Redox properties and cross-linking of the dithiol/disulphide active sites of mammalian protein disulphide-isomerase

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1. The redox properties of the active-site dithiol/disulphide groups of PDI were determined by equilibrating the enzyme with an excess of GSH + GSSG, rapidly alkylating the dithiol form of the enzyme to inactivate it irreversibly, and determining the proportion of the disulphide form by measuring the residual activity under standard conditions. 2. The extent of reduction varied with the applied redox potential; to a first approximation, the data fitted a model in which all the enzyme dithiol/disulphide groups are independent and equivalent and the equilibrium constant between these sites and the GSH/GSSG redox couple is 42 μM at pH 7.5. 3. The standard redox potential for PDI active-site dithiol/disulphide couples was calculated from this result and found to be -0.11 V ; hence PDI is a stronger oxidant and weaker reductant than GSH, nicotinamide cofactors, thioredoxin and dithiothreitol. 4. The redox equilibrium data for PDI with the GSH/GSSG redox couple showed sigmoidal deviations from linearity. The sigmoidicity could be modelled closely by assuming a Hill coefficient of 1.5. 5. This evidence of co-operative interactions between the four active sites in a PDI dimer was extended by studying the reaction between PDI and homobifunctional alkylating agents with various lengths between the reactive groups. A species whose electrophoretic mobility suggested it contained an intrachain cross-link was observed in all cases, whereas there was no evidence for cross-linking between the chains of the PDI homodimer. Most effective cross-linking was achieved with reagents containing five or more methylene spacer groups, implying a minimum distance of 1.6 nm (16 Å) between the active-site reactive groups within the two thioredoxin-like domains of the PDI polypeptide.

INTRODUCTION

cDNA sequence analysis of protein disulphide-isomerase (PDI) from rat liver (Edman *et al.*, 1985) and subsequently from many vertebrate sources (Parkkonen *et al.*, 1988) has indicated that each polypeptide contains two domains closely similar to thioredoxin. Thioredoxin is a small single-domain protein with a vicinal disulphide group in a reverse turn extending out from the bulk of the protein (Holmgren *et al.*, 1975). Modelling the structure of the thioredoxin-like regions of PDI indicated that these regions formed domains similar in overall conformation to thioredoxin (Freedman *et al.*, 1988). Both PDI and thioredoxin are capable of acting as catalysts of thiol/disulphide interchange processes; previous work has indicated that both, in appropriate conditions, can act as general thiol:protein-disulphide oxidoreductases catalysing reduction of protein disulphides, or as catalysts of the net oxidation of reduced proteins, or as disulphide-isomerases (Freedman, 1984; Holmgren, 1985; Pigiet & Schuster, 1986). In the following paper (Hawkins *et al.*, 1991) we compare quantitatively the activity of the two proteins as protein disulphide-isomerases.

An understanding of the catalytic properties of PDI and thioredoxin requires knowledge of the chemical properties of their vicinal dithiol/disulphide groups, since these are essential for activity and are presumed to participate directly in the catalytic process. In the preceding paper (Hawkins & Freedman, 1991) we examined the reactivities and pK of the active-site dithiol/disulphide groups of PDI and indicated that there is considerable similarity between PDI and thioredoxin at the mechanistic as well as structural levels. We examine here another chemical property of these active-site groups, namely their redox potential. This is of interest both in relation to the overall structure of PDI and thioredoxin, and also in relation to their

physiological functions. Differences in properties between PDI and thioredoxin might be due to the limited number of differences in sequence around the active sites, or to the fact that thioredoxin is a small single-domain enzyme whereas PDI is a homodimer that is an order of magnitude larger and contains in total four domains that are similar to thioredoxin plus other domains. The physiological function of PDI is to facilitate native disulphide bond formation in nascent and newly synthesized proteins within the lumen of the endoplasmic reticulum; the functions of thioredoxin are various and still subject to controversy, but in general it appears to act as a dithiol reductant within the cytoplasm (Holmgren, 1989), a compartment in which the thiol/disulphide redox potential is reducing and is determined by the equilibrium between GSH and GSSG (Meister, 1988).

In the present paper we show that the redox potential of the active-site dithiol groups of PDI is distinctly less negative than that of thioredoxin, and we provide some evidence of co-operativity between the PDI active sites. We further provide evidence of the minimum distance between the two active sites of a single PDI polypeptide, based on cross-linking experiments with homobifunctional reagents.

MATERIALS AND METHODS

Materials

The solvent dimethyl sulphoxide was obtained from Aldrich Chemical Co., and acrylamide and bis(acrylamide) were from Electran. Biochemicals were obtained from Sigma Chemical Co., and all other chemicals of AnalaR grade were from BDH Chemicals. A series of bifunctional reagents based on NN' -bis(iodoacetamide) (bis-IAM) with spacer chains of different lengths was generously supplied by Professor R. F. Luduena,

Abbreviations used: bis-IAM, NN' -bis(iodoacetamide); DTT, dithiothreitol; IAA, iodoacetic acid; IAM, iodoacetamide; PDI, protein disulphide-isomerase (EC 5.3.4.1); SRNAase, 'scrambled' RNAase.

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University of Texas, San Antonio, TX, U.S.A. A similar series based on bis(maleimide) was kindly donated by Professor R. B. Beechey, University College of Wales, Aberystwyth, Dyfed, U.K.

Purification of PDI

PDI was purified from bovine liver by the method of Lambert & Freedman (1983).

Assay of protein concentration

Protein was assayed with the use of Coomassie Blue, based on Bradford's (1976) original procedure, with the modification described by Sedmak & Grossberg (1977), with a standard curve based on BSA. For seven stock solutions of purified PDI assayed by this method, a concentration of 1.0 mg/ml was equivalent to $A_{280} = 1.02 \pm 0.17$ (mean $\pm$ s.d.); stock solutions were subsequently assayed by A_{280} .

Assay of PDI activity

PDI was assayed by its re-activation of 'scrambled' RNAase (SRNAase), which is fully oxidized but inactive owing to the presence of an undefined range of incorrectly formed disulphide bonds. PDI catalyses the isomerization of these disulphide bonds to those found in the native active enzyme. The assay is described in detail in the following paper (Hawkins *et al.*, 1991), including the preparation of SRNAase and the redefined units of PDI activity as μmol of substrate re-activated/min. For the preparation of SRNAase used here, K_m was $5.1 \mu\text{M}$ and V_{max} was $3.4 \mu\text{mol/min per g}$ of PDI.

Redox equilibration of PDI with glutathione

Homogeneous PDI ($5 \mu\text{M}$) was incubated in 50 mM-sodium phosphate buffer, pH 7.5, with 0.25 mM-GSH and 4.88 mM-GSSG (total glutathione concentration $[\text{GSH}] + 2[\text{GSSG}] = 10 \text{ mM}$) for 15 min at 20°C to reach equilibrium. Excess iodoacetic acid (IAA) was added (final concentration 100 mM), and after a further 5 min the incubation mixture was dialysed overnight at 4°C against buffer containing 50 mM-Tris/25 mM-KCl/5 mM-MgCl₂, pH 7.5. The dialysis residue was assayed for protein and PDI activity. The specific activity was expressed relative to control incubations in buffer alone, and represented the fraction of unmodified oxidized PDI at equilibrium (A).

The procedure was repeated with different proportions of GSH and GSSG while maintaining a constant total glutathione concentration of 10 mM; the range of GSH concentration was 0.25–2.0 mM. In control incubations PDI was equilibrated with GSSG alone (5 mM) or with GSH alone (10 mM).

Data analysis

When equilibrated with glutathione, the formation of intramolecular disulphide bonds in PDI could be expressed by the equation:

This gave an expression for the equilibrium constant, K_{eq} , as:

$$K_{\text{eq}} = \frac{[\text{PDI}_{\text{S}}^{\text{S}}][\text{GSH}]^2}{[\text{PDI}_{\text{SH}}^{\text{SH}}][\text{GSSG}]} \quad (1)$$

The fractional residual activity of unmodified PDI after

alkylation (A) represented the proportion of oxidized PDI at equilibrium. Then the proportion of reduced PDI at equilibrium (R) could be expressed as:

$$R = 1.0 - A = \frac{[\text{PDI}_{\text{SH}}^{\text{SH}}]}{[\text{PDI}_{\text{S}}^{\text{S}}] + [\text{PDI}_{\text{SH}}^{\text{SH}}]} \quad (2)$$

Combining eqns. (1) and (2):

$$R = \frac{[\text{GSH}]^2/[\text{GSSG}]}{K_{\text{eq}} + [\text{GSH}]^2/[\text{GSSG}]} \quad (3)$$

Eqn. (3) was equivalent to a Michaelis-Menten equation from which linear plots could be derived. The plot of $([\text{GSH}]^2/[\text{GSSG}])/R$ against $[\text{GSH}]^2/[\text{GSSG}]$ gave a value for K_{eq} from the negative intercept on the horizontal axis, and a plot of R against $R/([\text{GSH}]^2/[\text{GSSG}])$ gave a value for K_{eq} from the negative gradient.

The expression for the equilibrium constant [see eqn. (1) above] was used to investigate co-operativity between the active sites by re-arranging it in the form of a Hill equation; it used the same symbols as before for the proportions of oxidized PDI (A) and reduced PDI (R) at equilibrium, and included the Hill coefficient of co-operativity (h):

$$\frac{R}{A} = \left(\frac{[\text{GSH}]^2/[\text{GSSG}]}{K_{\text{eq}}} \right)^h \quad (4)$$

This equation could then be rewritten as:

$$\log(R/A) = h \cdot \log([\text{GSH}]^2/[\text{GSSG}]) - h \cdot \log K_{\text{eq}} \quad (5)$$

By plotting $\log(R/A)$ against $\log([\text{GSH}]^2/[\text{GSSG}])$, the Hill coefficient was obtained from the gradient of the line at the midpoint where $R = A$, and K_{eq} was obtained from the intercept.

Incubation of PDI with bifunctional reagents

The cross-linkers based on bis-IAM were soluble in aqueous buffer only at very low concentrations. They were soluble in dimethyl sulfoxide but unstable, so concentrated stock solutions (10 mM) were made up immediately before use. These were diluted 1 in 20 in aqueous buffer to give a final concentration of 0.5 mM in 5% dimethyl sulfoxide. A stock solution of the corresponding monofunctional reagent IAM (20 mM) was also made up in dimethyl sulfoxide. The cross-linkers were treated as though they had the same light-sensitivity as IAM, so all stock solutions and incubations were kept in the dark.

Homogeneous PDI ($17 \mu\text{M}$) was incubated with excess dithiothreitol (DTT) ($170 \mu\text{M}$) in 50 mM-Tris/HCl buffer, pH 7.5, at 30°C for 20 min. Then bis-IAM was added (final concentration 0.5 mM in 5% dimethyl sulfoxide) and the incubation was continued for a further 60 min. Cross-linking reactions were stopped by preparing samples immediately for SDS/PAGE in reducing conditions, which included excess DTT (50 mM). The incubation was repeated with a series of cross-linkers containing a different number of spacer methylene groups from three (bis-IAM-3) to ten (bis-IAM-10).

There were three control incubations. In one, PDI was incubated with 5% dimethyl sulfoxide alone. In another, PDI was incubated with DTT, and then 1.0 mM-IAM (i.e. double the concentration of bis-IAM) was added for a further 60 min. In the third incubation, PDI was incubated with DTT, then 1.0 mM-IAM was added for 60 min, and finally 0.5 mM-bis-IAM was added for a further 60 min.

PDI was also incubated with a series of cross-linkers based on bis(maleimide), with the same procedure as above.

SDS/PAGE

SDS/PAGE was performed with the discontinuous system of Laemmli (1970), with the use of mini-gels (8 cm × 6 cm) containing 7% (w/v) acrylamide. Protein samples were prepared for electrophoresis by boiling with 50 mM-DTT and 1% (w/v) SDS. Marker samples were prepared using untreated homogeneous PDI and a mixture of six proteins with molecular masses 29–205 kDa. After electrophoresis, the gels were stained for protein with Coomassie Blue R250.

RESULTS

Redox equilibrium of PDI with glutathione

The equilibrium constant, K_{eq} , for the equilibration of PDI with a GSH/GSSG redox couple was measured by incubating the enzyme with a mixture of GSH and GSSG at pH 7.5 until equilibrium was reached. The free thiol groups in reduced PDI were then blocked rapidly and irreversibly by adding excess IAA. After dialysis, the residual PDI activity was assayed, representing the concentration of oxidized PDI at equilibrium. The loss of activity compared with PDI incubated in buffer alone represented the fraction of reduced PDI at equilibrium (i.e. % of reduced PDI = 100 – % of residual activity). This procedure was repeated with different proportions of GSH and GSSG.

Several precautions were taken. The total concentration of glutathione species, i.e. $[GSH] + 2[GSSG]$, was kept constant (10 mM); it was much greater than the concentration of PDI dithiol/disulphide couples (10 μ M) so that the GSH/GSSG redox pair determined the redox potential of the mixture. A high

concentration of IAA (100 mM) was added to react rapidly with the free thiol groups present at equilibrium in GSH (maximum concentration 2 mM) and reduced PDI, and so trap accurately the fraction of reduced PDI. This concentration was considered adequate since the rate constant of this reaction is approx. $10 \text{ M}^{-1} \cdot \text{s}^{-1}$ at pH 8.7 and 25 °C (Creighton, 1986); the theoretical half-time for the reaction with 100 mM-IAA would be 1 s under these conditions and slightly longer under the incubation conditions of pH 7.5 and 20 °C used here.

The length of time required for PDI to equilibrate with the GSH/GSSG redox buffer was investigated under conditions corresponding to approx. 50% reduced PDI at equilibrium, where its redox state would be most sensitive to changes in GSH concentration, i.e. 0.5 mM-GSH and 4.75 mM-GSSG. Incubations for 10 min or 15 min before addition of IAA gave the same percentage of reduced PDI, so a 15 min incubation was used routinely. During the 15 min incubation at 20 °C, the concentration of GSH in the redox buffer decreased slightly as a result of air oxidation. The extent of oxidation was quantified by incubating each redox buffer at 20 °C without PDI and assaying for free thiol groups with 5,5'-dithiobis-(2-nitrobenzoic acid) reagent (Ellman, 1959). Over the range of GSH concentration used (0.25–2.0 mM) the extent of oxidation after 15 min was 9–15%; this was later taken into account in the determination of the equilibrium constant for PDI.

Two control incubations checked the minimum and maximum limits of PDI activity at equilibrium in the presence of GSSG alone or GSH alone respectively. When PDI was incubated with 5 mM-GSSG for 15 min and then free thiol groups were quenched with excess IAA, residual activity was 98%. This indicated that only 2% of PDI at equilibrium was inactivated by IAA. When incubated with 10 mM-GSH instead and then alkylated, residual PDI activity after 15 min was less than 1%, so that all the PDI present could be reduced and inactivated.

PDI incubated with a mixture of GSH and GSSG at different concentrations, with a total glutathione concentration ($[GSH] + 2[GSSG]$) of 10 mM and GSH concentrations from 0.25 to 2.0 mM, gave intermediate values for the fraction of reduced PDI at equilibrium. As the GSH concentration increased, the residual activity after alkylation decreased, indicating that more PDI was reduced at equilibrium. Assuming that only intramolecular disulphides are formed in PDI and that all the dithiol/disulphide couples are independent and equivalent, the equilibrium at each active site can be expressed by the equation:

giving an expression for the equilibrium constant, K_{eq} , as:

$$K_{eq} = \frac{[\text{PDI}_{\text{S}}^{\text{S}}][\text{GSH}]^2}{[\text{PDI}_{\text{SH}}^{\text{SH}}][\text{GSSG}]}$$

On the basis of this expression for K_{eq} , Fig. 1 relates the redox state of PDI at equilibrium to the relative concentrations of GSH and GSSG expressed as the ratio $[GSH]^2/[GSSG]$. Two different linear transformations of this approximately hyperbolic graph gave values for K_{eq} of 40 μ M and 57 μ M, averaging 49 μ M; good linear-regression correlation coefficients of 1.00 and 0.97 respectively could only be achieved by omitting the discrepant results obtained at 0.25 mM-GSH ($[GSH]^2/[GSSG] = 13 \mu$ M) and 0.30 mM-GSH ($[GSH]^2/[GSSG] = 21 \mu$ M).

These calculations assumed that the concentration of GSH and GSSG were constant during the incubation and did not allow for GSH oxidation during equilibration; the values of

Fig. 1. Redox equilibrium of PDI with glutathione

Homogeneous PDI (5 μ M) was incubated in 50 mM-sodium phosphate buffer, pH 7.5, with various concentrations of GSH and GSSG (combined GSH + 2GSSG concentration 10 mM) for 15 min at 20 °C, then excess IAA was added (final concentration 100 mM). After a further 5 min, the incubation was dialysed overnight at 4 °C and then assayed for protein and PDI activity. Specific activity was expressed relative to control incubations in buffer alone, and represented the fractional residual activity of unmodified oxidized PDI at equilibrium, A . Then $(1.0 - A)$ represents the fraction of reduced PDI at equilibrium, R (●). Experimental data are shown together with theoretical curves based on alternative models (Figs. 2 and 3). One theoretical curve is based on $K_{eq} = 49 \mu$ M with a Hill coefficient h of 1.0 (—), and the other on $K_{eq} = 53 \mu$ M with a Hill coefficient h of 1.5 (---). The values of $[GSH]^2/[GSSG]$ assume no GSH oxidation.

Fig. 2. Determination of the equilibrium constant for PDI and glutathione

Assuming PDI forms only intra-molecular disulphide bonds, reduced PDI at equilibrium (R) can be related to K_{eq} by the expression:

$$R = \frac{[GSH]^2/[GSSG]}{K_{eq} + [GSH]^2/[GSSG]}$$

Linear transformations of this equation use data from Fig. 1 to estimate K_{eq} in (a) from the negative intercept on the horizontal axis and in (b) from the gradient. The theoretical lines are obtained by linear regression analysis, based on values for $[GSH]^2/[GSSG]$ assuming no GSH oxidation (●—●) and 15% GSH oxidation (○—○). Estimated values for K_{eq} (correlation coefficients in parentheses) in (a) are 40 μ M (1.00) assuming no GSH oxidation and 30 μ M (1.00) assuming 15% GSH oxidation, and in (b) are 57 μ M (0.97) and 41 μ M (0.96) respectively. This analysis assumes independent equivalent sites on PDI with no co-operativity and omits the discrepant results obtained for the two lowest values of $[GSH]^2/[GSSG]$.

$[GSH]^2/[GSSG]$ were recalculated assuming a maximum GSH oxidation of 15% throughout, as indicated by the 5,5'-dithiobis-(2-nitrobenzoic acid) assays. The linear plots were redrawn and the corrected values for K_{eq} were 30 μ M and 41 μ M (correlation coefficients 1.00 and 0.96) respectively, remarkably consistent with the other values. Although the individual values of the $[GSH]^2/[GSSG]$ ratio decreased appreciably when oxidation of GSH was assumed, each linear plot shifted consistently so that the values for K_{eq} changed little (Fig. 2). The four values lie within the range 30–57 μ M with an average K_{eq} of 42 μ M.

The corresponding standard redox potential for PDI was derived from the equation:

$$E'_{0(PDI)} = E'_{0(glutathione)} - 0.03 \log K_{eq}$$

Based on the redox potential for glutathione of -0.24 V (Rost & Rapoport, 1964), the redox potential for the redox-active PDI disulphide groups is -0.11 V.

This determination of the equilibrium constant for PDI assumed that only intramolecular disulphide bonds were formed at equilibrium. A further precaution was taken to exclude the possibility that mixed disulphides with glutathione were formed instead. If this happens, the equilibrium includes only one molecule of GSH instead of two, i.e.:

The corresponding equilibrium constant is:

$$K_{eq} = \frac{[PDI_{SH}^{SSG}][GSH]}{[PDI_{SH}^{SH}][GSSG]}$$

A comparison of the two expressions for K_{eq} shows that the percentage of reduced PDI at equilibrium varies with the ratio $[GSH]/[GSSG]$ for both intramolecular and mixed disulphides, but also varies with $[GSH]$ for intramolecular disulphides only. This difference allows the two types of disulphides to be distinguished experimentally. When the ratio $[GSH]/[GSSG]$ is held constant while $[GSH]$ is changed, the percentage of reduced PDI at equilibrium will remain constant for mixed disulphides but will change for intramolecular disulphides. In two incubations with the same ratio $[GSH]/[GSSG] = 0.5$, the percentage of reduced PDI at equilibrium increased from 81% at 0.5 mM-GSH/1.0 mM-GSSG to 98% at 2.0 mM-GSH/4.0 mM-GSSG. These results are inconsistent with the presence of mixed disulphides and are very close to the theoretical values expected at equilibrium for intramolecular disulphides, i.e. 83% and 95% respectively; these theoretical values assume no GSH oxidation and are based on $K_{eq} = 49$ μ M. Identical theoretical values are obtained assuming 15% oxidation and using the appropriate K_{eq} ; for both values, the ratio $[GSH]/[GSSG]$ equals 0.41.

The experimental values for PDI at equilibrium deviate systematically from the best-fit hyperbolic curve based on $K_{eq} = 49$ μ M (Fig. 1); they follow a more sigmoidal curve, suggesting co-operativity between the four active sites in the PDI dimer. A Hill plot showed co-operativity with a Hill coefficient of $h = 1.5$ (linear-regression correlation coefficient = 0.99) assuming no GSH oxidation (Fig. 3); the derived value for $K_{eq} = 53$ μ M is very close to the value of 49 μ M obtained previously (Fig. 2). When the theoretical curve for the redox state as a function of $[GSH]^2/[GSSG]$ is recalculated according to this model ($K_{eq} = 53$ μ M, $h = 1.5$), it agrees more closely with the experimental data (Fig. 1). The value for the Hill coefficient was unchanged when 15% GSH oxidation was assumed (Fig. 3).

PDI and bifunctional reagents

This apparent co-operativity in PDI was investigated further with the use of bifunctional reagents to probe the spatial relationships between the active sites. PDI exists in solution as a dimer (Lambert & Freedman, 1983) with two active sites on each monomer (Edman *et al.*, 1985), so each disulphide group has a distinct relationship to each of the other three. The thiol-specific homobifunctional reagent bis-IAM was used to modify the active sites in PDI and evidence for cross-linking was obtained by SDS/PAGE in reducing conditions. Spacer chains of different lengths between the two reactive groups of the cross-linker monitored the distances between the active sites.

Fig. 3. Hill plot of PDI equilibrium with glutathione

The data are derived from the experimental results of Fig. 1 and the theoretical lines obtained by linear-regression analysis, based on values for $[GSH]^2/[GSSG]$ assuming no GSH oxidation (●—●) and 15% GSH oxidation (○—○). Both lines give a Hill coefficient of 1.50 with a correlation coefficient of 0.99. The derived values of K_{eq} are $53 \mu M$ (no GSH oxidation) and $39 \mu M$ (15% GSH oxidation).

Fig. 4. Reaction of PDI with bis-IAM reagents of different lengths

Homogeneous PDI ($17 \mu M$) was incubated with DTT ($170 \mu M$) in 50 mM-Tris buffer, pH 7.5, at $30^\circ C$ for 20 min. Then bis-IAM was added (final concentration $500 \mu M$ in 5% dimethyl sulfoxide) and the incubation continued for a further 60 min. Cross-linking reactions were stopped by preparing samples immediately for SDS/PAGE in reducing conditions, which included 50 mM-DTT. After electrophoresis on 7% slab gels, the samples were stained with Coomassie Blue. Samples in lanes 1–6 were incubated with bis-IAM containing successively shorter spacer chains of methylene groups, i.e. bis-IAM-10 in lane 1, bis-IAM-7 in lane 2, bis-IAM-6 in lane 3, bis-IAM-5 in lane 4, bis-IAM-4 in lane 5 and bis-IAM-3 in lane 6. Lane 7 contains molecular-mass markers. Controls in lanes 8–10 contained PDI untreated in lane 8, PDI incubated with IAM at double the concentration of cross-linker in lane 9 and PDI incubated with 5% dimethyl sulfoxide alone in lane 10.

Preincubation of PDI ($17 \mu M$) with excess DTT ($170 \mu M$) at pH 7.5 reduced each active-site dithiol group, exposing just one reactive thiol group at this pH (Hawkins & Freedman, 1991), so that each active site was available to form one unambiguous cross-link. The cross-linker was then added in excess ($500 \mu M$) over all the free thiol groups present. After 1 h, the reaction

was stopped by preparing the incubation immediately for SDS/PAGE in reducing conditions, which included excess DTT (50 mM). A series of cross-linkers was used with the number of spacer methylene groups varying between three and ten. For each cross-linker, electrophoresis separated two bands of approximately equal intensities (Fig. 4, lanes 1–6), one with the same mobility as untreated PDI (lane 8, 57 kDa) and one slightly faster. The monomer in this faster band remained more compact because an intra-chain cross-link connected the two active sites; the best yields were obtained with the cross-linkers containing five or more methylene groups as spacers (lanes 1–4).

Three control incubations all gave one band corresponding in mobility to untreated PDI. One incubation showed no effect of the organic solvent dimethyl sulfoxide used to solubilize the cross-linker and diluted in the incubation to 5% (lane 10). Another incubation included the monofunctional reagent IAM (at the same concentration of reactive groups as used in the experiment with bifunctional reagents) and showed that the chemical modification alone did not affect the mobility of PDI (lane 9). In the third control, PDI was incubated first with the monofunctional reagent at the same concentration as before; cross-linker was then added and the incubation continued. The absence of cross-links (results not shown) indicated that the same sites in PDI reacted with both mono- and bi-functional reagents.

Electrophoresis also separated several variable faint bands of much lower mobility for all cross-linkers, corresponding to a molecular mass of 125–190 kDa. It is unlikely that these represent specific inter-chain cross-links between active sites on different monomers, because regular clearly separated bands would be expected, representing possible dimers (114 kDa), trimers (171 kDa) or tetramers (228 kDa). A similar group of faint high-molecular-mass bands were found to be artifacts when tubulin was incubated with bis-IAM in the same way and analysed by SDS/PAGE (Luduena & Roach, 1981). Tubulin also formed mainly intra-chain linkages, and these extra faint bands appeared only when tubulin was denatured first with urea or SDS. The faint high-molecular-mass bands of PDI may similarly represent denatured protein.

Similar results were obtained with cross-linkers based on bis(maleimide) (not shown), but the intra-chain links were not clearly distinguishable as a well-defined band separate from native PDI.

DISCUSSION

Redox equilibrium of PDI with glutathione

In the presence of a glutathione redox buffer, reduced PDI equilibrated with oxidized PDI containing intra-molecular disulphide bonds, and the equilibrium constant, K_{eq} , was $42 \mu M$. This result was supported by earlier preliminary experiments with redox buffers based on glutathione or DTT. In one set of experiments, PDI was incubated with GSH/GSSG mixtures containing 0.5–1.0 mM-GSH (total $[GSH] + 2[GSSG] = 5 \text{ mM}$) before the addition of IAM (final concentration 2.5 mM). The results were analysed as before by two linear plots based on the equation for K_{eq} assuming intra-molecular disulphide bonds in oxidized PDI, and gave values for K_{eq} of $17 \mu M$ (correlation coefficient 1.00) and $7 \mu M$ (0.74). The concentration of IAM was too low to quench the PDI/glutathione equilibrium rapidly, and yet the results obtained are of the same order of magnitude as the accurate result obtained above with a much more rigorous procedure.

Similar experiments were set up using DTT instead of glutathione in order to avoid the accumulation of mixed disulphides (Creighton, 1978), and using IAA instead of IAM at

Table 1. Redox equilibria of proteins with glutathione

All these proteins formed intra-molecular disulphide bonds at equilibrium, i.e. $\text{protein} \frac{\text{SH}}{\text{S}} + \text{oxidant} \rightleftharpoons \text{protein} \frac{\text{S}}{\text{S}} + \text{reductant}$, with the equilibrium constant, $K_{\text{eq.}}$, represented by:

$$K_{\text{eq.}} = \frac{[\text{protein} \frac{\text{S}}{\text{S}}][\text{reductant}]}{[\text{protein} \frac{\text{SH}}{\text{S}}][\text{oxidant}]}$$

The results marked with an asterisk (*) were calculated; conversions from DTT values into glutathione values used $K_{\text{eq. (DTT/glutathione)}} = 1.2 \times 10^3$ M at pH 8.7 (Creighton & Goldenberg, 1984). References: (1), Holmgren (1984); (2), Walters & Gilbert (1986); (3), Lin & Kim (1989); (4), Creighton (1977); (5), Creighton (1978); (6), Creighton & Goldenberg (1984); (7), Cappel & Gilbert (1988); (8), Clancey & Gilbert (1987).

Protein	Source	Redox buffer	pH	$K_{\text{eq.}}$	Protein/ glutathione $K_{\text{eq.}}$ (M)
PDI	Bovine liver	Glutathione	7.5		4.2×10^{-5}
Thioredoxin	<i>E. coli</i>	NADPH/NADP ⁺	7.0	48 (1)	2* (2)
		Glutathione	8.7		10 (3)
RNAase A	Bovine pancreas	DTT	8.7	1.3×10^{-3} (4)	1.6*
First disulphides		DTT	8.7	0.4×10^{-3} (4)	0.48*
Second disulphides					
Trypsin inhibitor	Bovine pancreas	DTT	8.7	3.8×10^{-2} (5)	46*
First disulphides		Glutathione	8.7		1.3 (6)
Second disulphides		Glutathione	8.7		0.15 (6)
Third disulphide (Cys-14-Cys-38)		Glutathione	8.7		187 (6)
Fatty acid synthase	Chicken liver	Glutathione	8.0		1.5×10^{-2} (2)
Hydroxymethylglutaryl-CoA reductase	Rat liver	Glutathione	7.1		0.55 (7)
Fructose-1,6-bisphosphatase	Spinach chloroplasts	DTT	8.0	0.12 (8)	140*
		DTT	8.0	0.39 (8)	470*

the higher concentration of 100 mM in order to inactivate free thiol groups at equilibrium rapidly. The mixtures of oxidized and reduced DTT contained a maximum concentration of 1.0 mM reduced DTT in a total thiol concentration of 150 mM. The residual activity, representing unmodified oxidized PDI at equilibrium, was always less than 1%. PDI was fully reduced at equilibrium even when the ratio of reduced to oxidized DTT was as low as 1:1500; assuming a maximum of 1% oxidized PDI at equilibrium at this ratio, the equilibrium constant must be below 6.7×10^{-6} . This result is understandable when the equilibrium constant for PDI/DTT is now derived from that accurately determined for PDI/glutathione and the literature value for glutathione/DTT (Creighton, 1986), using the relationship:

$$K_{(\text{PDI/DTT})} = \frac{K_{(\text{PDI/glutathione})}}{K_{(\text{glutathione/DTT})}}$$

Two values for the equilibrium constant for glutathione/DTT at pH 7.0 (Creighton, 1986) give an average value of 11×10^3 M; the derived value for the equilibrium constant for PDI/DTT is 3.6×10^{-9} M. The midpoint of PDI reduction at equilibrium would be reached with an excess of oxidized DTT over reduced DTT of more than 10^8 , a ratio impossible to achieve experimentally.

These two preliminary results are therefore consistent with the value for $K_{\text{eq.}}$ obtained in the experiment described here in detail, which took several careful precautions, i.e. the total glutathione content of the equilibrium mixture was kept constant, PDI was incubated long enough to reach equilibrium, reduced PDI at

equilibrium was inactivated rapidly, oxidized PDI was shown to form intra-molecular, not mixed, disulphide bonds, and the derivation of $K_{\text{eq.}}$ showed a negligible effect of GSH oxidation during equilibration.

The standard redox potential for the PDI active-site disulphide groups, derived from this equilibrium constant, was -0.11 V. This potential is considerably less negative than those of familiar thiol/disulphide and dithiol/disulphide couples, e.g. those of cysteine/cystine and GSH/GSSG are in the range -0.22 to -0.24 V, close to that of riboflavin, and those of dihydro-lipoate/lipoate and reduced/oxidized DTT are both -0.32 V, equal to that of the nicotinamide cofactors (Jocelyn, 1972; Szajewski & Whitesides, 1980). The standard redox potential of thioredoxin is -0.26 V (Holmgren, 1968), intermediate between the values for the mono- and di-thiols quoted here. Thus PDI is a significantly weaker reductant than all these couples, and conversely a stronger oxidant. Recently a direct equilibration of thioredoxin with PDI showed thioredoxin to be significantly the stronger reductant (Lundström & Holmgren, 1990).

It is instructive to compare the state of the redox equilibria of PDI and thioredoxin when equilibrated with a mixture of GSH and GSSG at concentrations present in the cytoplasm. Literature data (Meister, 1988) suggest that typical concentrations are $[\text{GSH}] = 10$ mM and $[\text{GSSG}] = 0.02$ mM; when equilibrated with these concentrations of glutathione, thioredoxin would be approximately equally distributed between reduced and oxidized forms (30% oxidized) whereas PDI would effectively be completely reduced (less than 0.001% oxidized). It is also instructive

to compare the PDI and thioredoxin redox properties with those of structural disulphide bonds in proteins. Equilibrium constants for protein disulphide groups with the GSH/GSSG redox couple in various conditions are listed in Table 1. The strongest disulphide bond listed (strongest reductant in the dithiol state, highest value for K_{eq} with GSH and GSSG) is one of those in bovine pancreatic trypsin inhibitor ($K_{eq} = 187$ M) and the weakest is that of fatty acid synthase (15 mM), apart from that determined here for PDI (42 μ M). Thus the standard redox potential of the PDI active site is more oxidizing than that of any of the protein disulphide groups listed here or more comprehensively elsewhere (Gilbert, 1990).

PDI co-operativity and reaction with bifunctional reagent

The determination of K_{eq} required a series of incubations of PDI with different proportions of GSH and GSSG. The results showed co-operativity on a Hill plot (Fig. 3) and fitted a sigmoidal curve based on $K_{eq} = 53$ μ M with a Hill coefficient equal to 1.5 (Fig. 1). This co-operativity could reflect interactions between the two active sites on each monomer (Edman *et al.*, 1985) and/or between the four active sites of the homodimer in solution (Lambert & Freedman, 1983). Distances between these active sites were investigated by incubating PDI with the thiol-specific bifunctional reagent bis-IAM containing spacer chains of different lengths between the two reactive groups. Analysis by SDS/PAGE showed that PDI preferentially formed intra-chain linkages with no clear linkages between monomers (Fig. 4).

Intra-molecular linkages with bis-IAM were formed with cross-linkers containing three to ten methylene groups, corresponding to total chain lengths of 1.3–2.2 nm (13–22 Å) (Fig. 4). The extent of linkage was affected by chain length; there was little with three or four spacer groups (lanes 5 and 6) but considerably more with five [length 1.6 nm (16 Å), lane 4]. These results suggest that the two active sites on the monomer are at least 1.6 nm (16 Å) apart and are separated from those on the other subunit by more than 2.2 nm (22 Å).

Redox environment of the lumen of the endoplasmic reticulum

Very little is known about the redox potential effective within the lumen of the endoplasmic reticulum, or about the factors that determine it. Within the cytoplasm, the thiol/disulphide redox properties are dominated by the relatively high concentration of GSH, and the GSH/GSSG redox potential is linked to that of the nicotinamide nucleotides via glutathione reductase. A distinct pool of glutathione has been identified within mitochondria (Meredith & Reed, 1982), but it is not known whether glutathione is found within the endoplasmic-reticulum lumen. The permeability properties of the endoplasmic-reticulum membrane are such that it would not be freely permeable to GSH or GSSG on account of their size and charge (Nilsson *et al.*, 1973), and no specific translocators for these species have been identified. In general, the endoplasmic-reticulum lumen is thought to provide a more oxidizing environment than that of the cytoplasm (Isaacs & Binkley, 1977), and the redox properties of PDI determined here are consistent with this interpretation. Thus, if the redox potential were identical with that of the cytoplasm, many secretory-protein disulphide groups would be thermodynamically unstable and the equilibrium redox state of PDI would be almost completely reduced; these would not be favourable conditions, kinetically or thermodynamically, for PDI to catalyse native protein disulphide formation by interconverting between the dithiol, the disulphide and the mixed disulphide state. In studies *in vitro*, formation of protein disulphide groups occurs in nascent secretory proteins translated in the presence of microsomal vesicles, provided that excess reduced DTT is absent from the translation system (Scheele & Jacoby, 1982, 1983; Kaderbhai

et al., 1984) and provided that PDI is present in the microsomal interior (Bulleid & Freedman, 1988).

This sharpens the question of the effective redox potential within the endoplasmic-reticulum lumen of whole cells and how it is maintained. Overall, the formation of disulphide groups in a nascent protein is an oxidation. Ziegler & Poulsen (1977) proposed the existence of a specific cystamine oxidase that would generate cystamine within the endoplasmic-reticulum lumen, so that an oxidizing cystamine/cysteamine couple would exist there and provide the net oxidant for the formation of protein disulphide groups without equilibrating with the cytosolic GSH/GSSG coupled; however, little direct evidence has emerged in favour of this attractive hypothesis. Another possibility is that oxidized forms of vitamin K within the endoplasmic-reticulum membrane may provide a local distinct source of oxidizing equivalents (Vermeer, 1990); the vitamin K redox cycle is known to occur in the endoplasmic reticulum of many tissues and to proceed uncoupled from the γ -carboxylation of glutamic acid residues with which it is generally associated (Suttie, 1985; Vermeer, 1990). Quinone standard redox potentials are in the region of +0.10 V, distinctly more oxidizing than that of the PDI active site, so that the transfer of oxidizing equivalents is thermodynamically plausible from O_2 to a quinone to PDI to a nascent protein. However, these remain areas of speculation. The overall oxidant for the formation of protein disulphide groups and the dominant redox components within the endoplasmic-reticulum lumen, other than PDI itself, remain to be identified.

Note added in proof (received 14 February 1991)

In a very recent publication an independent estimate has been made of the equilibrium constant between the active-site disulphide groups of PDI and the GSH/GSSG redox couple by means of an entirely different method (Lyles & Gilbert, 1991). The value obtained was 0.06 mM, very close to that found here, confirming that the redox potential of the PDI active sites is significantly more oxidizing than that of thioredoxin.

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