Comparison of the activities of protein disulphide-isomerase and thioredoxin in catalysing disulphide isomerization in a protein substrate

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1. The activities of protein disulphide-isomerase (PDI) and thioredoxin in catalysing disulphide bond isomerization in a protein substrate were compared by using the standard assay, namely the re-activation of 'scrambled' RNAase. 2. The specific activity of PDI was 25-fold greater than that of thioredoxin. 3. The greater efficiency of PDI compared with thioredoxin is considered to be due more to the presence of multiple catalytic domains in PDI than to differences in their active-site sequences. 4. Data and procedures were defined for expressing enzyme activity in standard units, i.e. μmol of active RNAase generated/min.

INTRODUCTION

Protein disulphide-isomerase (PDI) is a major soluble component of the endoplasmic-reticulum lumen, and a significant body of evidence indicates that it acts as the catalyst of native disulphide bond formation in biosynthesis of secretory and cell-surface proteins. The cellular and subcellular distribution of the enzyme, its developmental properties and its catalytic activities *in vitro* are all consistent with this role (reviewed in Freedman, 1984; Freedman *et al.*, 1989). The activity of PDI as a catalyst of protein folding *in vitro* is remarkable for its wide specificity; it has been shown to be active towards a wide range of protein substrates in three overall reactions: (i) protein disulphide formation (from reduced or partially reduced proteins in oxidizing conditions), (ii) protein disulphide reduction (using low- M_r thiol compounds to reduce protein disulphides) and (iii) protein disulphide isomerization (where both breakage and formation of disulphide groups occur, permitting the system to select the set of disulphide bridges consistent with the most stable folded conformation). The chemical process catalysed in all three overall reactions is thiol/disulphide interchange, the attack of a thiol group (as the thiolate ion, S^-) on a disulphide bond. PDI action has been studied in most detail with a handful of protein substrates: reduced RNAase A (Givol *et al.*, 1964; Lang & Schmid, 1988), reduced bovine pancreatic trypsin inhibitor (Creighton *et al.*, 1980) and reduced procollagen (Forster & Freedman, 1984; Koivu & Myllylä, 1987) for studies of disulphide formation, 'scrambled' RNAase (SRNAase) (Fuchs *et al.*, 1967; Lambert & Freedman, 1983b) for studies of disulphide isomerization, and insulin for studies of disulphide reduction (Lambert & Freedman, 1983b; Morin & Dixon, 1985). The refolding and re-activation of SRNAase has been most widely used as an assay for PDI (Hillson *et al.*, 1984).

Sequencing of cloned PDI DNA from vertebrates (Parkonnen *et al.*, 1988) and yeast (R. Farquhar, N. Honey, S. J. Muraut, P. Bossier, R. B. Freeman & M. F. Tuite, unpublished work) shows the presence of two regions within the PDI polypeptide similar to the small bacterial oxidoreductase thioredoxin. The active site of thioredoxin is a vicinal dithiol/disulphide couple formed by two cysteine residues in the sequence-Trp-Cys-Gly-

Pro-Cys-Lys-; PDI contains, in two regions analogous to thioredoxin, the sequence-Trp-Cys-Gly-His-Cys-Lys-. Thioredoxin is a very effective catalyst of protein disulphide reduction (Holmgren, 1985), and it can also catalyse native disulphide formation from reduced RNAase A and disulphide isomerization in SRNAase (Pigiet & Schuster, 1986).

In the present paper we have sought to compare quantitatively the activities of thioredoxin and PDI in the standard assay of disulphide isomerization, the re-activation of SRNAase. This has provided the opportunity to revise the units of activity used for this assay, so that activities may be quoted in standard enzyme units. We find that PDI is considerably more active than thioredoxin as a catalyst of protein disulphide isomerization.

MATERIALS AND METHODS

Materials

Tris was obtained from Boehringer Mannheim, yeast RNA (as the sodium salt) from Koch-Light Laboratories, ultrapure urea from Bethesda Research Laboratories, and Sephadex G-25 (medium grade) from Pharmacia. Sigma Chemical Co. supplied Brilliant Blue G, dithiothreitol, sarcosine hydrochloride, BSA (98–99% pure) and bovine pancreatic RNAase A (proteinase-free). Folin's reagent and all other chemicals of AnalaR grade were obtained from BDH Chemicals.

Purification of enzymes

PDI was purified from bovine liver by the method of Lambert & Freedman (1983a). Thioredoxin, purified from *Escherichia coli*, was kindly donated by Professor A. Holmgren, Karolinska Institute, Stockholm, Sweden.

Preparation of SRNAase substrate

SRNAase is fully oxidized but inactive as a catalyst of RNA hydrolysis owing to the formation of a complex mixture of species containing an undefined variety of (mainly) intramolecular disulphide bonds. It was prepared by allowing reduced RNAase to re-oxidize under denaturing conditions as described

Abbreviations used: PDI, protein disulphide-isomerase (EC 5.3.4.1); SRNAase, 'scrambled' RNAase; TKM buffer, 50 mM-Tris/25 mM-KCl/5 mM-MgCl₂, pH 7.5.

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originally by Haber & Anfinsen (1962) and was based on a previous procedure (Hillson *et al.*, 1984).

RNAase was reduced by incubation at 25 mg/ml (1.8 mM) in 50 mM-Tris/HCl buffer, pH 8.6, containing 130 mM-dithiothreitol and 10 M-urea. The flask was flushed gently with N_2 , sealed, and left in the dark for 20–24 h at room temperature (20 °C). The reaction mixture was then acidified to pH 4.0 with acetic acid and separated from the reductant by gel filtration through Sephadex G-25 with 0.1 M-acetic acid containing 8 M-urea. Assays for free thiol groups with 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman, 1959) showed it to be fully reduced, and protein recovery was 70–80 %.

Reduced RNAase was re-oxidized under denaturing conditions at a concentration of 0.5–2.0 mg/ml, sufficiently low to favour the formation of intramolecular disulphide bonds; subsequent analysis of the final product by non-reducing SDS/PAGE showed it to be almost completely monomeric. The eluate from Sephadex G-25 chromatography was diluted with a concentrated solution of Tris/sarcosine hydrochloride in 8 M-urea to bring the buffer to a final concentration of 8 M-urea/0.1 M-Tris/0.1 M-sarcosine hydrochloride, adjusted to pH 8.5, and left in the dark at room temperature for 5–6 days. Sarcosine was included to react preferentially with cyanate ions, which would otherwise inactivate RNAase by carbamoylation (Stark *et al.*, 1960). Cyanate is formed by isomerization of urea (Dirnhuber & Schutz, 1948), and a significant concentration relative to the protein could develop during re-oxidation over this length of time. Re-oxidation was considered complete when 5,5'-dithiobis-(2-nitrobenzoic acid) assays showed less than 0.1 mol of free thiol group/mol of RNAase.

The solution of fully reoxidized SRNAase was acidified to pH 4.0 with acetic acid and concentrated by precipitation with 100 %-satd. $(NH_4)_2SO_4$. In the presence of 8 M-urea this requires approx. 45 g/100 ml at room temperature. After centrifugation at 18000 *g* for 30 min, the pellets were resolubilized in 50 mM- NH_4HCO_3 and then acidified to pH 4.0 (the pellets did not redissolve directly in acetic acid). The solution was desalted by gel filtration through Sephadex G-25 with 10 mM-acetic acid. Maximum solubility of the desalted SRNAase was 2.5–3 mg/ml. Recovery was approx. 50 % of the starting material.

SRNAase was routinely prepared in bulk from 250 mg of RNAase and stored in small portions for several months. Its effectiveness as a substrate for PDI varied considerably with the storage conditions. In frozen solution, it lost little effectiveness after 4 months at –20 °C, but lost it completely after 7 months at –20 °C or 4 months at –80 °C. SRNAase stored better as a freeze-dried powder and was fully effective after 3 months at both 4 °C and –20 °C. After 16 months, however, it had deteriorated at –20 °C more rapidly than at 4 °C, containing approx. 25 % of the original re-activatable substrate at –20 °C and approx. 50 % at 4 °C. Overall, its effectiveness decreased when stored in solution or at a lower temperature. Consequently, SRNAase was routinely freeze-dried from 10 mM-acetic acid and stored at 4 °C.

Characterization of the SRNAase substrate

The effectiveness of SRNAase as a substrate for PDI varied with each preparation, as noted previously by Koivu & Myllylä (1986). A titration of activity against SRNAase concentration provided values of K_m and V_{max} for each preparation, and SRNAase was used routinely at a concentration of $2K_m$ in the PDI assay. Variation in PDI activity becomes less dependent on the SRNAase concentration as the latter increases, but at the same time the background rate in the absence of PDI increases to become a significant component of the activity, so a balance was achieved with this concentration of SRNAase. For the

preparation used here, $K_m = 5.1 \mu M$ and $V_{max} = 3.4 \mu mol/min$ per g of PDI, when assayed with homogeneous bovine liver PDI.

Assay of protein concentration

The concentration of RNAase was measured by A_{280} ; with solutions containing known amounts of freeze-dried protein by weight, a concentration of 1.0 mg/ml corresponded to $A_{280} = 0.550$. This conversion was also used for reduced RNAase and SRNAase.

The assay for PDI concentration used Coomassie Blue reagent (Sedmak & Grossberg, 1977) and for thioredoxin used Folin's reagent (Lowry *et al.*, 1951). Both methods used BSA as the standard protein.

Assay of PDI activity

The assay of PDI activity followed the procedure of Lambert & Freedman (1983b), in which the re-activation of SRNAase by PDI is monitored by a spectrophotometric assay of RNAase activity. The two-stage assay involves first a PDI incubation with SRNAase, from which samples are withdrawn and assayed for RNAase activity by a continuous spectrophotometric method.

The buffer solution used throughout was 50 mM-Tris/25 mM-KCl/5 mM-MgCl₂, pH 7.5 (TKM buffer). The stock solution of SRNAase contained freeze-dried protein dissolved in distilled water at 10 times the required concentration of $2K_m$ (see above) and was kept at 4 °C for 1 week only; sufficient acetate ions remained after freeze-drying to acidify this solution to pH 3–4 and so prevent disulphide bond isomerization.

PDI was activated by preincubation with dithiothreitol in 180 μl for 2 min at 30 °C. Then 20 μl of SRNAase was added to give a final volume of 200 μl with final concentrations of 10 μM -dithiothreitol and 10.2 μM -SRNAase ($2K_m$ for this preparation). From this PDI incubation, 10 μl samples were withdrawn at five time points between 0.5 and 16 min and added to a cuvette containing 250 μg of highly polymerized RNA in 3.0 ml of TKM buffer. RNAase activity was assayed by the decrease in A_{260} on a Perkin-Elmer 356 spectrophotometer in dual-wavelength mode with a slit width of 2.5 nm, recording A_{260} relative to A_{280} . This mode is essential only for membrane samples with high background absorbance, so these samples of soluble protein could be assayed equally well with any sensitive single-wavelength spectrophotometer capable of recording a small change of $A_{260} < 0.1$ against a high background of A_{280} (~2 units).

Re-definition of PDI activity in standard units

PDI activity was measured by the rate at which the SRNAase substrate is re-activated. At each time point when a sample was removed from the PDI incubation, its RNAase activity was measured by the initial linear change in A_{260}/min . The increase in RNAase activity as a function of time, derived from a series of such assays, was linear up to 16 min. The PDI activity was calculated as the increase in RNAase activity per min, i.e. the change in A_{260}/min per min; linear-regression analysis with a correlation coefficient > 0.99 was considered acceptable.

This direct measurement of PDI activity was then converted into standard units of activity, i.e. μmol of RNAase generated/min in the PDI incubation. The conversion factor related the measurement of RNAase activity in the assay to the concentration of RNAase present in the assay incubation, which in turn was related to the quantity of RNAase in the PDI incubation. The conversion from a value of RNAase activity into its concentration was obtained by assaying homogeneous RNAase. From triplicate assays for each of four stock solutions, a 10 nM solution of RNAase had an activity of 0.55 ± 0.03

(mean $\pm$ S.D.) A_{260} unit/min. This concentration of RNAase in the assay was then converted into the quantity of RNAase in the PDI incubation by a factor F , which depended on the volumes used. When the assay sample ($A \mu\text{l}$) was withdrawn from the PDI incubation (original volume $V \mu\text{l}$) and assayed in the cuvette ($C \mu\text{l}$), $F = CV/A$. Thus $1 \mu\text{mol}$ of RNAase generated in the PDI incubation corresponds to RNAase activity measured in the assay as a change in A_{260} of $5.5 \times 10^7/F$ units A_{260}/min .

Consequently, PDI generating RNAase in the incubation at a rate of $1 \mu\text{mol}/\text{min}$ represents 1 standard unit of activity and is equivalent to an increase of RNAase activity in the assay giving a change in A_{260} of $5.5 \times 10^7/F$ units A_{260}/min per min.

In our routine procedure, where a $10 \mu\text{l}$ sample is withdrawn from $200 \mu\text{l}$ of original incubation and assayed in 3.0 ml of RNA, PDI activity of $1 \mu\text{mol}/\text{min}$ in the incubation corresponds to an increase in RNAase activity in the assay that gives a change in A_{260} of $920 A_{260}$ units/min per min.

Previously, PDI activity was defined directly in spectrophotometric units, and 1 unit of PDI activity generated RNAase activity at the rate of $1 A_{260}$ unit/min per min (Ibbetson & Freedman, 1976). This definition was rigidly dependent on the volumes used in the procedure, whereas the advantage of the standard unit of activity is that these volumes can be altered. Previous calculations of specific PDI activity were based on an incubation volume of $1000 \mu\text{l}$, so they can be converted from 184 'old' units/g into $1 \mu\text{mol}/\text{min}$ per g, e.g. an 'old' specific activity of 1000 units/g is equivalent to $5.4 \mu\text{mol}/\text{min}$ per g.

RESULTS AND DISCUSSION

Purified thioredoxin was assayed for PDI activity, by incubating SRNAase with different quantities of the enzyme from 1.5 to $9.0 \mu\text{g}$. The results in Fig. 1 show that thioredoxin re-activates inactive SRNAase, confirming the previous work by Pigiet & Schuster (1986) and demonstrating that thioredoxin can act in the same way as PDI, catalysing the rearrangement of incorrect disulphide bonds in a protein substrate. The results also show that the rate of re-activation increases linearly with the quantity of thioredoxin present, giving a quantitative estimate of its specific activity as $0.0725 \mu\text{mol}/\text{min}$ per g of protein.

Purified PDI was also assayed with the same substrate, with similar quantities of the enzyme (Fig. 1). PDI was considerably more active than thioredoxin and showed a similar linear relationship with the quantity of enzyme present. Its specific activity was $1.94 \mu\text{mol}/\text{min}$ per g of protein, 25-fold greater than thioredoxin.

Since PDI contains two active sites per polypeptide compared with one for thioredoxin (Edman *et al.*, 1985), it would be more informative to compare their specific activities based on the

concentration of active sites. Then the specific activity of PDI is $55.3 \text{ mmol}/\text{min}$ per active half-mol, based on a molecular mass of 57 kDa (Edman *et al.*, 1985), and the specific activity of thioredoxin is $0.87 \text{ mmol}/\text{min}$ per active mol, based on a molecular weight of 12 kDa (Holmgren, 1985). On this basis, PDI is about 50 times more efficient than thioredoxin at catalysing disulphide interchange in SRNAase.

Both PDI and thioredoxin also catalyse the re-activation of RNAase from the reduced denatured protein (Givol *et al.*, 1964; Pigiet & Schuster, 1986). More recent work has compared this activity in thioredoxin with two gonadotropic hormones, lutropin and follitropin (Boniface & Reichart, 1990). A comparison of these four catalysts shows considerable differences in their catalytic activity (Table 1). PDI is the most efficient, catalysing maximum re-activation of rRNAase in the least time with the lowest molar ratio of enzyme to substrate even when only partially purified; thioredoxin is the least efficient and the hormones are intermediate. These differences may be reflected in

Fig. 1. Re-activation of SRNAase by PDI and thioredoxin

Assays at 30°C in $200 \mu\text{l}$ of TKM buffer, pH 7.5, contained $10 \mu\text{M}$ -dithiothreitol and $10.2 \mu\text{M}$ -SRNAase plus purified bovine liver PDI ($\circ$) or purified *E. coli* thioredoxin ($\bullet$). Most results are the means of duplicate assays. The theoretical lines are obtained by linear-regression analysis. The derived specific activity of PDI is $1.94 \mu\text{mol}/\text{min}$ per g of protein (correlation coefficient 1.00) and that of thioredoxin is $0.0725 \mu\text{mol}/\text{min}$ per g of protein (0.99).

Table 1. Comparative rates of re-activation of reduced RNAase

Reduced RNAase was re-activated at pH 7.4 and 25°C . PDI was only partially purified and was assayed at 37°C . References: (1), Givol *et al.* (1964); (2), Pigiet & Schuster (1986); (3), Boniface & Reichart (1990).

Enzyme	Source	Concn. of reduced RNAase (μM)	Concn. of enzyme (μM)	Molar ratio enzyme/reduced RNAase	Approx. time for maximum re-activation	Reference
PDI	Bovine liver	5.8	0.14	1:40	20 min	(1)
Thioredoxin	<i>E. coli</i>	23	500	22:1	50 h	(2)
		29	500	17:1	30 h	(3)
Lutropin	Bovine	29	1	1:29	30 h	(3)
Follitropin	Sheep	29	1	1:29	80 h	(3)

PDI	- <u>Trp</u> -Cys-Gly- <u>His</u> -Cys- <u>Lys</u> -
Thioredoxin	- <u>Trp</u> -Cys-Gly- <u>Pro</u> -Cys- <u>Lys</u> -
Lutropin β -subunit	- <u>His</u> -Cys-Gly- <u>Pro</u> -Cys- <u>Arg</u> -
Follitropin β -subunit	- <u>His</u> -Cys-Gly- <u>Lys</u> -Cys- <u>Asp</u> -

Fig. 2. Comparison of the active-site sequences of PDI, thioredoxin and lutropin and follitropin β -subunits

These sequences refer to the same source of each enzyme as Table 1. Variable residues are underlined. References: PDI, Yamauchi *et al.*, (1987); thioredoxin, Holmgren (1968); lutropin and follitropin β -subunits, Boniface & Reichart (1990).

their active-site sequences; of the six amino acid residues, only three are completely conserved (Fig. 2). However, the greatest difference in catalytic activity, i.e. between PDI and thioredoxin, is also associated with the least difference in the active-site sequence (one residue only), suggesting there are other more relevant intrinsic differences between them. A further hint that the active site can tolerate a range of residues in the position preceding the second cysteine residue (see Fig. 2) is the finding by Gleason *et al.* (1990) that a mutant thioredoxin with the active-site sequence -Cys-Gly-Ser-Cys- is indistinguishable in kinetic properties from the wild-type.

If the difference in catalytic activity between thioredoxin and PDI is not simply a result of the limited local sequence difference, then other factors must be considered. As shown in the first paper of this series (Hawkins & Freedman, 1991), PDI and thioredoxin both contain essential cysteine residues of unusually low pK (6.7) and hence unusually high nucleophilic reactivity at neutral pH; this has been proposed as the attacking residue in the disulphide reductase activity of the thioredoxin (Kallis & Holmgren, 1980). Lundström & Holmgren (1990) have shown that both thioredoxin and PDI are reducible by bovine thioredoxin reductase and that they have comparable activities as catalysts of reduction of disulphide bonds in insulin. Where PDI and thioredoxin differ is in standard redox potential, as shown in the preceding paper (Hawkins *et al.*, 1991). PDI activity requires catalysis of both the breakage and formation of disulphide bonds, and it may be that the more oxidizing redox properties of the PDI active sites make them more favourable for the overall isomerization reaction.

A further important difference in properties between the proteins, which may contribute to the greater catalytic efficiency of PDI in protein disulphide-isomerization, is the presence of multiple catalytic domains in PDI. It contains two domains *a* and *a'*, both similar to the thioredoxin polypeptide in amino acid sequence (Edman *et al.*, 1985) and probably also closely similar to it in conformation (Freedman *et al.*, 1988). Since PDI exists in solution as a homodimer (Lambert & Freedman, 1983a), four active-site domains are located close together and may act co-operatively; evidence bearing on this is presented in the preceding paper (Hawkins *et al.*, 1991). By comparison, thioredoxin consists of only one such domain and is present in dilute solution as a monomer (Holmgren *et al.*, 1975).

Overall, the activities of all these proteins as catalysts of disulphide formation and isomerization are unspectacular, to say the least. On the basis of a polypeptide molecular mass of 57 kDa, the specific activities for PDI determined here convert into catalytic-centre activities of below 1 min^{-1} . This may be compared with data for the oxidation of reduced bovine pancreatic trypsin inhibitor, where the catalytic-centre activity is 3 min^{-1} (Creighton *et al.*, 1980) and for the reduction of insulin, where it is 12 min^{-1} (Lambert & Freedman, 1983b). SRNAase is a more complex substrate and its re-activation requires formation

of a greater number of native disulphide groups via an unknown number of individual thiol/disulphide exchange steps, so the lower catalytic-centre activity observed here is not surprising in itself. PDI is, however, a genuine catalyst; it is active in sub-stoichiometric amounts, turns over many molecules of substrate per molecule of enzyme, and in the case of bovine pancreatic trypsin inhibitor passes the protein substrate through the same sequence of intermediates as in the uncatalysed reaction (Creighton *et al.*, 1980). In appropriate conditions, it can catalyse native disulphide bond formation and folding at rates close to those observed in the cell (Koivu & Myllylä, 1987). As a catalyst, it does not instruct the formation of 'correct' disulphide bridges, but facilitates the selection by the substrate protein of the set consistent with the most stable conformation; this is best illustrated by studies on the disulphide isomerization in the RNAase S protein, where the set of disulphide bridges formed in the presence of PDI is dependent on whether the short S peptide is present and non-covalently associated with the protein (Kato & Anfinsen, 1969).

In secretory cells, formation of native disulphide bonds occurs in nascent or newly discharged polypeptides within the lumen of the endoplasmic reticulum. The process is complete within 1–5 min of the termination of translation (Bruckner *et al.*, 1981; Peters & Davidson, 1982). PDI is abundant in this compartment, comprising perhaps 10% of the total resident protein population of the endoplasmic-reticulum lumen; other proteins (immunoglobulin-heavy-chain-binding protein or BiP, endoplasmic reticulum chaperone) are also abundant here (Koch, 1987). The relatively low catalytic performance of PDI *in vitro* is presumably compensated by its high abundance at the cellular site of protein disulphide formation; it is not clear whether any of the other components, or some local supramolecular organization, also contribute to the catalytic effectiveness of PDI in the cellular context.

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