UV CD. It was found that the changes in the ellipticity of the protein at 225 nm were complete within 10 s, the 'dead time' of this type of manual mixing experiment (results not shown). It was also shown that the presence of an equimolar concentration of riboflavin had no significant effect on the rate of changes in the far-UV CD on refolding. These results indicated that the formation of native secondary structure during refolding was rapid. From a combination of fluorescence and CD studies, therefore, it would appear that the refolding denatured RfBP regains all of its



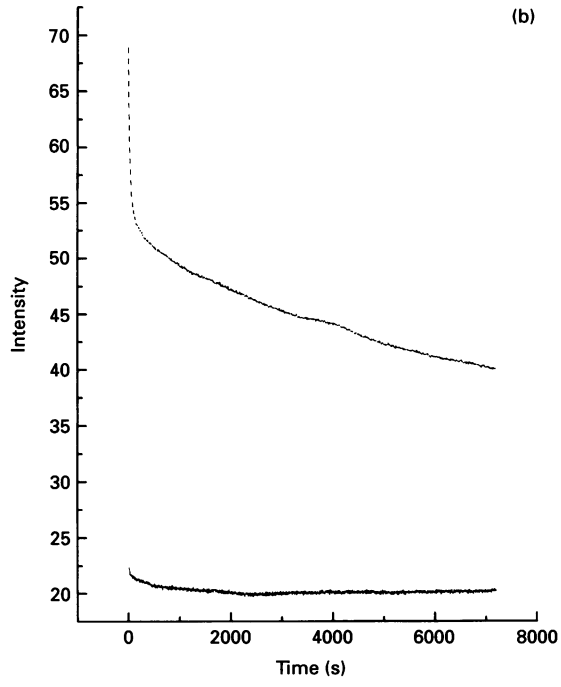


Figure 1 The refolding of RfBP monitored by changes in protein fluorescence

RfBP (disulphide bonds intact) was denatured by incubation in 6 M GdnHCl and refolding initiated by a 60-fold dilution into 50 mM sodium phosphate buffer, pH 7.0, as described in the text. The excitation and emission wavelengths were 290 nm and 350 nm respectively. (**a**) Refolding of RfBP in the absence of riboflavin. The solid and dashed lines represent the control sample incubated in 0.1 M GdnHCl and the refolding protein respectively. Under these conditions the fluorescence of the denatured RfBP corresponded to 340 units. (**b**) Refolding of RfBP in the presence of an equimolar concentration of riboflavin. The solid and dashed lines have the same significance as in (**a**). Under these conditions the fluorescence of the denatured RfBP corresponded to 320 units.

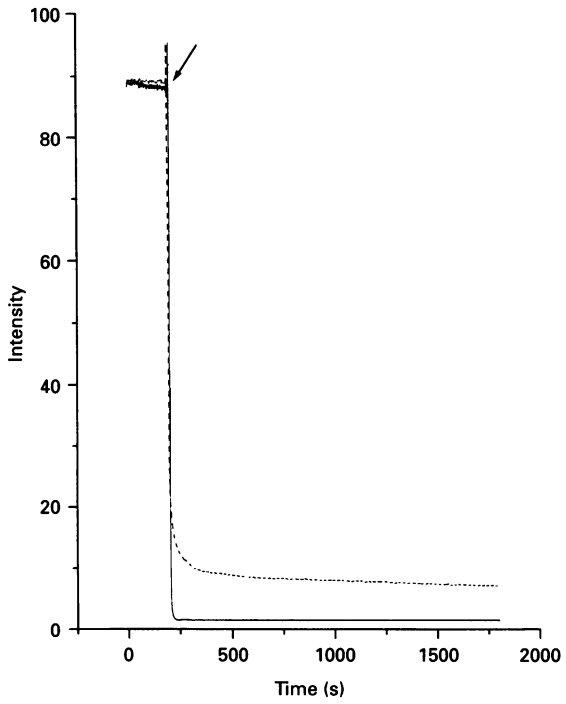


Figure 2 The recovery of riboflavin-binding activity during the refolding of RfBP

The fluorescence of riboflavin (1.4 μM) was monitored; at the time shown an equimolar concentration of RfBP was added, as described in the text. The excitation and emission wavelengths were 370 nm and 520 nm respectively. The broken line represents changes observed when RfBP, which had been previously incubated in 6 M GdnHCl, was diluted 60-fold into buffer at the time shown to initiate refolding. The solid line represents the changes observed when a control sample of RfBP was added to the riboflavin in buffer containing 0.1 M GdnHCl.

secondary structure, and most of its tertiary structure, within a few seconds, with the acquisition of the native tertiary structure taking somewhat longer (a few minutes). Analysis of the early events in the process will require the use of rapid reaction techniques.

Reduction and reoxidation of apo-RfBP

After reduction of apo-RfBP in denaturing conditions, and gel filtration to remove excess DTT and urea, reaction with Nbs_2 revealed a sulphydryl content of 20 ± 3 per molecule of RfBP, in reasonable agreement with the value of 18 expected from the primary structure of the protein. The CD spectrum obtained of the reduced protein was identical to that previously published [8]. The wavelength of maximum emission from the fluorescence spectrum was 349 nm. Previous work had suggested that the emission maximum of the reduced species was 354 nm [8]. It is possible that the $NaBH_4$ reduction method employed by Allen et al. [8] is responsible for this discrepancy. From these studies it is clear that reduced RfBP has a lower content of secondary structure and more highly exposed tryptophan side chains than native RfBP, but it still has some folded structure. The reduced RfBP was unable to bind riboflavin, as shown by the failure of the reduced RfBP to quench riboflavin fluorescence. Control experiments showed that the presence of GSH and/or GSSG at the concentrations used in these studies had no effect on the binding properties of native RfBP. Treatment of the reduced apo-RfBP with GSH/GSSG alone led to complete oxidation of

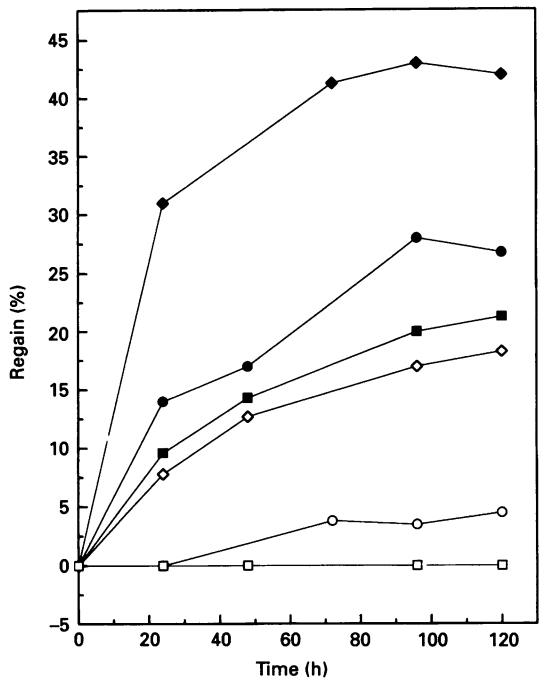


Figure 3 The regain of riboflavin-binding activity during the reoxidation of reduced RfBP

Binding activity was assessed by the ability to quench riboflavin fluorescence as described in the text. ○, Reoxidation in the presence of GSH/GSSG alone; □, reoxidation in the presence of GSH/GSSG and 2 mol of BSA/mol of RfBP; ◇, ■, ●, and ◆ represent reoxidation in the presence of GSH/GSSG at 0.1, 0.2, 0.5 and 5 mol of PDI/mol of RfBP respectively.

the sulphydryl groups, as shown by the lack of reaction with Nbs_2, after dialysis to remove excess GSH and GSSG. The overall structural properties of the reoxidized RfBP as measured by CD and fluorescence showed wide variations between material obtained in replicate experiments, presumably indicating that formation of a specific set of disulphide bonds had not occurred.

The recovery of binding activity during reoxidation is expressed as a percentage of the quenching of riboflavin fluorescence observed with a control sample of native RfBP of similar concentration to the reoxidizing RfBP (0.04 mg/ml) incubated at 4 °C in the GSH/GSSG mixture for an identical period of time. Less than 5% quenching was observed with the reoxidized RfBP, indicating that no significant binding ability had been regained.

The effect of inclusion of PDI during the reoxidation was studied. We found that reproducible data could only be obtained if all buffers were flushed with O_2-free nitrogen for 2 min immediately prior to the reoxidation experiments. We also found that recovery of activity was at least 10-fold higher at 4 °C than at 20 °C, and increased with increasing concentrations of PDI. Under the optimum conditions we have explored (i.e. a 5-fold molar ratio of PDI added to RfBP prior to addition of GSSG at 4 °C) approximately 45% of the binding ability of native apo-RfBP could be regained (Figure 3). In contrast, a maximum of 4.5% recovery was achieved with the GSH/GSSG redox system in the absence of PDI. At lower molar ratios of PDI: RfBP, lower degrees of recovery of activity were observed (Figure 3). Addition of BSA to reoxidizing RfBP in place of PDI yielded no active protein (Figure 3), indicating that the effect of PDI is specific and does not represent a non-specific protein effect. In agreement with the studies of Lilie et al. [13] on the refolding of reduced Fab, we found that the effect of PDI is very dependent on the time of addition of PDI to the reoxidation mixture, with yields of reactivation being decreased substantially if the PDI is not added until 10 s after the start of the reoxidation.

Because of the presence of large amounts of PDI, we have not been able to measure structural properties of the reoxidized RfBP.

DISCUSSION

In spite of having nine disulphide bridges, RfBP appears to lose all detectable secondary and tertiary structure after incubation in 6 M GdnHCl. This is analogous to the situation with another disulphide-bonded protein, lysozyme [22]. As long as the nine disulphide bonds remain intact, denatured RfBP will refold completely and very quickly after the removal of the denaturing agent, without any other additional agents, i.e. chaperone proteins, being necessary. It would appear, from the protein fluorescence refolding data, that the presence of riboflavin during the refolding process somehow impeded the final acquisition of tertiary structure, albeit very slightly, and this had no effect on the binding of riboflavin. It could be that the binding pocket, or the riboflavin-binding element, is formed relatively early in the refolding process, and the binding of riboflavin at this stage places constraints on small adjustments in the local tertiary structure. The situation is reminiscent of that described by Mucke and Schmid [23] who found that under certain conditions the presence of intact disulphide bonds decelerated the folding of ribonuclease T1, presumably by restricting local structural rearrangements. On the other hand, when RfBP is reduced, i.e. the disulphide bonds broken, the correct disulphide bonds do not reform spontaneously under oxidizing conditions, as shown by the lack of recovery of binding activity.

The extent of formation of the correct disulphide bonds during the reoxidation of reduced RfBP was clearly greatly enhanced by the presence of PDI. The use of PDI in reoxidation experiments increased the yield of correctly reoxidized RfBP by at least 10-fold to a value approaching 50%; in our experiments, optimum regain of activity was obtained at the highest ratio of PDI: RfBP used, namely 5:1. These findings are similar to those reported by Lilie et al. [13], who found that PDI, in concentrations comparable with or greater than those of reduced Fab, increased refolding and recovery of antigen binding activity. Given that the abundance of PDI in the lumen of the endoplasmic reticulum is high, and its concentration may be in the millimolar range, the relatively high concentration required to achieve a high yield of reactivation is not surprising. The results presented here are also comparable with those of Lilie et al. in that the enzyme-facilitated reactivation is very slow but that the presence of PDI from the initiation of refolding is crucial; in this case, although the process continues over 3 days, if PDI is not present during the first 10 s of refolding, its effect is much less apparent.

These kinetic observations provide some structural insight into the events following the transfer of unfolded, reduced RfBP under non-denaturing conditions. Most of the material rapidly forms disulphide-bonded species, which are non-native in conformation. These species are presumably non-native in disulphide-pairing, are probably compact in conformation, may be aggregated and may contain inter-chain disulphides. This process is essentially irreversible, and only 5% of this material spontaneously isomerizes to the native state in 72 h. PDI, when present from the outset in stoichiometric amounts, can interact with the refolding species and divert it into a productive folding

pathway. The details of this action cannot be established on the basis of these studies, and it is premature to speculate on the exact role of PDI when it is complexed with RfBP folding intermediates. However, the slow rate of the catalysed isomerization suggests that it is limited (i) by slow conformational steps in the misfolded protein substrate, which are required to make buried non-native disulphides accessible for thiol-disulphide interchange, and (ii) by the very large number of isomerizations required to generate the nine native disulphides. It has been suggested [18] that in RfBP the breaking of only one (unidentified) disulphide bond can lead to loss of the binding activity of the protein. In the reverse process, correct formation of this single bond could be the crucial, final step in reactivation; this would be comparable with the case of bovine pancreatic trypsin inhibitor (BPTI), where formation of the final disulphide requires rate-limiting conformational and disulphide isomerizations in folding intermediates [24]. Alternatively, correct formation of this disulphide at any early stage may stabilize crucial refolding intermediates and facilitate the subsequent formation of native disulphide bonds.

The refolding and reoxidation process studied *in vitro* is clearly quite distinct from that occurring during biosynthesis. *In vivo*, translation, translocation into the endoplasmic reticulum and folding of disulphide-bonded proteins occur on similar timescales of 10–1000 s [25], and additional components such as molecular chaperones (BiP, Grp94, etc) are present [26]. In multi-domain proteins, formation of native disulphides within an individual domain can occur before more C-terminal domains are fully translated or translocated [27,28]; generation of disulphides within incomplete domains may be random, or biased by transient formation of local structural elements. There is at present no information on the domain organization of RfBP and the number of disulphides within each domain. While PDI is undoubtedly a useful tool *in vitro* for enhancing yields in the reactivation of reduced unfolded proteins by the isomerization of non-native disulphides, it is clear that its role *in vivo* can only be understood in the context of the other processes and factors involved in the production of correctly folded proteins within the lumenal compartment.

However, given that the oxidation of reduced, unfolded RfBP to give reasonable yields of active protein can only be achieved in the presence of PDI, studies of the type described here can give important insights into the interplay between protein folding and disulphide bond formation in secreted proteins. This is particularly true for those proteins, such as RfBP, where reoxidation of the reduced proteins using mixtures of GSH and GSSG gives very poor yields of active material.

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