Catalysis of Thiol/Disulfide Exchange: Single-Turnover Reduction of Protein Disulfide-Isomerase by Glutathione and Catalysis of Peptide Disulfide Reduction†

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ABSTRACT: Protein disulfide-isomerase, a protein localized to the lumen of the endoplasmic reticulum of eukaryotic cells, catalyzes the posttranslational formation and rearrangment of protein disulfide bonds. As isolated from bovine liver, the enzyme contains 0.8 free sulfhydryl group per mole of protein monomer and 3.1 disulfide bonds. Single-turnover experiments in which the disulfide bonds of the native enzyme are reduced by glutathione reveal three distinct reduction steps corresponding to the sequential reduction of the three disulfide bonds. The fastest disulfide to be reduced undergoes a change in the rate-determining step with increasing GSH concentration from a step which is second-order with respect to GSH concentration to a step which is first-order in GSH concentration. The disulfide which is reduced at an intermediate rate displays kinetics that are first-order in GSH concentration, and the slowest disulfide to be reduced exhibits kinetics which are second-order in GSH concentration. The enzyme catalyzes the steady-state reduction of a disulfide-containing hexapeptide (CYIQNC) by GSH. Initial velocity kinetic experiments are consistent with a sequential addition of the substrates to the enzyme. Saturation behavior is not observed at high levels of both substrates (K_m for GSH $\gg$ 14 mM, K_m for CYIQNC $\gg$ 1 mM). Only one of the three disulfides appears to be kinetically competent in the steady-state reduction of CYIQNC by GSH. The second-order thiol/disulfide exchange reactions catalyzed by the enzyme are 400–6000-fold faster than the corresponding uncatalyzed reactions.

Protein disulfide-isomerase (PDI)[1] is a 56 800-kDa protein which appears to be located primarily in the lumen of the endoplasmic reticulum of eukaryotic cells (Freedman, 1984; Hillson et al., 1984). PDI is thought to catalyze thiol/disulfide exchange reactions involved in the posttranslational formation of disulfide bonds (Freedman, 1984; Hillson et al., 1984; Creighton et al., 1980). Recently, several very diverse functions have also been proposed for PDI. The protein has been identified as a thyroid hormone binding protein (Cheng et al., 1987), as the β-subunit of prolyl hydroxylase (Obata et al., 1988; Koivu et al., 1987), and as a component of the N-linked glycosylation apparatus of the Golgi (Geetha-Habib et al., 1988). The sequence of the protein is highly conserved; the rat (Edman et al., 1985) and human (Morris & Varandani, 1988) enzymes show 94% sequence identity.

Many disulfide-containing proteins spontaneously refold and oxidize to the native structure either in air or in the presence of thiol/disulfide redox buffers (Anfinsen & Scheraga, 1975; Saxena & Wetlaufer, 1970; Creighton, 1978); however, the in vitro attainment of the correct structure and biological activity is often slow (minutes to hours) compared to disulfide bond formation in vivo (minutes to seconds) (Bergman & Kuehl, 1979; Peters & Davidson, 1982). Consequently, a catalyst (PDI) is required to facilitate the correct formation of disulfide bonds in vivo.

Upon exposing a reduced, denatured protein to oxidizing agents, disulfide bonds are formed much more rapidly than structure and biological activity are recovered (Saxena & Wetlaufer, 1970; Hantgan et al., 1974). The rapid formation of disulfide bonds often precedes a slow rearrangement to the native disulfide-bonded structure (Creighton, 1978). Protein disulfide-isomerase was initially isolated by its ability to catalyze the rearrangment of incorrectly oxidized ribonuclease A to the native molecule (Epstein et al., 1963; Freedman, 1984; Hillson et al., 1984). The enzyme was also independently isolated by its ability to catalyze the GSH-dependent reduction of insulin (glutathione-insulin transhydrogenase activity) (Varandani, 1978) and by its ability to catalyze thiol/disulfide exchange reactions involving a variety of protein and nonprotein thiols and disulfides (thiol-protein oxidoreductase activty) (Morin et al., 1978). Subsequently, these three activities were shown to reside in the same protein (Bjelland et al., 1983). The protein is distinctly different from similar activities observed from cytoplasmic preparations (Mannervik & Axelsson, 1980).

Protein disulfide-isomerase is not a particularly effective catalyst, and rather high concentrations of the protein are required to observe catalytic activity (Morin et al., 1978; Jauhianien et al., 1987; Lang & Schmid, 1988). The broad specificity of the enzyme (Morin et al., 1978; Lambert & Freedman, 1983) and the lack of a well-defined substrate have hampered detailed kinetic and mechanistic studies. While examining PDI catalysis of thiol/disulfide exchange between glutathione and defined peptide disulfide substrates, we were surprised to find that PDI shows no evidence for saturation behavior with these substrates and that only one of the three disulfides of PDI participates in turnover, at least for reactions involving the model peptide examined. The potential mechanistic and physiological consequences of catalysis by PDI are discussed.

Experimental Procedures

Materials. Glutathione (GSH), NADPH, the peptide CYIQNC, and glutathione reductase (yeast, type IV) were

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[1] Abbreviations: PDI, protein disulfide-isomerase; CYIQNC, Cys-Tyr-Ile-Gln-Asn-Cys disulfide; GSH, glutathione; GSSG, glutathione disulfide; DTNB, 5,5′-dithiobis(2-nitrobenzoate); TNB^-, thionitrobenzoate; NEM, *N*-ethylmaleimide; DTT, dithiothreitol.

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obtained from Sigma. The peptide CYIQNC was >95% pure by C_{18} reversed-phase HPLC (linear gradient of 8/92/0.1 to 40/60/0.1 acetonitrile/water/trifluoroacetic acid over 30 min). Reagents for polyacrylamide gel electrophoresis were from Bio-Rad. DTT was from Boehringer Mannheim. All other reagents were analytical reagent grade or better. Protein disulfide-isomerase was prepared from bovine liver by the method of Hillson et al. (1984). The purified protein was >95% homogeneous on SDS–polyacryamide gel electrophoresis. When assayed at pH 7.5, 25 °C (0.2 M potassium phosphate buffer with 2 mM EDTA), using 3.7 mM GSH and 35–70 μM bovine insulin, the specific activity was 0.11 μmol of GSSG formed min^{-1} (mg of protein)$^{-1}$. At a GSH concentration of 7.4 mM, the specific activity was 0.2 μmol of GSSG formed min^{-1} (mg of protein)$^{-1}$. This is comparable to the activity of 0.2 μmol of GSSG min^{-1} mg^{-1} observed by Lambert and Freedman (1983) at 7.4 mM GSH.

Methods. GSH concentrations were determined by the method of Ellman (1959), and NADPH concentrations were determined by the absorbance at 340 nm using $\epsilon = 6.23$ mM^{-1} cm^{-1}. Protein was estimated by the method of Bradford (1976) or by the absorbance at 280 nm ($E^{0.1\%} = 0.94$). Spectrophotometric measurements were performed with a Beckman DU7 or DU70 spectrophotometer equipped with a thermostated cell compartment maintained at 25.0 °C.

Sulfhydryl Titrations. The reaction of native PDI with DTNB is very slow ($t_{1/2}$ > 60 min at pH 7.5 with 0.8 mM DTNB). The number of free sulfhydryl groups in the enzyme as isolated was consequently measured by the reaction of the protein (0.76 mg/mL) in 3 M guanidine hydrochloride [pH 7.0, 0.1 M tris(hydroxymethyl)aminomethane] with DTNB (0.8 mM). After correction for the absorbance due to contaminating TNB^- in the DTNB solution (<20% of the total absorbance change), the concentration of protein sulfhydryl groups was calculated by using an extinction coefficient of 1.36×10^4 M^{-1} cm^{-1} for TNB^- (Ellman, 1959). For thiol titration of the fully reduced enzyme, PDI (0.9 mg/mL) was incubated for 30 min in 6 M guanidine hydrochloride (0.2 M potassium phosphate, pH 7.5) with 2 mM DTT. Excess DTT was removed by centrifugal gel filtration. Protein concentration in the effluent was determined by the absorbance at 280 nm, and the concentration of protein thiols was determined with DTNB as before. Modification with NEM was accomplished by incubating PDI with NEM (5 mM) for 3 h at pH 7.5 (0.2 M potassium phosphate), 22 °C. After removal of excess NEM by centrifugal filtration (G-25), residual protein thiol was titrated with DTNB under denaturing conditions.

Kinetics of PDI Reduction by GSH. The stoichiometric reduction of PDI by GSH was followed in a continuous spectrophotometric assay in which the GSSG resulting from reduction of the enzyme by GSH was trapped with excess NADPH and glutathione reductase. The disappearance of NADPH at 340 nm was observed spectrophotometrically. A typical experiment contained 36 units/mL glutathione reductase, 0.16 mM NADPH, 23 μM PDI, and the appropriate concentration of GSH in 0.2 M potassium phosphate buffer/5 mM EDTA at pH 7.5. To remove contaminating GSSG in the GSH solution, the GSH was preincubated with excess NADPH and glutathione reductase. The reaction was initiated by the addition of the GSH solution (5 μL) to the temperature-equilibrated solution of PDI (260 μL). For the higher concentrations of GSH, mixing was accomplished by introducing the GSH from a microliter syringe with a spring-loaded t-shaped mixing device. Recording could be initiated within 2–3 s of mixing using this device.

Although buffers were degassed with argon prior to use, there was still some autoxidation of GSH to GSSH which resulted in a slow, linear decrease in the NADPH absorbance during the experiment. The absorbance was followed until the rate of GSH autoxidation paralleled that observed in the absence of PDI. Generally, the autoxidation of GSH resulted in a total absorbance change which was approximately one-third to half of the total absorbance change measured in the presence of PDI. Doubling the concentration of glutathione reductase (at the highest GSH concentration used) had no effect on the observed kinetics of PDI reduction, ensuring that the rate-limiting process in the assay was reduction of PDI by GSH.

Steady-State Kinetics of CYIQNC Reduction. The steady-state reduction of CYIQNC by GSH was observed by coupling the formation of GSSG to the oxidation of NADPH catalyzed by glutathione reductase (Chandler & Varandani, 1975). The appropriate concentration of GSH was preequilibrated with NADPH (0.14 mM) and glutathione reductase (12 units/mL) to remove contaminating GSSG. After temperature equilibration, a small aliquot of CYIQNC (15 μL) was added to the cuvette (340 μL), and the initial decrease in absorbance at 340 nm from the nonenzymatic reduction of CYIQNC by GSH was observed for 0.5–1.5 min. The initial concentration of the disulfide of CYIQNC changed less than 20% (generally 5–10%) during the measurement of both the nonenzymatic rate and the rate in the presence of PDI. The reaction was then initiated by the addition of PDI (60 μg/mL, 1.0 μM). The approximately linear change in absorbance at 340 nm was observed for 0.5–2 min after the addition of PDI. The velocity observed in the presence of PDI was corrected for the nonenzymic background reaction.

At concentrations of peptide below 50 μM and high concentrations of GSH (>1 mM), the absorbance change due to pre-steady-state reduction of the enzyme in the absence of CYIQNC became significant enough (0.007 absorbance unit/PDI disulfide) to preclude accurate measurements of velocity at lower peptide concentrations. The increasing rate of the nonenzymatic reaction between GSH and CYIQNC precluded the use of higher concentrations of peptide and GSH. In all cases, the observed rate in the presence of PDI was at least 50% greater (usually 100% greater) than the background rate. All measurements were performed at least in duplicate experiments on different days.

Redox State of PDI under Turnover Conditions. PDI (26 μM) was incubated with 4 mM GSH, 0.43 mM CYIQNC, 1 mM NADPH, and 23 units/mL glutathione reductase at pH 7.5 (0.2 M potassium phosphate) for 40 s (approximate time for the steady-state assay under these conditions). After centrifugal gel filtration of Sephadex G-75, the isolated PDI was incubated with 1 mM GSH in the presence of 0.2 mM NADPH and glutathione reductase, and the reduction of the intermediate and slowly reduced disulfides was observed spectrophotometrically as before. Controls included PDI which had been fully reduced (4 mM GSH for 9 min in the presence of 1 mM NADPH and glutathione reductase) and PDI treated in the same manner but in the absence of GSH (fully oxidized control). Rate constants and absorbance changes were evaluated by the data analysis procedures described below. Protein concentrations were determined by the absorbance at 280 nm after gel filtrations and corrected for a small (10%) contribution of glutathione reductase to the absorbance. For the fully oxidized control, the magnitude of the absorbance change was approximately 80% of that expected on the basis of the measured protein concentration. Rate constants for the

intermediate and slow reduction process agreed well (±8%) with those determined in independent experiments.

Data Analysis. The rate constants for the triphasic reduction of PDI by GSH were determined by nonlinear least-squares fitting to a six-parameter equation for a triexponential decrease in absorbance:

$$A_t = \Delta A_a e^{-k_a t} + \Delta A_b e^{-k_b t} + \Delta A_c e^{-k_c t} \quad (1)$$

where A_t is the absorbance at time t and each ΔA_n term represents the absorbance change occurring with rate constant k_n. After 7–10 half-lives for PDI reduction, the absorbance decrease becomes linear with time due to the slow autoxidation of GSH. The total observed absorbance decrease was corrected at each time point for the autoxidation of GSH by subtracting this linear absorbance decrease from the absorbance observed at earlier times. This correction generally amounted to 20–30% of the absorbance change observed from PDI reduction. At low concentrations of GSH, the reduction of the PDI disulfide reduced at the slowest rate was comparable to the rate of GSH autoxidation and could not be accurately observed.

Initial estimates of k_{slow} and the absorbance change due to the slow process were obtained from the slopes and intercepts of the linear portion of a plot of log $(A_{cor} - A_\infty)$ against time where A_{cor} represents the corrected value of the absorbance and A_∞ is defined as zero by the correction procedure. At each time point, the absorbance change due to the slow exponential reduction process was then subtracted from the total observed absorbance change. After subtracting out the slow exponential, the residual absorbance was plotted as log $(A_{cor} - A_\infty)$ vs time, and estimates of k_{med} and the associated absorbance change were made from the linear portion of this curve as before. After subtraction of the additional absorbance change resulting from the reduction described by k_{med} from the observed absorbance change at each time point, k_{fast} was evaluated from a linear plot of log $(A_{cor} - A_\infty)$ vs time. These initial estimates for k_n and ΔA_n were used in subsequent evaluation of the rate constants and absorbance changes by nonlinear least-squares fitting to eq 1. The least-squares values for the rate constants and absorbance changes were generally near (±20%) the initial estimates. Although there was considerable transformation of the primary data before arriving at estimates for the individual parameters, the rate constants frequently differed by 5–10-fold for each phase so that reliable estimates could be obtained.

For initial velocity studies, the data were fit to eq 2 by unweighted, nonlinear least squares. Initial estimates of the parameters were obtained from appropriate replots of the slopes and intercepts of the double-reciprocal plots against the reciprocal of the concentration of the nonvaried substrate (Cleland, 1970). All nonlinear least-squares was performed using a Gauss–Newton elimination method (Oestriecher & Pinto, 1987).

Results

Titration of Thiols and Disulfides. At pH 7.5, native PDI reacts very slowly with DTNB, suggesting the presence of no reactive free sulfhydryl groups. However, in the presence of 3 M guanidine hydrochloride, titration of the enzyme with DTNB reveals 0.8 equiv of thiol per PDI subunit (M_r 56 800). DTNB titration of PDI which has been incubated in 6 M guanidine hydrochloride with 2 mM DTT at pH 7.5 for 30 min indicates the presence of 7.1 mol of sulfhydryl per PDI subunit. Thus, as isolated, PDI possesses one free sulfhydryl group and three disulfide bonds. Alkylation of native PDI with

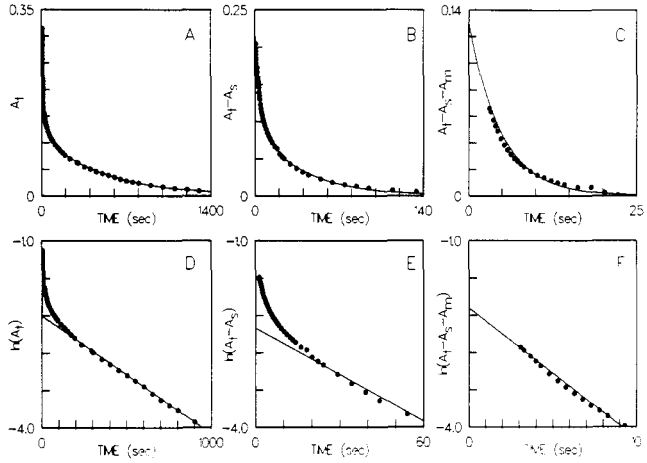


FIGURE 1: Triphasic reaction kinetics for the reduction of the disulfide bonds of PDI by GSH. The absorbance changes upon reduction of PDI (17 μM) with GSH (1.0 mM) in the presence of excess glutathione reductase and NADPH at pH 7.5 (0.2 M potassium phosphate), 25.0 °C, are shown as a function of time. Panels A–C show the absorbance change directly, and panels D–F show the natural logarithm of the absorbance changes. The data have been corrected for a slow linear decrease in absorbance due to autoxidation of GSH (see text). The total reaction time course is shown in panels A and D. Panels B and E show the residual change in absorbance after subtraction of the slow change in absorbance (ΔA = 0.11, k_{slow} = 0.0019 min^{-1}; panels A and B). Panels C and F show the residual change in absorbance after subtraction of both the slow change in absorbance and the intermediate change in absorbance (ΔA = 0.090, k_{med} = 0.025 min^{-1}). The residual absorbance change in panels C and F is described with ΔA = 0.13 and k_{fast} = 0.21 min^{-1}. The curves in panels A–C were drawn with the triexponential function shown in eq 1 using the rate constants and absorbance changes indicated above.

5 mM NEM at pH 7.5 (0.2 M potassium phosphate), 22 °C for 3 h results in complete (>95%) reaction of the free cysteine residue as determined by DTNB titration of the NEM-modified enzyme isolated by gel filtration. The NEM-alkylated enzyme retains >85% of the activity of the unmodified enzyme in an assay with the peptide CYIQNC.

Kinetics of Reduction by GSH. The reduction of the three disulfides of PDI by GSH can be followed continuously by trapping the product, GSSG, with a rapid glutathione reductase catalyzed reduction of GSSG in the presence of NADPH. After correction for a slow, constant rate of autoxidation of GSH to GSSG, three distinct kinetic phases are observed during the reduction of PDI by GSH (Figure 1). The average absorbance changes (14 experiments) during the 3 phases of reduction corresponded to the reduction of 1.04 ± 0.17, 0.95 ± 0.22, and 1.07 ± 0.18 equiv of disulfide per PDI monomer (M_r 56 800) during the fast, medium, and slow reductions, respectively.

The observed first-order rate constants for the three reductions of PDI are shown as a function of the GSH concentration in Figures 2–4. The fastest reduction process exhibits a second-order dependence on the concentration of GSH at low GSH concentrations (Figure 2), but at higher GSH concentrations, the reaction becomes first-order in GSH. A plot of the overall second-order rate constant for the fast reaction (k_{obs}/[GSH]) against the concentration of GSH is hyperbolic and approaches a first-order behavior at high concentrations of GSH (Figure 2, inset). The reduction which occurs at an intermediate rate exhibits a first-order dependence on GSH concentration over the entire range of GSH concentrations employed (Figure 3). The slowest reduction, however, is second-order in GSH concentration under all conditions examined (Figure 4). Rate constants are summarized in Table I.

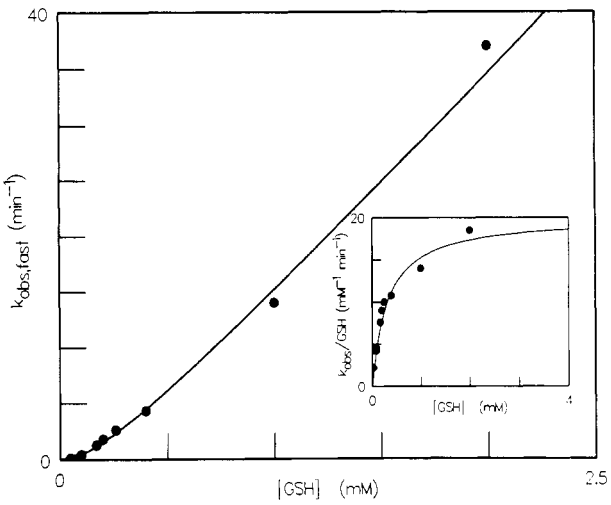


FIGURE 2: Dependence of the observed first-order rate constant for the most rapid reduction of PDI on the concentration of GSH. All experiments were performed at pH 7.5 (0.2 M potassium phosphate), 25.0 °C. Rate constants were determined as discribed in Figure 1. The inset shows the apparent second-order rate constant (k_{obs}/[GSH]) as a function of the GSH concentration. The solid curves in the main figure and inset were drawn by using the equation and rate constants for k_{fast} shown in Table I.

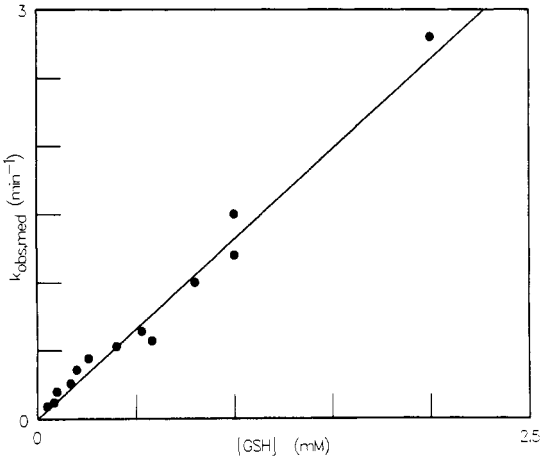


FIGURE 3: Dependence on the observed first-order rate constant for the intermediate-rate reduction of PDI on the concentration of GSH. All experiments were performed at pH 7.5 (0.2 M potassium phosphate), 25.0 °C. Rate constants were determined as described in Figure 1. The line was drawn by using a second-order rate constant of 1.3×10^3 M^{-1} min^{-1} (k_{med} Table I).

Table I: Kinetic Constants for the Reduction of PDI by GSH at 25.0 °C, pH 7.5

process	rate equation	kinetic constants
fast	$k_{obs} = k_1k_3[GSH]^2/(k_2 + k_3[GSH])$	$k_1k_3/k_2 = (6 \pm 1) \times 10^7$ M^{-2} min^{-1} $k_1 = (2.0 \pm 0.1) \times 10^4$ M^{-1} min^{-1}
intermediate	$k_{obs} = k_{med}[GSH]$	$k_{med} = (1.3 \pm 0.1) \times 10^3$ M^{-1} min^{-1}
slow	$k_{obs} = k_{slow}[GSH]^2$	$k_{slow} = (8 \pm 1) \times 10^4$ M^{-2} min^{-1}

Steady-State Kinetics of the PDI-Catalyzed Reduction of CYIQNC by GSH. PDI catalyzes the GSH-dependent reduction of the disulfide-containing hexapeptide CYIQNC. In the presence of glutathione reductase and NADPH, the steady-state formation of GSSG upon reduction of CYIQNC by GSH can be observed continuously (Chandler & Varandani, 1975). HPLC of the reaction mixture shows that under these conditions the peptide disulfide is converted smoothly to the dithiol peptide without accumulation of peptide glutathione mixed disulfides (data not shown).

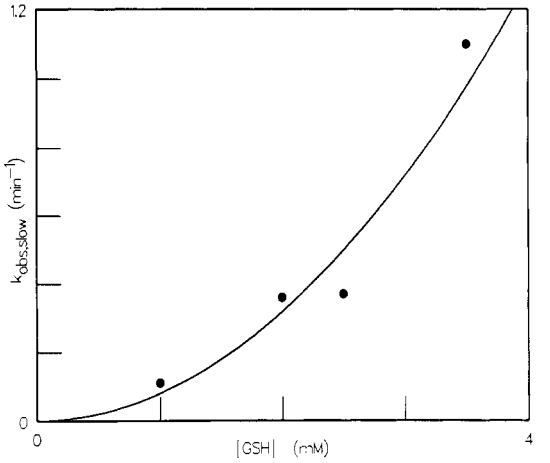


FIGURE 4: Dependence on the observed first-order rate constant for the slowest reduction of PDI on the concentration of GSH. All experiments were performed at pH 7.5 (0.2 M potassium phosphate), 25.0 °C. Rate constants were determined as described in Figure 1. The solid curve was drawn by using a thirrd-order rate constant of 8×10^4 M^{-2} min^{-1} and the equation shown for k_{slow} in Table I.

The PDI-catalyzed reduction of CYIQNC by GSH is accompanied by a significant nonenzymatic reaction between CYIQNC and GSH. The rate constant for the uncatalyzed, second-order reaction of CYIQNC and GSH is 20 M^{-1} min^{-1} at pH 7.5 (data not shown). For a fixed level of CYIQNC, this nonenzymatic reaction becomes comparable to the catalyzed reaction as the GSH concentration is increased. As GSH approaches saturating concentrations (when the second-order reaction of the enzyme and CYIQNC becomes rate-limiting, see below), further increases in GSH concentration increase the background faster than the catalyzed reaction. This effect limits the maximum concentration of GSH which can be employed. At low concentrations of CYIQNC and GSH, the pre-steady-state, stoichiometric reduction of PDI (1.4 μM in the assay) becomes significant in comparison to the measured absorbance change during the catalyzed reaction. This limits the lower concentrations of GSH and CYIQNC which can be employed. In all cases, CYIQNC and GSH concentrations were limited to conditions where the noncatalyzed rate is less than 50% of the catalyzed rate and where the initial stoichiometric reduction of PDI by GSH accounts for less than 20% of the observed absorbance change. Both of these effects contribute to the higher than normal error associated with the steady-state kinetic measurements.

Inital velocity experiments at varying concentrations of CYIQNC and GSH indicate an intersecting pattern on double-reciprocal plots (Figure 5). A replot of the y intercepts of the double-reciprocal plots against 1/[CYIQNC] (Figure 5, inset) reveals that this substrate does not exhibit saturation behavior. Both GSH and CYIQNC concentrations are significantly below the true K_m's for these substrates.

The initial velocity data can be empirically described by the equation:

$$E_t/v = \phi_p/P + (1/G)(\phi_g + \phi_{gp}/P + \phi_{gg}/G) \quad (2)$$

where E_t is the total enzyme concentration, v is the observed velocity, P represents the concentration of the peptide CYIQNC, G is the concentration of GSH, and the various ϕ_n's represent empirical constants. The reactions of the enzyme can be described entirely by second-order reactions of the substrates with the enzyme. At a fixed concentration of CY-

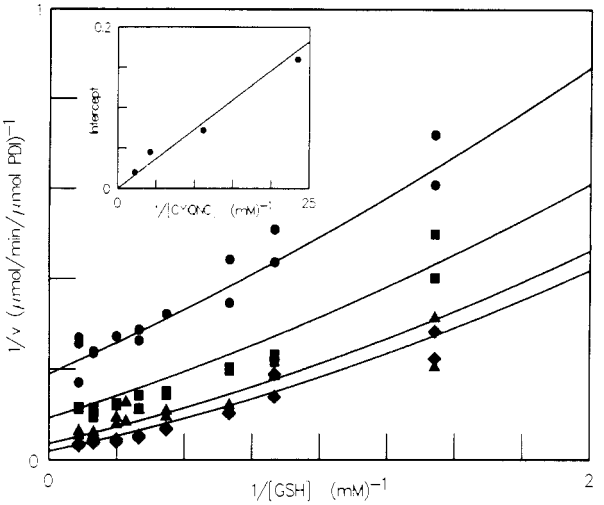


FIGURE 5: Initial velocity kinetics of the reduction of CYIQNC by GSH catalyzed by PDI. All experiments were performed at pH 7.5 (0.2 M potassium phosphate), 25 °C. CYIQNC concentrations were (●) 0.043, (■) 0.089, (▲) 0.23, and (♦) 0.43 mM. The curves were drawn by using eq 2 and the kinetic constants shown in Table II. The inset shows the intercepts of the double-reciprocal plots as a function of the reciprocal of the CYIQNC concentration.

Table II: Rate Constants for the Steady-State Reduction of CYIQNC by GSH Catalyzed by PDI

kinetic constant[a]	rate constants[b]	value[c]
$1/\phi_p$	k_5	$(1.2 \pm 0.1) \times 10^5$ M^{-1} min^{-1}
$1/\phi_g$	$k_3k_9/(k_3 + k_9)$	$(9 \pm 2) \times 10^3$ M^{-1} min^{-1}
$1/\phi_{gp}$	k_5k_9/k_6	$(1.5 \pm 0.3) \times 10^8$ M^{-2} min^{-1}
$1/\phi_{gg}$	$k_1k_3k_9/(k_3k_7 + k_2k_9)$	$(3 \pm 1) \times 10^7$ M^{-2} min^{-1}

[a] Steady-state constants are defined in eq 2. [b] Rate constants are defined in Scheme I. [c] Values were determined from nonlinear least-squares fitting to eq 2 and are reported ± the standard deviation of the estimate.

IQNC, apparent saturation behavior is observed with increasing GSH concentration; the velocity will approach P/ϕ_p, and the apparent K_m for GSH will be $(\phi_g P + \phi_{gp})/\phi_p$. A similar argument applies to the observation of saturation behavior for CYIQNC at a fixed GSH concentration.

The term containing the quantity ϕ_{gg} results in curvature of the double-reciprocal plots. All of the parameters are reasonably well-defined except for the term ϕ_{gg}. The existence of this term is suggested by a potential mechanism for the reaction (see Discussion). Fits of the data with a form of the equation lacking the ϕ_{gg} term show only slightly higher residuals so that the use of an equation with an extra parameter cannot be fully justified on statistical grounds (Mannervik, 1982). Values for the kinetic constants are given in Table II.

Redox State of PDI under Turnover Conditions. At the higher GSH concentrations used in the initial velocity studies, the possibility exists that the more slowly reacting disulfides of PDI could become reduced during the initial velocity experiments and alter the kinetic behavior of the enzyme. When PDI is fully reduced by preincubation with GSH before initiation of the assay, the initial velocity is virtually identical with that observed when the assay is initiated by the addition of PDI with all disulfide bonds intact. PDI was fully reduced by incubation with 4 mM GSH and 1 mM NADPH at pH 7.5 in the presence of glutathione reductase for 5 min (>10 half-lifes for reduction of the most slowly reduced disulfide). In these controls, the GSH concentration in the assay was 0.8 or 4.0 mM, and the CYIQNC concentration was 0.1 or 0.43 mM. There was no indication of significant curvature with

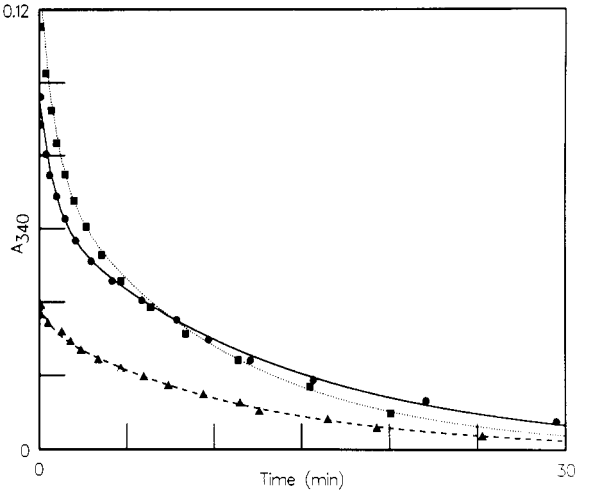


FIGURE 6: Disulfide bond status of PDI during initial velocity turnover conditions in the presence of GSH and CYIQNC. The intermediate and slow disulfide bonds of PDI were reduced in an assay containing 1.0 mM GSH, 0.2 mM NADPH, and 27 units/mL glutathione reductase after isolation of PDI from the preincubation medium by centrifugal gel filtration. After correction for GSH autoxidation, the absorbance change at 340 nm due to NADPH oxidation is shown. Absorbances are normalized to a PDI concentration of 18 μM. Curves are drawn with the rate constants k_{med} and k_{slow} and absorbance changes ΔA_{med} and ΔA_{slow} determined from nonlinear least-squares fitting to a biexponential function. (●) Turnover conditions: PDI preincubated at pH 7.5 (0.2 M potassium phosphate) with 4 mM GSH and 0.43 mM CYIQNC in the presence of a GSSG trap for 40 s and isolated by gel filtration (k_{slow} = 1.2 min^{-1}, k_{med} = 0.075 min^{-1}, αA_{slow} = 0.075, ΔA_{med} = 0.032). (■) Oxidized PDI: PDI preincubated at pH 7.5 (0.2 M potassium phosphate) with 0.43 mM CYIQNC in the presence of a GSSG trap for 40 s and isolated by gel filtration (k_{slow} = 1.2 min^{-1}, k_{med} = 0.087 min^{-1}, ΔA_{slow} = 0.076, ΔA_{med} = 0.052). (▲) Fully reduced PDI: PDI preincubated at pH 7.5 (0.2 M potassium phosphate) with 4 mM GSH in the presence of a GSSG trap for 9 min and isolated by gel filtration (k_{slow} = 1.5 min^{-1}, k_{med} = 0.10 min^{-1}, ΔA_{slow} = 0.006, ΔA_{med} = 0.033).

time in the assays regardless of the initial redox state of the PDI added to initiate the reaction. This indicates that the activity of the enzyme was not changing during the assay and that if reduction of the slow and intermediate disulfide bonds was occurring, it did not significantly influence the observed velocity.

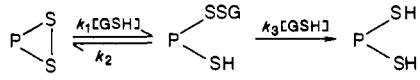
The redox states of the intermediate and slowly reduced disulfide bonds of PDI were probed directly during steady-state turnover. These two disulfide bonds are largely intact in PDI isolated from reaction mixtures containing both GSH (4 mM) and CYIQNC (0.43 mM) (Figure 6). Compared to the fully oxidized control, 40% and 83% of the intermediate and slowly reduced PDI disulfides were intact after 40 s under turnover conditions. On the basis of the measured rate constants for PDI reduction by GSH, these two disulfide bonds would be expected to be only 3% and 40% intact after 40-s reduction with 4 mM GSH. Thus, the presence of CYIQNC during turnover appears to maintain these two disulfides in the oxidized state. Because of the high PDI concentration required for these stoichiometric experiments, about 60% of the CYIQNC should have been reduced during the 40-s incubation under turnover conditions. This amount of substrate depletion is considerably higher than that which would occur at the much lower PDI concentrations used in the initial velocity kinetic experiments. Consequently, the disulfide bonds in PDI would be expected to be even more intact in the initial velocity experiments than this experiment would indicate. A control in which PDI was totally reduced by preincubation with GSH showed that <10% of the disulfide reduced at the intermediate rate and 43% of the disulfide reduced at the slowest rate were

present after gel filtration, most likely resulting from some autoxidation during isolation.

Discussion

Redox Status of the Native Enzyme. DTNB titration of the native and DTT-reduced PDI indicates that, as isolated, the enzyme contains one free thiol and three disulfide bonds. This observation is consistent with the sequence of the rat liver and human proteins which indicates a total of seven cysteine residues (Edman et al., 1985; Morris & Varandani, 1988). Alkylation of the free sulfhydryl group has no effect on activity, at least for the GSH reduction of CYIQNC or insulin. The rate constant for the reaction of the free sulfhydryl group of the enzyme is considerably slower than expected for the reaction of an exposed thiol and is uncharacteristic of "active-site" sulfhydryl groups in general. It would appear that this free sulfhydryl is of minimal functional utility, at least with regard to the thiol/disulfide exchange reactions which have been studied. Carmichael et al. (1979) and Hillson and Freedman (1980) detected inhibition of the enzyme by cadmium, and Freedman et al. (1988) showed that the enzyme was inhibited by iodoacetamide alkylation only after reduction by DTT (Freedman et al., 1988). All of these experiments point to an essential role for a thiol (or dithiol) in catalysis and the lack of participation of the odd, free sulfhydryl group.

Reduction of PDI with GSH. Reaction of stoichiometric concentrations of PDI with the predominant intracellular thiol, GSH, results in the sequential reduction of the three disulfide bonds. The kinetics of these single-turnover experiments are complex; however, they are entirely consistent with the generally assumed mechanisms of disulfide reduction which involve the formation of a mixed-disulfide intermediate (eq 3) (Creighton, 1986; Zhang & Snyder, 1988; Gilbert, 1989).

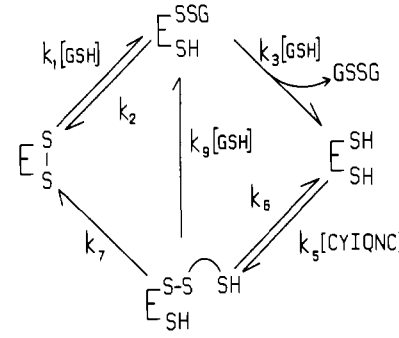


(3)

The most rapid reaction of PDI with GSH exhibits a change in the rate-determining step with increasing concentration of GSH (Figure 2). At low GSH concentrations, the reaction is second-order with respect to GSH concentration (third-order overall). For the mechanism depicted in eq 3, the rate-determining step at low GSH concentrations would be the step k_3 since $k_2 \gg k_3$[GSH]. In this case, the observed rate constant, k_{fast}, will be given by k_1k_3[GSH]$^2/k_2$. With increasing GSH concentrations, a point will be reached at which the mixed-disulfide intermediate partitions more rapidly toward reaction with GSH (k_3) than toward expulsion of GSH from the mixed disulfide (k_2). Under these conditions, the rate-determining step is the initial attack of GSH on the disulfide (k_1), and the reaction exhibits a first-order dependence on GSH concentration. For the most rapidly reduced disulfide of PDI, the intermolecular attack of GSH on the mixed disulfide (k_3) occurs at the same rate as the intermolecular attack of the thiol of the mixed disulfide (k_2) when the concentration of GSH is 0.3 mM. In this putative, mixed-disulfide intermediate, the "effective molarity" of the free thiol of PDI is 3×10^{-4} M, surprisingly low for an intramolecular reaction at the active site of an enzyme where "effective molarities" may approach 10^9 M (Jencks, 1975).

For the disulfide of PDI which is reduced at the intermediate rate, the reaction is first-order in GSH concentration over the range 0.01–4 mM. On the basis of kinetic information, it is not possible to decide which step in the mechanism shown in eq 3 is rate limiting. On the other hand, the rate-limiting step for the reduction of the most slowly reduced disulfide must be the intermolecular attack of GSH on the mixed-disulfide intermediate (k_3) since the reaction is second-order in GSH concentration from 1 to 4 mM GSH. In the absence of a change in the rate-determining step, it is only possible to surmise that the "effective molarity" of the sulfhydryl group of mixed-disulfide intermediate involved in the most slowly reduced PDI disulfide is greater than 4×10^{-3} M.

Scheme I: Potential Reaction Mechanism for the PDI-Catalyzed Reduction of CYIQNC by GSH



Steady-State Reduction of CYIQNC. PDI catalyzes thiol/disulfide exchange between a variety of protein and nonprotein substrates. The hallmark of this enzyme is a reasonable lack of specificity (Hillson & Freedman, 1980; Hillson et al., 1984). The initial velocity kinetics (Figure 5) observed at varying concentrations of CYIQNC and GSH clearly indicate an intersecting pattern of double-reciprocal plots characteristic of a sequential reaction mechanism involving ternary complex formation (Cleland, 1970). Lambert and Freedman (1983) have also mentioned an intersecting initial velocity profile for the PDI-catalyzed reduction of insulin by GSH; however, no kinetic constants were reported.

The simplest mechanism to account for the initial velocity kinetic behavior would involve the simultaneous binding (in an ordered or random fashion) of GSH and CYIQNC to the enzyme, thiol/disulfide exchange, and the release of products. Such a mechanism need not involve any participation by the disulfides of PDI in the reaction. However, Freedman et al. (1988) have shown that alkylation of the reduced protein destroys activity, implicating the participation of protein thiols in the reaction mechanism.

With respect to the nature of the reactions catalyzed and the participation of the disulfides of PDI in the reaction, the most likely mechanism would be a ping-pong mechanism in which the disulfide form of the enzyme is initially reduced by GSH followed by reduction of CYIQNC by the protein dithiol. However, this simple ping-pong mechanism is inconsistent with the observed initial velocity kinetics (a series of parallel lines would be required in Figure 5) (Cleland, 1970). A hybrid mechanism (Scheme I) with characteristics of a sequential mechanism at high GSH concentrations and characteristics of a ping-pong mechanism at low GSH concentrations is proposed. This scheme is also consistent with the ability of GSH to reduce PDI in the absence of CYIQNC, the participation of protein thiols and disulfide in the reaction, and the sequential character of the initial velocity kinetics.

The full steady-state equation describing the mechanism of Scheme I is complex with 18 terms, some of which are cubic in GSH concentration. Obviously, such a mechanism will describe the data; however, the use of such a complex mechanism is not required to account for the observed initial velocity kinetics. The equations simplify to a simple sequential model (eq 2) when the GSH concentration is sufficiently high that k_8[GSH] is much greater than k_7. The mechanism depicted in Scheme I is consistent with the mechanism proposed by

Creighton et al. (1980) for catalysis of the disulfide rearrangements of ribonuclease A.

According to Scheme I, initial reduction of an enzyme disulfide by GSH leads to a form of the enzyme [$E(SH)_2$] which can react with the peptide disulfide to form a mixed-disulfide intermediate. This intermediate either can break down by an intramolecular reaction involving the free enzyme thiol (k_7) or may be converted to the reduced peptide and a protein–SSG-mixed disulfide by reaction with GSH (k_9). At sufficiently high concentrations of GSH, the reaction will cycle through the steps defined by k_3, k_5, and k_9, a sequential mechanism. At a constant concentration of CYIQNC (P), the velocity of the reaction will continue to increase with increasing GSH concentration until the bimolecular reaction of the enzyme with CYIQNC becomes rate-limiting (k_5), accounting for the saturation by GSH at constant CYIQNC concentration. At high GSH concentration, all of the steps involved in turnover are bimolecular reactions of the enzyme with GSH or CYIQNC, accounting for the failure to observe a true V_{max} with ever-increasing levels of CYIQNC and GSH. Currently, there is no direct evidence for the accumulation of intermediates in which either glutathione or CYIQNC is bound covalently to PDI. Experiments directly addressing this question are in progress.

The initial velocity kinetic experiments provide no evidence for an initial noncovalent association (binding) of PDI with either substrate. This is not expected behavior from an enzyme with an active site designed to bind specific substrates; however, disulfide bonds in proteins do not occur in a defined sequence motif nor with a constant number of intervening amino acids between the two cysteine residues (Thornton, 1981). Therefore, PDI must be capable of catalyzing thiol/disulfide exchange rearrangements within a variety of peptide contexts so the lack of specificity against the peptide disulfide substrate is perhaps not too surprising.

The inability to "bind" glutathione is somewhat more surprising. Glutathione is not an anomalously bad substrate for PDI; in the reduction of insulin, cysteamine is a comparable substrate to GSH (Hillson et al., 1984).

Catalysis by PDI has traditionally required the use of relatively high concentrations of the protein (Lambert & Freedman, 1983). At a constant concentration of GSH (8 mM), the k_{cat} for insulin reduction is 11 min^{-1} (apparent K_m = 3 μM) (Lambert & Freedman, 1983). At this GSH concentration, CYIQNC is reduced with a k_{cat} of 70 min^{-1} (apparent K_m = 0.6 mM). Thus, the hexapeptide CYIQNC does not appear to be an abnormally poor substrate.

The single, free sulfhydryl group of PDI does not participate in the reduction of CYIQNC catalyzed by PDI; full alkylation with NEM has no significant effect on the activity. However, the participation of the multiple disulfides of PDI in the steady-state reduction of CYIQNC must be considered. If the steady-state reaction proceeded through a ping-pong mechanism which required the reduction of the PDI disulfides by GSH each catalytic cycle, only the most rapidly reduced disulfide would be kinetically competent; the rates of the reduction of the other two disulfides are simply too slow to support the observed rate.

At a GSH concentration of 2 mM, the half-life for reduction of the most slowly reduced PDI disulfide would be about 2 min so that some of this disulfide might be reduced by GSH during the course of a 0.5–2-min assay. For the PDI disulfide which is reduced at an intermediate rate by GSH, the half-life for reduction by GSH would be about 12 s. Reduction of these two disulfides during the initial velocity experiments could have two opposing effects on the observed activity of the enzyme. If after an initial reduction by GSH, either of the two disulfide/dithiols was capable of rapidly cycling around a sequential mechanism (k_3, k_5, k_9) without reverting to the protein disulfide form (k_7), it is conceivable that these other dithiol/disulfide centers could contribute to the observed turnover, at least at the higher GSH concentrations (>1–9 mM). Alternatively, extensive reduction of these two disulfides could significantly alter the structure and catalytic properties of the enzyme.

Under turnover conditions, the two more slowly reduced disulfides of PDI are maintained mostly in the disulfide form (Figure 6). There is no indication that the activity of the enzyme changes during the course of the reduction of CYIQNC by GSH even at high GSH concentrations (4 mM), and when all three PDI disulfides are reduced by GSH before initiation of the assay, the initial velocity is linear and identical with that observed when the assay is initiated by the addition of PDI with all three disulfide bonds intact. All of these observations suggest that there is no significant deleterious effect due to reduction of these disulfides during the assay.

Rate Enhancements. PDI is not a particularly effective or selective catalyst of thiol/disulfide exchange. The seond-order rate constant for the reduction of the most rapidly reduced disulfide of PDI by GSH (k_1) is only 1000-fold faster than the noncatalyzed reaction of GSH with the disulfide of CYIQNC. The second-order rate constant for the reaction of PDI with CYIQNC (k_5) is about 6000-fold faster than the reaction of GSH with CYIQNC, and the second-order rate constant for the reaction of GSH with enzyme-bound CYIQNC [$k_3k_9/(k_3 + k_9)$] is about 400-fold faster than the noncatalyzed reaction. Although the rate acceleration achieved by PDI is small compared to many other enzymes, the 400–6000-fold rate acceleration is substantial.

For the reactions in which PDI cysteines must behave as the leaving group, disulfide stain or a decreased pK_a of the leaving thiol (Gillbert, 1989) could account for the observed rate acceleration without having to invoke any specific binding interaction of a substrate with an active site. However, for participation of the cysteine residue of PDI as a nucleophile, the observed 1000-fold rate acceleration is more difficult to explain without invoking some type of interaction (albeit a weak one) between the enzyme and GSH and CYIQNC.

A number of previous studies have focused on the catalysis of oxidative protein folding and protein disulfide bond reduction (Creighton et al., 1980; Lang & Schmid, 1988). Creighton et al. (1980) observed that PDI catalyzed the oxidation and reduction of the dithiols/disulfides of proteins such as bovine trypsin inhibitor and ribonuclease A in the presence of oxidants and reductants such as DTT and glutathione. PDI behaved as a catalyst in that the concentrations of the various disulfide intermediates during protein folding were not altered but the rate of recovery of the native structure was accelerated. PDI catalysis was not observed under all conditions; generally, the steps found to be catalyzed involved not only thiol/disulfide exchange but also protein conformational changes, leading to the speculation that PDI may catalyze conformational transitions as well as thiol/disulfide exchange (Creighton, 1980).

In Vivo Role of PDI. Virtually nothing is known about the intracellular environment of the endoplasmic reticulum in which oxidative protein folding occurs—the source or oxidizing equivalents, the redox status, or the availability of catalysts other than PDI. Ziegler and Poulsen (1977) have proposed that a flavoprotein located on the endoplasmic reticulum may deliver cystamine as an oxidant. Thioredoxin, a small protein

with a single, redox-active dithiol/disulfide, also catalyzes the oxidative folding and rearrangement of disulfide-containing proteins (Pigiet & Schuster, 1986). Bulleid and Freedman (1988) have provided evidence that PDI-deficient microsomes do not function as efficiently in oxidative protein folding using an in vitro translation system, and PDI can be cross-linked to nacent immunoglobulin chains in vivo (Roth & Pierce, 1987). Both in vitro and in vivo results tend to support a role for PDI in disulfide bond formation (Bulleid & Freedman, 1988). However, recent reports have identified PDI as a thyroid hormone binding protein (Cheng et al., 1987), as the β-subunit of prolyl hydroxylase (Obata et al., 1988; Koivu et al., 1987), and as a component of the N-linked glycosylation apparatus of the Golgi (Geetha-Habib et al., 1988).

PDI is a major intracellular protein (0.4% of total cellular protein) (Hillson et al., 1984). If PDI were uniformly distributed throughout the cell, the concentration would be about 14 μM (0.1 mM cysteine equiv), comparable to the total cellular concentration of GSSG (Gilbert, 1989). However, since the protein is confined primarily to the lumen of the endoplasmic reticulum, the local concentration may approach the millimolar range. The high local concentration of this enzyme coupled with the necessity of promoting thiol/disulfide exchange between structurally dissimilar thiols and disulfides during posttranslational protein oxidation may necessitate an enzyme of "unusual" properties when gaged against the specific and rapid enzymes of metabolism.

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