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The reactivities and ionization properties of the active-site dithiol groups of mammalian protein disulphide-isomerase

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1. The number of reactive thiol groups in mammalian liver protein disulphide-isomerase (PDI) in various conditions was investigated by alkylation with iodo[^{14}C]acetate. 2. Both the native enzyme, as isolated, and the urea-denatured enzyme contained negligible reactive thiol groups; the enzyme reduced with dithiothreitol contained two groups reactive towards iodoacetic acid at pH 7.5, and up to five reactive groups were detectable in the reduced denatured enzyme. 3. Modification of the two reactive groups in the reduced native enzyme led to complete inactivation, and the relationship between the loss of activity and the extent of modification was approximately linear. 4. Inactivation of PDI by alkylation of the reduced enzyme followed pseudo-first-order kinetics; a plot of the pH-dependence of the second-order rate constant for inactivation indicated that the essential reactive groups had a pK of 6.7 and a limiting second-order rate constant at high pH of 11 $M^{-1} \cdot s^{-1}$. 5. Since sequence data on PDI show the presence within the polypeptide of two regions closely similar to thioredoxin, the data strongly indicate that these regions are chemically and functionally equivalent to thioredoxin. 6. The activity of PDI in thiol/disulphide interchange derives from the presence of vicinal dithiol groups in which one thiol group of each pair has an unusually low pK and high nucleophilic reactivity at physiological pH.

INTRODUCTION

Protein disulphide-isomerase (PDI) is a major component of the luminal content of secretory cells and is believed to function there as a catalyst of the formation of native disulphide bonds in newly synthesized extracellular proteins (for reviews see Freedman, 1984; Freedman *et al.*, 1989). The enzyme has been purified from mammalian liver; it is active towards a wide variety of protein substrates and catalyses oxidation and folding of reduced denatured proteins, reduction of protein disulphides by thiol compounds and, in appropriate conditions, isomerization of disulphide bonds in proteins (Anfinsen, 1973; Freedman *et al.*, 1989). The isomerization reaction, as in the standard assay with incorrectly disulphide-bonded ('scrambled') bovine pancreatic RNAase (SRNAase), requires either that the PDI is first reduced by a thiol or dithiol reductant or that a low concentration of such a reductant is present during the incubation with the 'scrambled' substrate. These are not required in reactions where PDI acts on a reduced protein substrate (Givol *et al.*, 1965; Fuchs *et al.*, 1967).

These findings imply that PDI contains reducible disulphide groups that participate in the catalytic process. This was also indicated by early studies which showed that PDI was inhibited by arsenite or Cd^{2+}, diagnostic inhibitors of enzymes with active-site dithiol groups (Ramakrishna Kurup *et al.*, 1966; Hillson & Freedman, 1980), and that it was inactivated by classical alkylating agents, but only after reduction (Fuchs *et al.*, 1967; Lambert & Freedman, 1984). These findings suggest that PDI contains one or more disulphide groups that, on reduction, generate thiol groups that are essential for the enzyme's activity. de Lorenzo *et al.* (1966) identified an essential cysteine residue in the sequence -Cys(Gly,His)-. Models for the involvement of thiol or dithiol groups in the action of PDI were proposed by Creighton *et al.* (1980).

The proposal that the action of PDI is dependent on vicinal dithiol group(s) (Ramakrishna Kurup *et al.*, 1966; Hillson & Freedman, 1980) was lent further weight by the determination of the sequence of rat PDI (Edman *et al.*, 1985). This showed that PDI contains two regions of sequence closely similar to that of the small protein thioredoxin, which catalyses disulphide reductions and participates in other redox processes (Holmgren, 1985) and contains a single essential dithiol/disulphide couple formed by cysteine residues in the sequence -Trp-Cys-Gly-Pro-Cys-Lys-. In all PDI sequences available to date (Parkkonen *et al.*, 1988) the similar sequence -Trp-Cys-Gly-His-Cys-Lys- appears twice, each time in a region of about 100 residues that shows 30% identity with *Escherichia coli* thioredoxin (Edman *et al.*, 1985; Freedman *et al.*, 1988). It appears highly likely that the two pairs of cysteine residues in PDI in positions analogous to the essential dithiol/disulphide couple in thioredoxin comprise the active-site groups of PDI.

Recent developments in cell biology have led to considerable interest in proteins that facilitate the folding and assembly of newly synthesized proteins (Rothman, 1989; Ellis, 1990). PDI is a protein for which such a cellular role is highly probable (Bulleid & Freedman, 1988; Freedman *et al.*, 1989), but it is also an enzyme with a well-defined activity. In order to understand better the active site of PDI, and hence to define its action as a catalyst of protein folding, we have undertaken further characterization of the properties of the cysteine residues of PDI. Part of this work has been reported previously in preliminary form (Hawkins *et al.*, 1986; Freedman *et al.*, 1988).

MATERIALS AND METHODS

Materials

Tris was obtained from Boehringer Mannheim, scintillation liquids PCS from Hopkin and Williams and Optiphase T from LKB, SRNAase from Miles Laboratories (no longer available) and iodo[2-^{14}C]acetic acid from Amersham International. Sigma Chemical Co. supplied Brilliant Blue G, dithiothreitol (DTT), BSA (98–99% pure), iodoacetic acid (IAA) and iodoacetamide

Abbreviations used: [^{14}C]IAA, iodo[2-^{14}C]acetic acid; DTT, dithiothreitol; IAA, iodoacetic acid; IAM, iodoacetamide; NEM, *N*-ethylmaleimide; PDI, protein disulphide-isomerase (EC 5.3.4.1); SRNAse, 'scrambled' RNAase.

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(IAM). All other chemicals of AnalaR grade were obtained from BDH Chemicals.

Purification of PDI

PDI was purified to homogeneity from bovine or rat liver as described by Lambert & Freedman (1983*a*) and Mills *et al.* (1983).

Assay of protein concentration

Protein was assayed with the use of Coomassie Blue, based on Bradford's (1976) original procedure. The modification described by Sedmak & Grossberg (1977) was used for bovine liver PDI with a standard curve based on BSA. Rat liver PDI was used at lower concentrations, and so was assayed with the more sensitive modification described by Read & Northcote (1981), using a standard curve based on purified rat liver PDI.

Assay of PDI activity

PDI was assayed by its re-activation of SRNAase; this substrate is fully oxidized but inactive owing to the presence of an undefined range of incorrectly formed disulphide bonds, and PDI catalyses the isomerization of these disulphide bonds to those found in the native active enzyme. The assay is described in detail in Hawkins *et al.* (1991*b*), including redefined units of PDI activity as μmol of substrate re-activated/min. Assays of purified rat liver PDI used a commercial preparation of SRNAase with K_m 2 μM and $V_{max.}$ 5.4 μmol/min per g of PDI, equivalent to $V_{max.}$ 1000 'old' units/g of PDI (Lambert & Freedman, 1983*b*). When this preparation became unavailable, assays of purified bovine liver PDI used SRNAase prepared by the procedure described in Hawkins *et al.* (1991*b*) with K_m 5.1 μM and $V_{max.}$ 3.4 μmol/min per g of PDI. Since the recorded activity would vary according to the kinetic properties of the SRNAase substrate, all PDI activities were expressed as a percentage of a control, which was either a parallel control incubation or the original activity before treatment.

Measurement of radioactivity

Radioactivity was counted in a liquid-scintillation counter (Beckman LS 7800) with the use of a standard counting programme for ^{14}C. The scintillant used for rat liver PDI samples was PCS, and that for bovine liver PDI samples was Optiphase T. A minimum 10000 c.p.m. was recorded to give results within 2σ percentage error, and all incubations were assayed in triplicate.

Extent of alkylation and inactivation of PDI

Homogeneous rat liver PDI (2 μM) was reduced by preincubation with excess DTT (50 μM) in 1.0 ml of 50 mM-sodium phosphate buffer, pH 7.5, at 20 °C for 10 min. A further excess of [^{14}C]IAA was then added (final concentration 500 μM; 1.4 Ci/mol) and the incubation was continued at 0 °C for different lengths of time from 5 min to 24 h. The reaction mixture was then dialysed overnight at 4 °C against buffer containing 50 mM-Tris/25 mM-KCl/5 mM-$MgCl_2$, pH 7.5, and the recovered PDI was assayed for protein, enzyme activity (expressed relative to control incubation in buffer alone) and incorporated radioactivity. Parallel incubations omitted DTT. These incubations were repeated in the presence of 8 M-urea and kept at 20 °C throughout. Some incubations were duplicated.

A further set of incubations used purified bovine liver PDI (5 μM) with [^{14}C]IAA (6.3 Ci/mol) at a final concentration of either 50 μM or 200 μM.

Rate of inactivation of PDI by alkylation over a range of pH

Homogeneous bovine liver PDI (1.25 μM) was reduced by preincubation with excess DTT (25 μM) in 50 mM-sodium phosphate buffer, pH 5.7, at 20 °C for 5 min. Enzyme activity was determined by diluting samples directly into the assay. Four samples were assayed in order to given an accurate value for 100% activity before alkylation began, i.e. at $t = 0$. A parallel preincubation mixture was cooled to 0 °C, then IAM was added (final concentration 250 μM), and the incubation was continued at 0 °C. Duplicate samples were removed and assayed for PDI activity at 30 min intervals up to 150 min. Dilution of each sample into the assay lowered the rate of alkylation sufficiently so that no further inactivation was detectable during the assay. The diluted IAM did not itself affect the assay. A semi-logarithmic plot [log(% of original activity) versus time] indicated pseudo-first-order kinetics of PDI inactivation; the pseudo-first-order rate constant for inactivation ($k_{app.}$) was derived from the plot and hence a second-order rate constant could be calculated ($k_2 = k_{app.}/[IAM]$ $M^{-1} \cdot s^{-1}$).

The rate of inactivation was determined over a range of pH from 5.7 to 8.5 (measured at 0 °C), with a change of buffer from phosphate to Tris at pH 8.5 only, and PDI activity was assayed at four or five time points at each pH. As the rate of inactivation increased at higher pH, assays were required at time intervals shorter than the 20 min duration of the assay and so were obtained from a series of parallel incubations instead of the same incubation throughout. The scatter introduced into the graph by this modification was resolved by determining the pseudo-first-order rate constant for each time point individually and averaging the results. For consistency, this treatment of the results was repeated for each pH.

PDI activity at high pH

Homogeneous bovine liver PDI (5.3 μM) was reduced by incubation with excess DTT (25 μM) in 50 mM-Tris/HCl buffer, pH 8.5, at 20 °C for 5 min and was then cooled to 0 °C. Duplicate samples were removed and assayed for PDI activity after a total incubation time of 10 min and 40 min. PDI was also incubated similarly without DTT and assayed after 40 min only. Control assays before incubation represented 100% activity. The assay was modified for reduced PDI; the samples already contained DTT and so the routine preincubation was DTT alone was omitted before addition of SRNAase. The incubations were repeated in Tris/HCl buffer at pH 8.9 and 9.4; all three pH values were measured at 0 °C.

RESULTS

Extent of alkylation of PDI by IAA

Vertebrate PDI sequences indicate the presence of six cysteine residues. Each of the two regions similar in sequence to thioredoxin contains a pair of neighbouring cysteine residues; two further residues are Cys-294 and Cys-325 in the b' domain.

PDI was treated with radiolabelled IAA in order to determine the number of free thiol groups exposed under various conditions, i.e. in the presence or in the absence of reductant, and in the presence or in the absence of denaturant. Purified PDI was incubated at pH 7.5 with 250-fold molar excess of [^{14}C]IAA, dialysed, and assayed for protein and ^{14}C incorporation. Very little radioactivity was incorporated; even after incubation for 24 h, significantly less than one carboxymethyl group per PDI polypeptide was incorporated (Table 1). When 8 M-urea was included in the incubation, a similar result was obtained for the denatured protein. These results suggest that the six cysteine residues of PDI form three disulphide bonds in the protein as isolated; the low degree of ^{14}C incorporation would be explained by non-specific modification of other amino acid residues (Creighton, 1984). Molecular O_2 was not excluded at any stage

Table 1. Extent of modification of PDI by [^{14}C]IAA in various conditions

Homogeneous rat liver PDI (2 μM) was incubated with or without DTT (50 μM) at pH 7.5 for 10 min at 20 °C. Then [^{14}C]IAA was added (final concentration 500 μM) and the incubation continued at 0 °C for 5 min, 6 h and 24 h. After dialysis overnight, the modified PDI was assayed for protein and ^{14}C incorporation. Parallel incubations in the presence of 8 M-urea were incubated at 20 °C throughout.

Length of incubation	Incorporation of [^{14}C]IAA (mol/mol of PDI polypeptide)			
	0 °C, no urea		0 °C, 8 M-urea	
	−DTT	+DTT	−DTT	+DTT
5 min	0.05	1.26	0.17	1.80
6 h	–	1.84	0.18	4.92
24 h	0.22	2.23	–	4.14

of the purification of PDI or during the labelling incubation, and these oxidizing conditions would favour disulphide formation.

When PDI was incubated first with 25-fold molar excess of DTT and then for 6–24 h with a further excess of [^{14}C]IAA, ^{14}C incorporation increased to 1.8–2.2 mol of IAA per PDI polypeptide in the absence of denaturant and to 4.1–4.9 mol of IAA in its presence. Thus reducing conditions exposed two cysteine residues reactive at pH 7.5 in the native protein, and a further two or three residues when it was denatured. The results do not show whether the effect of denaturation is to make further disulphide groups accessible to reduction or whether all three disulphide groups are reducible in the native protein but the thiol groups generated are not all accessible and/or reactive.

Alkylation and inactivation of PDI by IAA

The two reactive cysteine residues in the reduced protein might also be the residues essential for the enzyme's activity. Further work investigated this relationship by assaying the activity of PDI modified to various extents by alkylation with IAA.

The procedure was similar to the previous experiment, i.e. PDI was reduced by excess DTT at pH 7.5 and then incubated with a further excess of [^{14}C]IAA. After dialysis, samples were assayed for PDI activity as well as for protein and ^{14}C incorporation. Various extents of alkylation were obtained by varying the concentration of IAA and the time of incubation. The results for PDI purified from both rat and bovine liver show that, as ^{14}C incorporation increased, so also did the inactivation of PDI (Fig. 1). The enzyme was completely inactivated when it was alkylated by 2 mol of IAA/mol of PDI polypeptide. Therefore PDI activity absolutely requires the two cysteine residues that are reactive in the reduced protein at pH 7.5.

pH-dependence of rate of inactivation of PDI by IAM

Thiols are alkylated only as the thiolate ion, $-S^-$ (Lindley, 1960), so as the pH increases the two reactive cysteine residues in reduced PDI should be alkylated at a higher rate. The relationship established in Fig. 1 allowed inactivation to be used to monitor alkylation, so the variation with pH of the rate of inactivation of PDI was used to follow the pH-dependence of the reactivity of these cysteine residues. At several values of pH between 5.5 and 8.5, PDI was reduced by excess DTT and then incubated with a further excess of IAM. Samples were removed and assayed for PDI activity at four or five time points, and compared with the original activity before IAM was added. Semi-logarithmic plots of remaining activity against time of reaction were linear at each

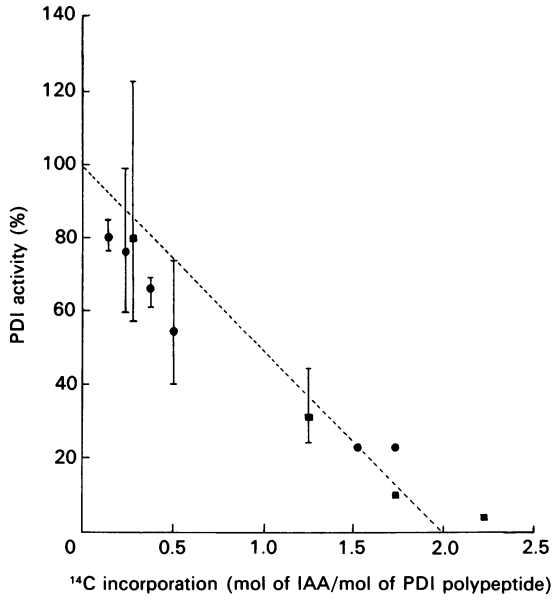

Fig. 1. Alkylation and inactivation of PDI

Homogeneous PDI (2–5 μM) was incubated with DTT (50 μM) at pH 7.5 for 10 min at 20 °C. Then [^{14}C]IAA was added (final concentration 50–500 μM) and the incubation continued at 0 °C for different lengths of time from 5 min to 24 h. After dialysis overnight, the modified PDI was assayed for protein, PDI activity and ^{14}C incorporation. PDI activity was expressed relative to control incubations in buffer alone, and ^{14}C incorporation as mol of IAA/mol of PDI polypeptide. Some incubations were duplicated. The source of PDI was bovine liver (●) or rat liver (■).

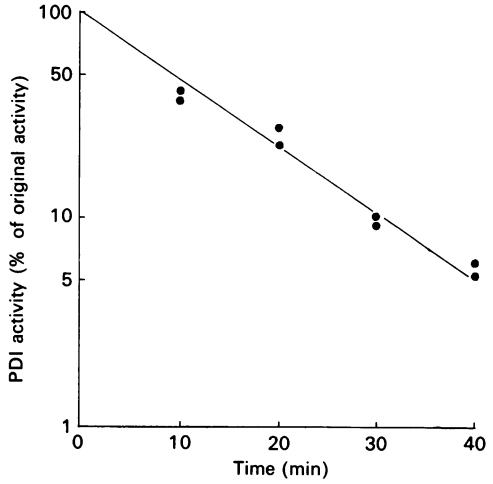

Fig. 2. Pseudo-first-order kinetics of PDI inactivation

Homogeneous bovine liver PDI (1.25 μM) was preincubated with DTT (25 μM) at pH 6.6 for 5 min at 20 °C, then cooled to 0 °C, and IAM was added (final concentration 250 μM). Duplicate samples were removed and assayed for PDI activity after 10 min and 30 min. A parallel incubation provided samples at 20 min and 40 min. The original activity (100 %) was determined by four assays after preincubation only. The line represents $k_{app.} = 7.7 \times 10^{-2} \pm 1.3 \times 10^{-2}$ min^{-1} (mean ± S.D.).

pH, giving single pseudo-first-order rate constants, as Fig. 2 shows for PDI inactivation at pH 6.6. The corresponding second-order rate constants were calculated and found to increase with pH as expected, giving a single pK of 6.7 (Fig. 3). Thus the

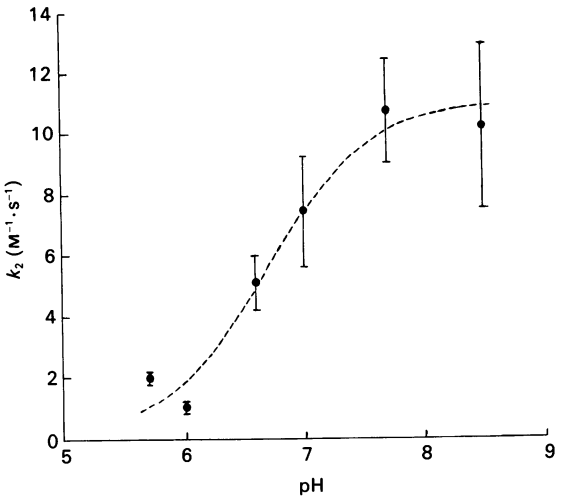

Fig. 3. Dependence on pH of rate of inactivation of bovine PDI by IAM

At each pH, the pseudo-first-order rate constant was determined by the procedure in Fig. 2, and used to calculate the second-order rate constant. The theoretical curve is based on $pK_a = 6.7$ and maximum $k_2 = 11.0\ M^{-1} \cdot s^{-1}$.

Table 2. Effect of high pH on PDI activity

Homogeneous bovine liver PDI (5.3 μM) was incubated with DTT (25 μM) at pH 8.5 for 5 min at 20 °C and then cooled to 0 °C. Duplicate samples were removed and assayed for PDI activity after a total incubation time of 10 min and 40 min. PDI was also incubated without DTT and assayed after 40 min only. Control assays before incubation represented 100% activity. The incubations were repeated at pH 8.9 and pH 9.4.

Conditions of incubation	Activity (% of original activity)		
	pH 8.5	pH 8.9	pH 9.4
No DTT, 40 min	98	99	102
+DTT, 10 min	104	33	4
+DTT, 40 min	102	4	0

essential reactive cysteine residues of PDI have a pK of 6.7 and react with IAM with a rate constant of 11 $M^{-1} \cdot s^{-1}$ when fully ionized.

The rate of inactivation by alkylation could not be determined at pH values higher than 8.5; at pH 8.9, PDI was gradually inactivated by preincubation with DTT alone, without addition of IAM. Protein thiol groups usually have a high pK, e.g. 8.6 for ficin (Holloway *et al.*, 1964), 8.8 for bovine pancreatic trypsin inhibitor (Creighton, 1975) and 9.8 for BSA (Tanford, 1962), but the possible presence of groups with this pK in PDI could not be investigated by this procedure.

Effect of high pH on PDI activity

The loss of activity in reduced PDI at pH greater than 8.5 was investigated further. At each pH, PDI was incubated with excess DTT and samples were removed and assayed for PDI activity after 10 min and 40 min; parallel incubations omitted DTT. Reduced PDI retained its original activity at pH 8.5, but retained only 4% after 40 min at pH 8.9 or after 10 min at pH 9.4; oxidized PDI remained stable throughout (Table 2). Therefore the loss of PDI activity was associated with its reduction and became more marked at higher pH. The chemical and structural properties of PDI inactivated by this high pH treatment have not been characterized.

DISCUSSION

The reactivity of PDI with the thiol-specific reagent [^{14}C]IAA confirmed earlier work (Fuchs *et al.*, 1967; Lambert & Freedman, 1984). Untreated PDI was not modified significantly, whereas reduced PDI contained two reactive groups per polypeptide (Table 1); the data of Fuchs *et al.* (1967) give the same result if recalculated on the basis of the current M_r for PDI of 57000 (Lambert & Freedman, 1983*a*; Edman *et al.*, 1985). In denaturing conditions, no reactive groups are detectable in the absence of prior reduction, but reduced denatured PDI contained up to five reactive groups. We have not directly demonstrated the identity of the groups modified in this work, but we assume that they are the thiol groups of cysteine residues (i) because of the known reaction preference of the reagent, (ii) because the modifications are only observed after treatment of the enzyme with reagents that reduce protein disulphides, (iii) because after alkylation of PDI in comparable conditions de Lorenzo *et al.* (1966) isolated a single modified peptide including the sequence carboxymethyl-Cys(Gly,His)- and (iv) because the maximum number of carboxymethyl groups incorporated is slightly less than the total number of cysteine residues per PDI polypeptide.

In previous work, we investigated the effects of alkylating agents on PDI activity (Lambert & Freedman, 1984). Incubation of PDI with IAA or *N*-ethylmaleimide (NEM) alone caused little inactivation, consistent with the low degree of alkylation (Table 1). However, after incubation of the enzyme with GSH or DTT, reaction with either IAA or NEM led to almost complete inactivation. These results were extended here by using [^{14}C]IAA to give a quantitative correlation of inactivation and extent of modification. Modification of the two groups that are reactive to IAA in reduced PDI at pH 7.5 led to complete inactivation. Thus PDI contains two essential thiol groups that are only available for modification in the reduced enzyme. The 'active-site' sequence -Cys(Gly,His)- detected by de Lorenzo *et al.* (1966) occurs twice in the full PDI sequence within the sequence -Trp-Cys-Gly-His-Cys-Lys-, which is duplicated within the polypeptide and highly conserved in PDI from various sources (Parkkonen *et al.*, 1988). We therefore conclude that the essential groups of PDI, whose exclusive modification at pH 7.5 leads to inactivation, are Cys-35 and Cys-379 respectively. The relationship between inactivation and extent of modification appears to be linear, implying that the two essential groups are independent, but this could not be established reliably because of the poor reproducibility in the determination of residual activity. The pH-dependence of the inactivation suggested that the essential groups of PDI have a pK of 6.7, unusually low for a thiol group. Furthermore these groups, like Cys-32 of thioredoxin, show high nucleophilic reactivity in the fully ionized state; compare the values of k_2 for the reduced protein and IAM of 11 $M^{-1} \cdot s^{-1}$ at 0 °C for PDI and 108 $M^{-1} \cdot s^{-1}$ at 25 °C for thioredoxin (Kallis & Holmgren, 1980) with 20 $M^{-1} \cdot s^{-1}$ at 25 °C for bovine pancreatic trypsin inhibitor (Creighton, 1975).

All these results can be interpreted in the light of comparable data for thioredoxin (Kallis & Holmgren, 1980). Native *E. coli* thioredoxin contains a single disulphide group, and is not readily alkylated in the presence or in the absence of denaturant. The disulphide of thioredoxin is reducible, but only one thiol group of the resultant dithiol is reactive towards alkylating agents at pH 7.5 in the absence of denaturant. The thioredoxin cysteine residues are Cys-32 and Cys-35 in the sequence -Trp-Cys-Gly-Pro-Cys-Lys-, and only Cys-32 is alkylated in these conditions; both react at higher pH or at pH 7.5 in the presence of denaturant.

Alkylation of Cys-32 inactivates thioredoxin by preventing it from interconverting between the dithiol and disulphide state. Studies of the rate of modification of Cys-32 and Cys-35 of thioredoxin as a function of pH show that Cys-32 has a pK of 6.7 whereas Cys-35 has the more typical thiol pK of 8.7.

The chemical properties of PDI thus show close similarities to those previously established for thioredoxin. We interpret the PDI data as follows. PDI, as isolated, contains three disulphide bonds, two of which are in domains similar to thioredoxin at positions analogous to the thioredoxin disulphide. When these disulphides are reduced to the dithiol state, the more *N*-terminal of each pair of cysteine residues has an unusually low pK, and hence is predominantly in the thiolate ($-S^-$) state at pH 7.5. Since it is the thiolate anion that is reactive as a nucleophile towards alkylating agents, the two cysteine residues of PDI that are analogous to Cys-32 of thioredoxin are exceptionally reactive at pH 7.5; little modification of other residues occurs. Since the modification of these two cysteine residues blocks the ability of the two dithiol groups to recycle to the disulphide state, the enzyme is completely inactivated by alkylation at these two sites.

The source of the unusually low pK of the essential thiol group of thioredoxin (Cys-32), and the analogous cysteine groups of PDI, has been the subject of some speculation. Holmgren (1985) proposed stabilization of the thiolate of thioredoxin Cys-32 by ionic interaction with the side chain of Lys-36; lysine residues are present in comparable positions adjacent to both active-site dithiol groups of PDI. However, there are no structural data to suggest that such an interaction occurs, and replacing this residue with glutamic acid in a mutant thioredoxin did not change its response to pH (Gleason *et al.*, 1990). An alternative source for stabilization of the anion might be interaction with the positive pole of the α-helix that runs from residues 32 to 49 (Hol, 1985). This theory is supported by the work of Katti *et al.* (1990), which refined more closely the three-dimensional structure of oxidized thioredoxin; the Cys-32 S atom in the active-site disulphide lies directly over the positive pole of this helix, but is more than 0.8 nm (8 Å) from Lys-36. Model-building studies on the PDI regions that are similar in sequence to thioredoxin show that these regions can fold to give domains closely similar in structure to thioredoxin (Freedman *et al.*, 1988). Hence the source of the unusually low pK is likely to be the same for both proteins.

Together, the modelling results and the data reported here on the reactivity of essential thiol groups in PDI suggest strong structural and functional similarity between PDI and thioredoxin. They imply that, to a first approximation, each of the thioredoxin-like regions of PDI can function as an independent oxidoreductase with an interconvertible dithiol/disulphide group. Since there are two such domains per PDI polypeptide, and since native PDI is a homodimer in solution, there are four such groups per active PDI molecule. The close structural and functional similarity between the thioredoxin and PDI active sites is further supported by the finding that the two active-site disulphide groups in PDI are reducible by bovine thioredoxin reductase (Lundström & Holmgren, 1990). Further studies will be required to picture the interaction between PDI, with multiple active sites, and substrate proteins, with multiple disulphide bonds. There is no information on the structural dispositions of the domains nor any data to show whether the active sites show processivity or co-operativity. In the following paper (Hawkins *et al.*, 1991*a*), we examine the redox potential of the essential dithiol/disulphide groups of PDI and report preliminary evidence for interactions between the multiple active sites of the PDI homodimer.

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