Determination of the Reduction–Oxidation Potential of the Thioredoxin-like Domains of Protein Disulfide-Isomerase from the Equilibrium with Glutathione and Thioredoxin†

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ABSTRACT: Protein disulfide-isomerase (PDI) contains two thioredoxin-like domains with the active-site sequence: Cys-Gly-His-Cys. Reduction of the two active-site disulfides in PDI by NADPH and bovine thioredoxin reductase was not reversible by addition of excess $NADP^+$, consistent with a redox potential (E_0') above −200 mV. Redox states of PDI and a mutated *Escherichia coli* thioredoxin, P34H Trx, were determined by quantitative analysis of cysteine residues by alkylation in equilibrium mixtures of oxidized and reduced forms of the two proteins. From the known E_0' of P34H Trx (−235 mV), an E_0' value of −190 ± 10 mV was calculated for PDI. Similarly, with defined redox buffers of glutathione, the redox-active dithiols in PDI were shown to have an equilibrium constant of 3 mM (E_0' = −175 ± 15 mV). The results showed that PDI has a high redox potential and therefore is a good oxidant of nascent protein thiols. Direct transfer of reducing equivalents from PDI to $NADP^+$ via thioredoxin reductase during protein disulfide formation seems unlikely due to the unfavorable equilibrium. The thioredoxin domains in PDI have a widely different redox potential compared with that of thioredoxin. A Pro to His exchange in the active site contributes to half of the change; the other half remains to be identified in the structure of PDI.

Disulfide formation in eukaryotic proteins takes place in the endoplasmic reticulum and is assumed to be catalyzed by the luminal enzyme, protein disulfide-isomerase (PDI[1]) (Freedman et al., 1989). This enzyme (M_r 57 000) contains two domains with vicinal cysteine residues in the sequence (Cys-Gly-His-Cys), which is similar to that of the active site of thioredoxins (Cys-Gly-Pro-Cys) [Edman et al., 1985; for review, see Holmgren (1989) and Eklund et al. (1991)]. Recent results with mutated PDI indicate that both active sites operate independently (Vouri et al., 1992). In addition, bovine PDI contains two additional cysteines outside the two dithiol active sites (Pihlajaniemi et al., 1987; Yamauchi et al., 1987; Toyoshima et al., 1987). A thiol–disulfide interchange reaction mechanism whereby PDI catalyzes the formation of disulfides from thiols in reduced polypeptides is implied. However, the detailed reaction mechanism and the ultimate electron acceptor of reaction 1 are as yet not known.

$$\text{protein-}(SH)_2 \xrightarrow{PDI} \text{protein-}S_2 + 2H^+ + 2e^- \qquad (1)$$

Hawkins et al. (1991) determined the reduction–oxidation (redox) potential of PDI using different redox buffers of GSH and GSSG. A value of −110 mV (K_{eq} = 42 μM) was obtained by measuring the activity of the enzyme after alkylation. This redox potential is higher than that of any known protein disulfide [for a review, see Gilbert (1989)] and is very different from that of *Escherichia coli* thioredoxin (K_{eq}(with glutathione) = 10 M, (Lin & Kim, 1989). Obviously this implies that PDI is a strong oxidant of nascent protein thiols. PDI can also catalyze disulfide reduction and isomerization depending on the initial substrates and surrounding redox potential (Freedman et al., 1984; Lundström & Holmgren, 1990; Lundström et al., 1991). In particular, PDI exhibits insulin disulfide reductase activity like thioredoxin in the presence of thioredoxin reductase and NADPH (Lundström & Holmgren, 1990). PDI also shows glutathione insulin transhydrogenase or oxidoreductase activity (Chandler & Varandani, 1975). In a linked assay, PDI transfers electrons and protons from GSH to insulin disulfides in the presence of a glutathione-regenerating system with glutathione reductase and NADPH.

In a previous study (Krause et al., 1991), we used site-directed mutagenesis to change the active site of *E. coli* thioredoxin to mimick PDI by replacing Pro 34 with His. The mutated thioredoxin (P34H Trx) had a redox potential 35 mV higher (−235 mV) than that of wild-type (wt) Trx (−270 mV). The equilibrium between oxidized PDI and reduced wt Trx at equimolar concentrations was previously estimated by intrinsic tryptophan fluorescence, and the result was incomplete (90%) oxidation of reduced thioredoxin corresponding to an equilibrium constant close to 100 (Lundström & Holmgren, 1990) and, thus, a redox potential higher than −200 mV (not −230 mV as given previously). This observation indicated that some thiols in PDI had a redox potential lower than −110 mV as reported by Hawkins et al., (1991). We have therefore studied the redox potential of PDI in equilibrium with the previously described P34H Trx, making use of its elevated E_0' (Krause et al., 1991), and in redox buffers of glutathione by measuring free SH groups in PDI after alkylation with iodoacetate.

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[1] PDI, protein disulfide-isomerase; Trx, *E. coli* thioredoxin; P34H Trx, *E. coli* thioredoxin with Pro 34 mutated to His; DTT, dithiothreitol; TR, thioredoxin reductase from calf thymus; Trx-S_2 and Trx-$(SH)_2$, oxidized and reduced thioredoxin; PDI-S_2 and PDI-$(SH)_2$, oxidized and reduced states of one thioredoxin-like active site in PDI; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); E_0', oxidation–reduction potential or redox potential; wt, wild type.

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MATERIALS AND METHODS

Materials. [^{14}C]- and [^{3}H]iodoacetic acid were purchased from Amersham (Sweden AB). Unlabeled sodium iodoacetate, guanidine hydrochloride, DTT, NADPH, NADP$^+$, and Na_2SeO_3 were from Sigma Chemical Co. (St. Louis, MO). GSH and GSSG were purchased from Boeringer Mannheim. NAP 5 desalting columns and FPLC equipment were from Pharmacia LKB Biotechnology, and microconcentrators (Centricon 10) were purchased from Amicon. All other chemicals were of analytical grade.

Radioactivity Determinations. Radioactivity was determined in a Packard Tri Carb liquid scintillation spectrometer. Less than 200 μL of sample was added to 3 mL of scintillation liquid (Instagel, Packard, Meriden, CT) and counted over 10 min with background subtraction.

Enzyme Preparations and Protein Concentration Determinations. The enzymes used in this investigation were prepared according to previously published procedures: *E. coli* P34H Trx and wt Trx, Krause et al. (1991); PDI, Lundström and Holmgren (1990); and thioredoxin reductase, Luthman and Holmgren (1982). Protein concentrations were determined spectrophotometrically by the absorbance at 280 nm with subtraction of the absorbance at 310 nm. Molar extinction coefficients for this corrected absorbance at 280 nm were 47 300 M^{-1} cm^{-1} for PDI and 13 700 M^{-1} cm^{-1} for wt and P34H Trx. The concentration of calf thymus thioredoxin reductase (M_r 116 000) was determined by activity in a DTNB reduction assay in which 1200 A_{412}/min in 1 mL was assumed to correspond to 1 mg of the enzyme, as described (Luthman & Holmgren, 1982).

Preparation of Fully Reduced PDI and P34H Trx. An aliquot (less than 100 μL) of either Trx or PDI stock solution was incubated in the presence of 10 mM DTT for 30 min at 37 °C. The reduced protein sample was then applied to a column of Sephadex G-25 (NAP 5, Pharmacia LKB Biotechnology), which previously had been equilibrated with degassed and N_2-equilibrated 0.10 M potassium phosphate (pH 7.0) and 1 mM EDTA. To avoid any contamination by DTT in the reduced protein, the column was eluted by slight modification of the manufacturer's recommended procedure: 100 μL of sample was applied and the column was eluted first with 400 μL of buffer followed by 600 μL in which reduced Trx or PDI was collected. The protein concentration in the reduced proteins was checked by absorbance readings at 280 nm and correlated with the thiol content, which was determined with DTNB (Ellman, 1959). Reduced PDI contained 5.7–5.8 SH groups per PDI molecule (M_r 57 000), and reduced P34H Trx contained 1.7–1.8 SH groups per molecule in all of the experiments. The reduced and desalted proteins were used within a few days, and the thiol content was determined daily to exclude oxidation. Both proteins were stable for several days at around 1 mg/mL in the presence of EDTA. Incorporation of radioactive iodoacetate into the fully reduced proteins gave the expected specific radioactivity for the fully reduced proteins.

Preparation of Stock Solutions of [^{14}C]- and [^{3}H]-Iodoacetate. Solid [^{14}C]- or, alternatively, [^{3}H]-labeled iodoacetic acid was dissolved in a solution containing 0.10 M potassium phosphate buffer (pH 7.0), 1 mM EDTA, and 0.50 M unlabeled sodium iodoacetate. The recovery of radioactive material from the ampule was checked by liquid scintillation, and the pH of the final solution was checked with indicator paper. To obtain labeled iodoacetate solutions with the appropriate specific activity, the radioactive stock solutions were diluted with potassium phosphate buffer containing 0.50 M unlabeled sodium iodoacetate. The specific activity of iodoacetate in the experiments was between 5×10^4 and 5×10^6 cpm/μmol.

Determination of Redox Potentials in Thioredoxin and PDI by NADPH/NADP$^+$ Equilibrium Assays. The equilibrium between NADPH and Trx or PDI was essentially studied as described previously (Krause et al., 1991). The concentration of calf thymus thioredoxin reductase was 74 nM, that of thioredoxin and P34H Trx was 15 μM, and that of PDI was 6.0 μM. The initial concentration of NADPH was 25 μM. After reduction of thioredoxin or PDI, the reaction was reversed by the addition of a high concentration of NADP (1 mM) to both the sample and the reference cuvettes. The redox potential of the equilibrium reaction was calculated according to the Nernst formula:

$$E_0'(\text{Trx-S}_2/\text{Trx-(SH)}_2) = E_0'(\text{NADP}^+/\text{NADPH}) + \frac{RT}{nf} \ln \frac{[\text{NADP}^+][\text{Trx-(SH)}_2]}{[\text{NADPH}][\text{Trx-S}_2]}$$

Reduction of disulfides in PDI by P34H Trx was performed by first reducing 9.3 μM P34H Trx by NADPH in the presence of a low concentration of *E. coli* thioredoxin reductase (10 nM) and then adding 5 μM PDI, as described previously (Lundström & Holmgren, 1990).

Preparation of Solutions of Reduced and Oxidized Glutathione. Reduced glutathione was dissolved in water and titrated to pH 5.0 with sodium hydroxide, and the concentration of GSH was determined with DTNB according to Ellman (1959). The concentration of oxidized glutathione in stock solutions of GSH and GSSG was determined with glutathione reductase and NADPH (Carlberg & Mannervik, 1985).

Alkylation of Equilibrium Mixtures and Separation of Alkylated Proteins. Defined amounts of oxidized or reduced PDI, wt Trx, P34H Trx, or GSH and GSSG were added to degassed, N_2-equilibrated 0.10 M potassium phosphate (pH 7.0) and 1 mM EDTA in a final volume of 100 μL. The redox buffers of glutathione were composed as follows, and a 1 M standard state was assumed: $-\log [GSH]^2/[GSSG] = 4.3$, 4.75×10^{-3} M GSSG, and 0.50×10^{-3} M GSH; $-\log [GSH]^2/[GSSG] = 3$, 1.00×10^{-3} M GSSG, and 1.00×10^{-3} M GSH; $-\log [GSH]^2/[GSSG] = 2$, 2.50×10^{-3} M GSSG, and 5.00×10^{-3} M GSH; $-\log [GSH]^2/[GSSG] = 1.2$, 0.63×10^{-3} M GSSG, and 6.25×10^{-3} M GSH; $-\log [GSH]^2/[GSSG] = 0.1$, 0.20×10^{-3} M GSSG, and 5.00×10^{-3} M GSH. After incubation for 20 or 30 min at 37 °C, the reaction mixtures were alkylated in guanidine hydrochloride using iodoacetate in excess of the thiols by the following procedure: 20 μL of 0.50 M radioactive iodoacetate was added first, followed by 120 μL of 6 M guanidine hydrochloride in 0.20 M Tris Cl buffer (pH 8.0). After 5 min at 25 °C, the sample was centrifuged for 10 min and then loaded on a 22-mL gel filtration column (FPLC, Superdex 75, Pharmacia LKB Biotechnology). The column was eluted with 0.10 M potassium phosphate buffer (pH 7.0), 1 mM EDTA, and 0.15 M NaCl at 0.5 mL/min with fractions of 0.5 mL. The elution profile was followed by absorbance at 278 nm for protein content, and the presence of radioactivity was determined in 100 μL of each fraction. The column was washed with approximately 2 column vol of buffer between each run to remove all radioactivity.

Determination of Redox State by Radioactivity and Absorbance. Fractions containing PDI or thioredoxin were

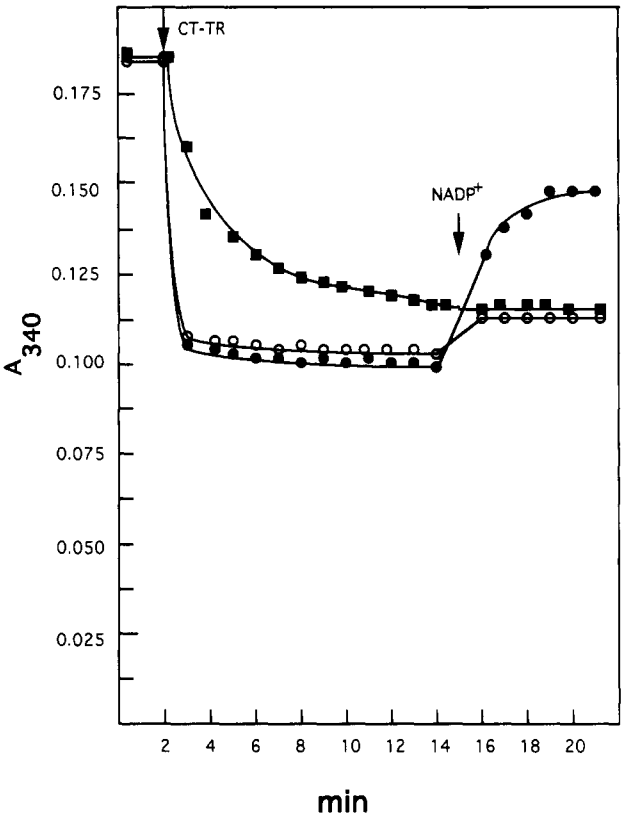

FIGURE 1: Equilibrium reactions between NADPH and thioredoxin and PDI: ●, 15 μM wt Trx; ○, 15 μM P34H Trx; ■, 6 μM PDI. NADPH (25 μM) was added and the concentration was followed by the absorbance at 340 nm. Calf thymus thioredoxin reductase (74 nM) was added as indicated by the arrow. After reduction of disulfides in Trx, P34H Trx, and PDI, excess $NADP^+$ (1 mM) was added and the formation of NADPH was recorded.

pooled separately and concentrated by centrifugation in Centricon 10 microconcentrators. After concentration, the absorbance at 280 nm was measured, and aliquots were taken to measure the radioactivity. To calculate the specific activity, the content of carboxymethylcysteine residues in the protein as determined by radioactivity measurements in cpm was divided by the absorbance value at 280 nm, which represented protein content.

RESULTS

Equilibrium between NADPH and PDI. The redox-active disulfides of oxidized PDI were completely reduced by near-stoichiometric amounts of NADPH in the presence of thioredoxin reductase from calf thymus:

$$\text{PDI-S}_2 + \text{NADPH} + \text{H}^+ \overset{\text{TR}}{\rightleftharpoons} \text{PDI-(SH)}_2 + \text{NADP}^+ \quad (2)$$

As seen from Figure 1, subsequent addition of excess $NADP^+$ to the cuvette (1 mM) did not reverse the reaction. Control experiments using wt Trx-S_2 and P34H Trx-S_2 gave the expected reversible reaction under the same conditions (Figure 1), demonstrating a different reactivity for PDI.

The fact that reaction 2 did not appear reversible could be caused either by a structural limitation of reduced PDI to act as a substrate for thioredoxin reductase or by an unfavorable reaction equilibrium due to a high redox potential of PDI. To rule out the first explanation, experiments were carried out by adding a catalytic amount (0.6 μM) of Trx to promote the establishment of a putative equilibrium; this did not change the result. The reaction was also not reversible at high pH values (up to pH 9). Taken together, these results indicate that the equilibrium of reaction 2 is shifted far to the right and that the redox potential of PDI is higher than that of P34H Trx (−235 mV). Since the E_0' value of the NADPH/$NADP^+$ couple is −315 mV (Gilbert, 1989), the estimated redox potential of PDI is greater than or equal to −200 mV from these experiments.

Reduced P34H Trx Can Reduce Oxidized PDI. The two disulfides in oxidized PDI are substrates for wt Trx-$(SH)_2$ (Lundström & Holmgren, 1990). To test whether this is also the case for P34H Trx, PDI was reduced by the complete thioredoxin system from *E. coli* as described (Lundström & Holmgren, 1990). The P34H Trx behaved like its wt counterpart (data not shown).

Redox Equilibrium between PDI and Thioredoxin. In previous experiments, we determined the redox state of wt Trx by measuring the tryptophan fluorescence and reported that equimolar initial concentrations of oxidized thioredoxin-like domains of PDI and reduced Trx resulted in at least 90% reduction of PDI. Here, we have determined the equilibrium between PDI and wt Trx (E_0' = −270 mV) or P34H Trx (E_0' = −235 mV) starting from both sides of reaction 3:

$$\text{PDI-S}_2 + \text{Trx-(SH)}_2 \rightleftharpoons \text{PDI-(SH)}_2 + \text{Trx-S}_2 \quad (3)$$

We determined the redox status of PDI and Trx in equilibrium mixtures by quantitative alkylation of free thiols with radioactive iodoacetate.

Several different concentrations of reduced or oxidized PDI were mixed with reduced or oxidized wt or P34H Trx and then incubated for 30 min at 37 °C. After incubation, the samples were alkylated with a final concentration of 40 mM [^{3}H]iodoacetate in 3 M guanidine hydrochloride for 5 min at 25 °C and centrifuged, and Trx was separated from PDI by gel filtration on a Superdex 75 FPLC column.

The elution profiles of equilibrium mixtures containing reduced PDI and one of the oxidized thioredoxins are shown in Figure 2. PDI concentrations refer to the concentration of thioredoxin-like domains in PDI. When the thioredoxins had been incubated with reduced PDI, more radioactivity could be detected in the P34H Trx peak as compared with wt Trx (Figure 2A,B). The amount of disulfide reduced in Trx by one concentration of PDI was also dependent on the amount of Trx (compare A with B and C with D in Figure 2), reflecting an equilibrium situation. If the reduction of Trx by one preparation of reduced PDI was dependent on contaminating amounts of DTT in the PDI preparation, the reduction of Trx would be stoichiometric and only depend on the amount of reduced PDI. In control experiments in the absence of reduced PDI (E and F), very little radioactivity was incorporated into either of the oxidized thioredoxins. Only the preparation of wt thioredoxin contained any measurable amount of radioactivity. On the basis of these data, we conclude that at least one dithiol in PDI is in a measurable equilibrium with Trx, and in particular with P34H Trx.

From a series of experiments with P34H Trx and PDI, we started reaction 3 from both sides and analyzed the redox state of the proteins at equilibrium in more detail. After gel filtration, the fractions containing PDI and P34H Trx were pooled separately and concentrated by centrifugation in microconcentrators. The protein concentration in each pool was then related to the radioactivity content, and this was taken as the equilibrium redox state of the proteins (Table I).

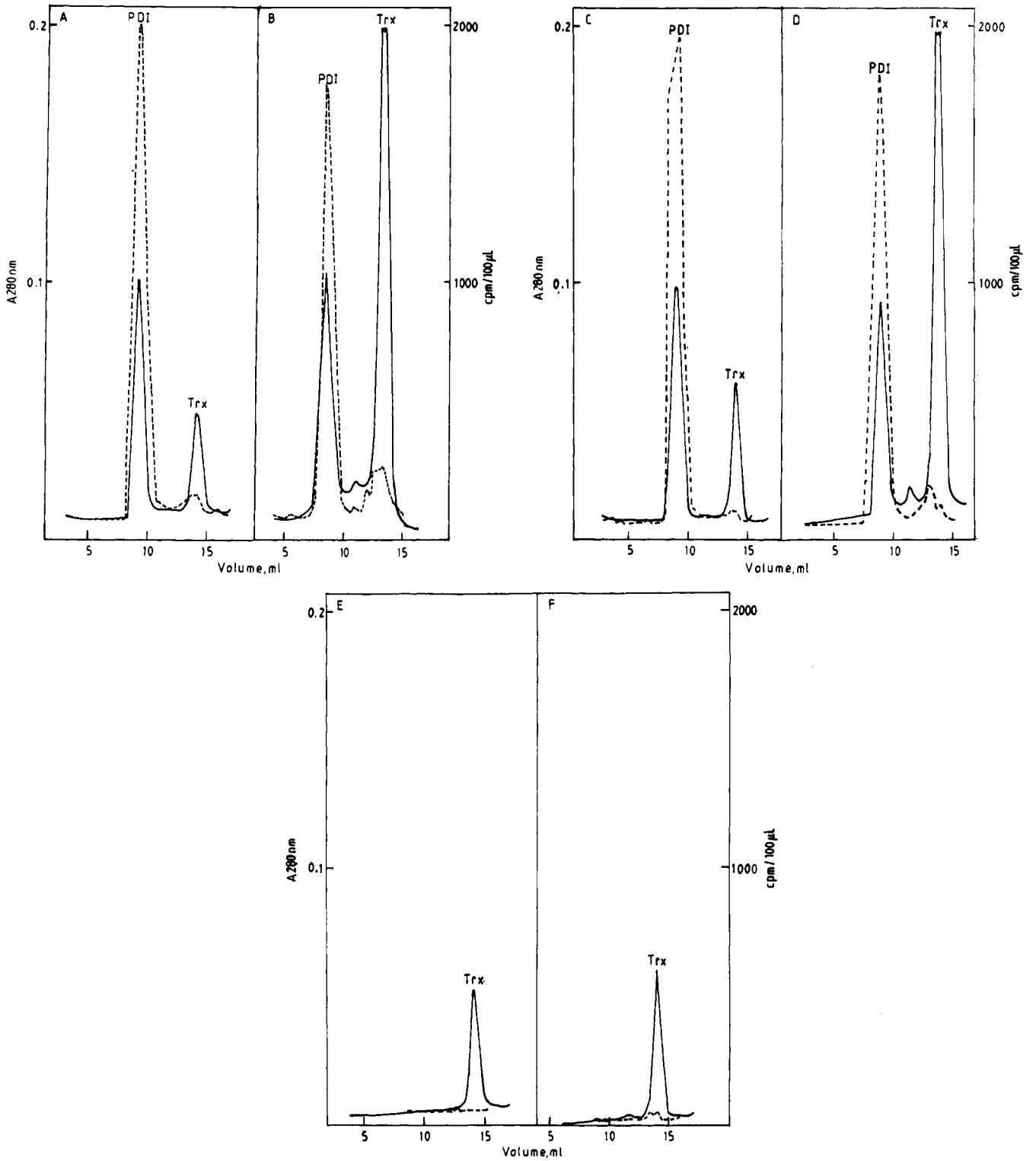

FIGURE 2: Elution profiles of equilibrium reaction mixtures of reduced PDI and oxidized thioredoxin after alkylation by iodoacetate: —, absorbance at 280 nm; - - -, radioactivity. The initial concentrations were (A) 85 μM $PDI(SH)_2$ and 85 μM P34H-S_2 Trx, (B) 85 μM $PDI(SH)_2$ and 273 μM P34H Trx-S_2, (C) 85 μM $PDI(SH)_2$ and 85 μM Trx-S_2, (D) 85 μM $PDI(SH)_2$ and 273 μM Trx-S_2, (E) 85 μM P34H Trx-S_2 only, and (F) 85 μM Trx-S_2 only. $PDI(SH)_2$ refers to thioredoxin-like domains of PDI.

The experiment described in Table I is typical for a large number of similar experiments using varied protein concentrations. There was good agreement between the results of equilibrium reactions starting from reduced PDI and oxidized thioredoxin and the results of reactions starting from the reverse side, but slight oxidation by oxygen in air could be observed as a decrease in the total amount of thiols before and after the incubation. The concentration of PDI-$(SH)_2$ (one thioredoxin-like domain of PDI) was calculated by subtracting the amount of radioactivity in "oxidized" PDI and then relating the remaining radioactivity to that of completely reduced PDI (see the paragraph about equilibrium between PDI and glutathione for further discussion about structural thiols). From the concentration of the reduced components, the amounts of PDI-S_2 and P34H Trx-S_2 were deduced. The equilibrium constant of reaction 3 was calculated for exper-

Table I: Redox State of PDI and P34H Trx at Equilibrium

	starting concentrations							
	200 μM P34H-$(SH)_2$	200 μM P34H-$(SH)_2$ and 200 μM PDI-$(S)_2$[c]		200 μM PDI-$(SH)_2$	200 μM P34H-S_2 and 200 μM PDI-$(SH)_2$		200 μM P34H-S_2 and 200 μM PDI-S_2	
redox state at equilibrium	P34H	PDI	P34H	PDI	PDI	P34H	PDI	P34H
cpm[d]/50 μL	679	848	135	2484	2472	94	482	29
A_{280}	0.041	0.063	0.056	0.156	0.174	0.059	0.084	0.052
cpm/A_{280}	16560	13460	2410	15923	14207	1593	5738	558
mol of SH/mol	1.8	4.7	0.25	5.6	5.0	0.17	2.0	0.06
% redn	100[a]	84	14.6	100[a]	89	9.6	34	3.4
[PDI-$(SH)_2$] (μM)		152		200	166		0[b]	
[P34H-$(SH)_2$] (μM)	200		29.2			19		0[b]

[a] Defined as 100% reduced P34H Trx or thioredoxin-like domains in PDI. [b] Defined as 100% oxidized P34H Trx or thioredoxin-like domains in PDI. [c] PDI-$(SH)_2$ and PDI-S_2 refer to thioredoxin-like domains in PDI. [d] The specific activity of the iodoacetate was 2700 cpm/nmol.

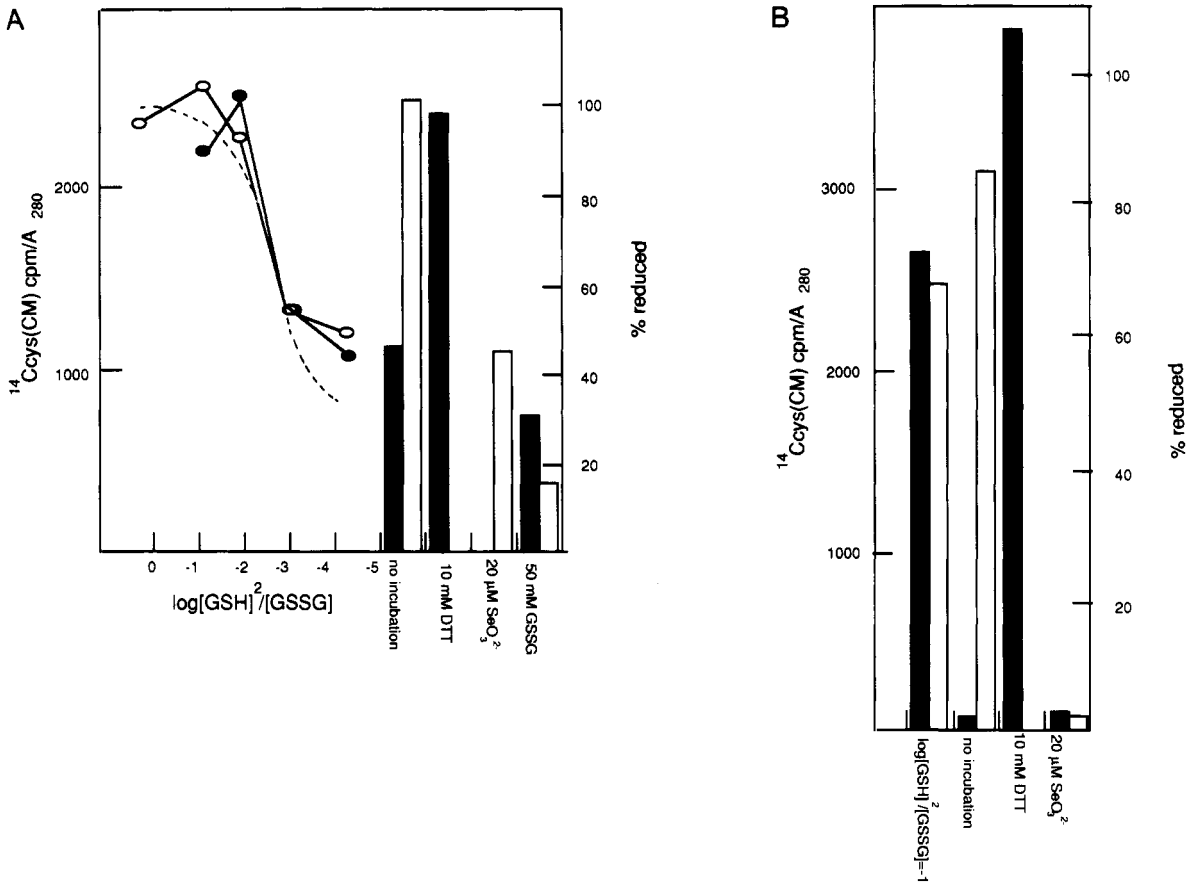

FIGURE 3: Redox state of PDI and P34H Trx thiols in different redox buffers of glutathione. After incubation of reduced and desalted proteins (open symbols) or oxidized proteins (filled symbols) for 20 min at 37 °C in different redox conditions, PDI (A) and P34H Trx (B) were alkylated by excess [^{14}C]iodoacetate. In A, a curve is drawn (- - -) assuming a midpoint titration value for [GSH]^2/GSSG of 3.1 mM and 6 mol of SH groups/mol of PDI in fully reduced protein and 2 SH groups in oxidized PDI. The alkylated proteins were separated on a column of Superdex 75, and the redox state of the proteins was determined by relating the incorporation of radioactivity to the protein concentration. The specific activity of the iodoacetate was 128 cpm/nmol, and 200 μL from each pool was counted.

iments that resulted in measurable equilibrium concentrations for all four components (see Table I):

$$K_{eq} = \frac{[\text{Trx-S}_2][\text{PDI-(SH)}_2]}{[\text{Trx-(SH)}_2][\text{PDI-S}_2]}$$

This gave a K_{eq} value for the reaction of 31 (n = 5, SD = 20) or an E_0' value for PDI of −190 ± 10 mV. There were no contradictory results in experiments where the reaction had essentially proceeded completely in one direction. In particular, when reduced PDI was incubated in the presence of a high concentration of P34H Trx-S_2, the equilibrium redox state of PDI was the same as in oxidized PDI.

Redox Equilibrium between PDI and Glutathione. The equilibrium between PDI and glutathione was studied in a manner similar to that used for the equilibrium between PDI and Trx. Reduced PDI or oxidized PDI at a final concentration of about 15 μM was incubated for 20 min at 37 °C in different redox buffers of GSH and GSSG. The equilibrium mixtures containing 100 μL were alkylated by the addition of 20 μL of 0.50 M [^{14}C]iodoacetate followed by 120 mL of 6 M guanidine hydrochloride in 0.20 M Tris (pH 8.0). Under these conditions, iodoacetate is present in at least 20-fold excess over reduced glutathione and more than 100-fold excess over PDI thiols. After incubation for 5 min at 25 °C and centrifugation for 10 min, the samples were applied to the column of Superdex 75, and the alkylated PDI was collected

and analyzed for absorbance and radioactivity.

In Figure 3A, the redox state of PDI is plotted against log $[GSH]^2/[GSSG]$ for the glutathione redox buffer, assuming a dithiol reaction mechanism. It is apparent from this figure that about 40% of PDI thiols remained in the reduced state in the most oxidizing redox buffer used. Only incubation of PDI in the presence of 50 mM GSSG partially protected these thiols from alkylation (staples). The results are equilibrium values since the redox state of PDI was independent of whether the starting material was reduced and desalted or oxidized PDI (Figure 3). Controls (staples) included in the figure are the following: proteins not incubated with GSH and GSSG; incubation with 10 mM DTT, which generates fully reduced protein; and incubation with 20 μM selenite, which leads to oxidation. Selenite is a nonstoichiometric oxidant of the active-site disulfide/dithiol of Trx (Kumar et al., 1992) and, as seen from these results, also of dithiols of PDI.

The redox titration curve in Figure 3A shows a midpoint titration value at log $[GSH]^2/[GSSG] = -2.5$ or 3.1 mM. Given an E_0' value of −250 mV (Gilbert, 1989; also see the Discussion) for the GSH/GSSG redox couple, and assuming a dithiol mechanism, this corresponds to an E_0 for PDI of −175 mV according to

$$E_0'(\text{PDI-S}_2/\text{PDI(SH)}_2) = E_0'(\text{GSSG/GSH}) + \frac{RT}{nF} \ln \frac{[\text{GSSG}][\text{PDI-(SH)}_2]}{[\text{GSH}][\text{PDI-S}_2]}$$

The redox state of PDI incubated in the redox buffer containing 4.75 mM GSSG and 0.5 mM GSH or $-\log [GSH]^2/[GSSG] = 4.3$ ($[GSH]^2/[GSSG] = 50$ μM), which according to Hawkins et al. (1991) would generate 50% active PDI after alkylation, was similar to the results for oxidized PDI. We believe that the E_0' value derived from the titration curve is that of the thioredoxin-like active sites of PDI. This conclusion is based on the following observations: (1) stock solutions of PDI, which were alkylated without incubation in any redox buffer, contained a carboxymethylcysteine level of about 40% of fully reduced PDI. We have shown previously (Lundström & Holmgren, 1990) that, as isolated, PDI contains two oxidized thioredoxin-like active sites, which are reducible with NADPH and thioredoxin reductase. This was confirmed for the preparation used in the experiments described here. The detection of thiols in oxidized PDI can only be explained by alkylation of two cysteine residues outside the thioredoxin-like active sites. In the absence of a denaturing agent, these cysteines were not accessible to DTNB, but a low degree of alkylation by iodoacetate could be detected (data not shown). (2) Reduced PDI, which was incubated with a low concentration of selenite, was isolated in the same redox state as oxidized PDI. This implies that selenite specifically oxidized the Trx-like active sites of PDI.

Equilibrium between P34H Trx and Glutathione. Thioredoxin (wt) from *E. coli* is difficult to reduce by glutathione (Lin & Kim, 1989) due to its low redox potential. The experiment shown in part B of Figure 3 is similar to that in part A, but P34H Trx was analyzed instead. A redox buffer of glutathione, where $[GSH]^2/[GSSG] = 0.1$ M, led to about 70% reduced P34H Trx at equilibrium and a redox potential for P34H Trx of −220 mV, which is slightly higher than the previously determined redox potential for P34H of −235 mV from the equilibrium with NADPH (Figure 3B). No radioactivity could be detected in reduced Trx after incubation with selenite, confirming that the oxidation of reduced Trx by selenite is nonstoichiometric and complete (Kumar et al., 1992).

DISCUSSION

In this work, we have determined the redox potential of PDI using two independent methods: (a) a study of the equilibrium between PDI and the dithiol/disulfide of the mutant thioredoxin P34H, and (b) a determination of the redox state of PDI in different redox buffers of the monothiol glutathione. Both methods depend on quantitative analysis of cysteine residues by alkylation with radioactive iodoacetate. The redox potential of P34H Trx (−235 mV) has been accurately determined by equilibrium with NADPH using thioredoxin reductase (Krause et al., 1991).

The results obtained by the first method showed that there is a redox equilibrium between PDI and P34H Trx. The mean value of the K_{eq} of the equilibrium between PDI and P34H Trx was 31, determined from both sides of the reaction. This value is 2–3 orders of magnitude lower than that would be expected if all dithiols/disulfides in PDI had E_0' values of −110 mV. In essence, any measurable reduction of P34H Trx (or wt Trx) by PDI is a clear indication of an E_0' for PDI lower than −170 mV ($K_{eq} = 150$). This is a considerably lower redox potential than the one reported (−110 mV) by Hawkins et al. (1991) and indirectly by Lyles and Gilbert (1991).

Our results also showed that PDI which has been incubated in a redox buffer of glutathione with an E_0' value of −120 mV contained two oxidized thioredoxin-like domains per PDI molecule. These two active-site disulfides titrated at −175 mV. The latter redox potential is slightly higher than the one calculated from the equilibrium reaction between PDI and P34H Trx, possibly because the two redox scales of a dithiol/disulfide and a monothiol-like glutathione may not perfectly agree. At least two of the six thiols in PDI were detected in the reduced state after incubation in all redox buffers used, but they could be partially protected from alkylation by iodoacetate after preincubation with a high concentration of GSSG, indicating a slow reactivity in the native protein. Experiments by Gilbert et al. (1991) resulted in 1.4 ± 0.3 and 1.8 ± 0.2 mol of SH/mol of PDI in recombinant material. In contrast, Hawkins and Freedman (1991) found 0.22 mol of SH/mol of PDI in their preparation.

Determination of the redox state of thiols in proteins by alkylating agents has been widely used, especially in the study of folding pathways of proteins [e.g., Creighton (1984, 1986)]. When an equilibrium situation is studied, one aims at alkylating all thiols involved at approximately the same rate to avoid a shift of the equilibrium. Weissman and Kim (1991) have pointed out the risk of using alkylating agents when trapping folding intermediates of bovine pancreatic trypsin inhibitor (BPTI), since the thiol–disulfide exchange in the unfolded protein is expected to occur at the same rate as the alkylation of thiols in the protein. However, their case is an intramolecular reaction with high local concentrations of thiols ($C_{eff} = 50$ mM), whereas we are studying bi- or trimolecular events. In our case, the alkylating agent was in at least 100-fold molar excess over protein thiols and at least 20-fold excess over glutathione. Iodoacetate was added first at 80 mM followed by the denaturing agent at pH 8.0. The alkylation reactions were performed under denaturing conditions to alkylate all thiols, and iodoacetate is known to be specific (Creighton, 1986).

The discrepancy in redox potential values of PDI obtained in this study and by others (Hawkins et al., 1991) can in part be explained by the use of different buffers, pH, temperature, and standard redox potentials of glutathione. By comparing

the equilibrium constants with glutathione directly, 3.1 mM and 42 μM, respectively, the difference in redox potential is 60 mV. A possible alternative explanation for the remaining 60 mV is a requirement for the reduced state of the "structural cysteines" in PDI for activity. Then, the involvement of any of these thiols in a mixed disulfide with glutathione, alkylation with iodoacetate, or formation of an intramolecular disulfide would change the activity and properties of PDI. We note that the PDI preparation used by Hawkins et al. (1991) contained no free SH groups, whereas our preparation contained 2 mol of SH/mol of PDI. Indeed, when stock solutions of our PDI preparation were incubated in a glutathione redox buffer, where $[GSH]^2/[GSSG] = 65$ μM, and then alkylated with iodoacetate in the absence of guanidine hydrochloride, the activity in a disulfide formation assay was significantly reduced (data not shown).

This does not explain the reported conformance to a true dithiol mechanism and cooperativity between active site (Hawkins et al., 1991). Results by Vuori et al. (1991) using site-directed mutagenesis indicated instead two equivalent and independent active sites in PDI. Another difference may be in the preparation of PDI. Sequence analysis of PDI prepared by the method of Lambert and Freedman (1983) suggested two species in the preparation as a result of partial cleavage in the C-terminal, which resulted in a 3-fold higher activity of the truncated protein (Hu & Isou, 1992). In effect, we have no simple explanation for the two different redox potentials obtained for PDI.

Lyles and Gilbert (1991) reported 50% of PDI activity in a glutathione redox buffer with a redox potential of −130 mV, but optimal PDI activity was found in a glutathione buffer of −180 mV (1 mM GSH and 0.2 mM GSSG). Thus, PDI activity is highest close to the redox potential of the two thioredoxin-like domains when they can undergo rapid thiol–disulfide interchange with the environment. A similar relation between reduced and oxidized glutathione was recently quantified in the endoplasmic reticulum ($E_0' = -164$ to −195 mV) (Hwang et al., 1992). This, taken together with our results, may provide an essential clue to the mechanism of PDI catalysis and to the nature of how PDI is reoxidized in the presence of a reducing polypeptide *in vivo*. Results regarding the role of the DsbA and DsbB proteins in disulfide bond formation *in vivo* in *E. coli* (Bardwell et al., 1991, 1993) presents an alternative hypothesis on how redox equivalents are transduced across the cytoplasmic membrane.

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