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Articles

Catalysis of the Oxidative Folding of Ribonuclease A by Protein Disulfide Isomerase: Dependence of the Rate on the Composition of the Redox Buffer†

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ABSTRACT: The velocity of the oxidative renaturation of reduced ribonuclease A catalyzed by protein disulfide isomerase (PDI) is strongly dependent on the composition of a glutathione/glutathione disulfide redox buffer. As with the uncatalyzed, glutathione-mediated oxidative folding of ribonuclease, the steady-state velocity of the PDI-catalyzed reaction displays a distinct optimum with respect to both the glutathione (GSH) and glutathione disulfide (GSSG) concentrations. Optimum activity is observed at [GSH] = 1.0 mM and [GSSG] = 0.2 mM. The apparent k_{cat} at saturating RNase concentration is 0.46 ± 0.05 μmol of RNase renatured min^{-1} (μmol of PDI)$^{-1}$ compared to the apparent first-order rate constant for the uncatalyzed reaction of 0.02 ± 0.01 min^{-1}. Changes in GSH and GSSG concentration have a similar effect on the rate of both the PDI-catalyzed and uncatalyzed reactions except under the more oxidizing conditions employed, where the catalytic effectiveness of PDI is diminished. The ratio of the velocity of the catalyzed reaction to that of the uncatalyzed reaction increases as the quantity $[GSH]^2/[GSSG]$ increases and approaches a constant, limiting value at $[GSH]^2/[GSSG]$ greater than 1 mM, suggesting that a reduced, dithiol form of PDI is required for optimum activity. As long as the glutathione redox buffer is sufficiently reducing to maintain PDI in an active form ($[GSH]^2/[GSSG] > 1$ mM), the rate acceleration provided by PDI is reasonably constant, although the actual rate may vary by more than an order of magnitude. PDI exhibits half of the maximum rate acceleration at a $[GSH]^2/[GSSG]$ of 0.06 ± 0.01 mM.

Protein disulfide isomerase (PDI)[1] was initially isolated by Anfinsen and his colleagues (Goldberger et al., 1963) on the basis of the ability of the protein to catalyze the oxygen or glutathione disulfide dependent renaturation of RNase A from the reduced enzyme (Epstein et al., 1963). PDI (M_r 57 800) is localized primarily in the endoplasmic reticulum as a luminal, peripheral membrane protein (Lambert & Freedman, 1983). The enzyme exhibits broad specificity and, in addition to catalyzing disulfide bond formation and rearrangement in proteins, accelerates thiol/disulfide exchange reactions involving numerous protein and nonprotein substrates (Hillson et al., 1984; Morin & Dixon, 1985; Varandani et al., 1975). This lack of specificity is uncharacteristic of conventional enzymes but is in keeping with the necessity for catalyzing the formation, rearrangement, and reduction of disulfide bonds between cysteine residues in a number of sequence contexts.

Surprisingly, PDI has also been shown to function as the β-subunit of prolyl hydroxylase (Pihlajaniemi et al., 1987), one of the subunits of a triglyceride transfer complex of the endoplasmic reticulum (Wetterau et al., 1990), a thyroid hormone binding protein (Yamauchi et al., 1987), and as a component of the glycosylation apparatus of the ER–Golgi complex (Geetha-Habib et al., 1988). This has naturally led to disagreement about the in vivo role of this protein. Homology is observed between PDI and thioredoxin, suggesting that PDI contains two thioredoxin-like domains, each with a dithiol/disulfide pair (Edman et al., 1985; Freedman et al.,

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[1] Abbreviations: PDI, protein disulfide isomerase; RNase A, bovine pancreatic ribonuclease A; BPTI, bovine pancreatic trypsin inhibitor; GSH, glutathione; GSSG, glutathione disulfide; DTT, dithiothreitol.

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1988). PDI is a substrate for thioredoxin reductase, and two disulfides are reduced in an NADPH-dependent fashion (Lundstrom & Holmgren, 1990). The coding sequence of PDI from several species shows the presence of six conserved cysteine residues (Edman et al., 1985; Yamauchi et al., 1987; Morris & Varandani, 1988). The disulfide bonds show different kinetics for reduction by GSH, suggesting that in spite of the sequence homology, the two thioredoxin domains do not have identical properties (Gilbert, 1989).

Studies of the mechanism of the uncatalyzed, oxidative folding of small proteins have led to the conclusion that the specific disulfide bonds observed in the native structure do not form in an ordered, sequential fashion (Creighton, 1978). Extensive nonnative disulfide bond formation invariably precedes the regain of native structure and enzyme activity (Hantgan et al., 1974). These nonnative disulfides must be rearranged by thiol/disulfide exchange to the native disulfides (Anfinsen et al., 1961; Saxena & Wetlaufer, 1970; Ahmed et al., 1975; Creighton, 1978; Scheraga et al., 1984). In the absence of PDI, the uncatalyzed oxidative renaturation of RNase proceeds through a number of disulfide-containing intermediates, which may be converted to the native molecule by one (Creighton, 1979; Wearme & Creighton, 1988) or a number (Konishi et al., 1982) of intra- and/or intermolecular thiol/disulfide rearrangements.

There have been few studies of the PDI-catalyzed oxidative renaturation of reduced proteins (Creighton et al., 1980; Tang et al., 1988; Lang & Schmid, 1988) and no systematic study of the effects of the redox buffer composition on catalysis by PDI. PDI is usually assayed by observing the rearrangement of scrambled RNase (oxidized in the presence of denaturants) to the native enzyme in the presence of low concentrations of thiols (Hillson et al., 1984). Lambert & Freedman (1983) found that PDI catalysis of the rearrangement of scrambled RNase exhibits a K_m of 4 μM for DTT and 0.4–0.6 mM for monothiols such as GSH and cysteamine and is inhibited by DTT concentrations greater than 30 μM, presumably by overreducing the disulfide bonds in the substrate. Using a very limited range of dithiothreitol and glutathione disulfide concentrations, Creighton et al. (1980) showed that PDI does not appreciably change the nature of the disulfide-containing intermediates during the oxidative folding of BPTI. They also suggested that PDI selectively catalyzes thiol/disulfide exchange processes, which may be linked to conformational changes in the protein substrate.

Changes in the composition of the folding redox buffer (GSH and GSSG) during oxidative renaturation of RNase could have two effects on catalysis by PDI. As demonstrated by Konishi et al. (1982) for the uncatalyzed reaction, changes in the glutathione redox buffer alter the distribution of the nonnative thiol/disulfide redox isomers of RNase. If only one or a few of these redox isomers are substrates for PDI-catalyzed refolding, alterations in the redox buffer composition could alter the rate of the reaction by changing the availability of the proper substrate for PDI. Additionally, changes in the glutathione redox buffer composition could affect the redox state of PDI itself so that the activity of the enzyme would be expressed only under the appropriate redox conditions. The optimum GSH and GSSG concentrations need not be the same for the catalyzed and uncatalyzed reactions. By examining the PDI-catalyzed oxidative refolding of reduced RNase A over a wide range of GSH and GSSG concentrations, we have found effects of the glutathione redox buffer that can be attributed to alterations in the availability of PDI substrates and to changes in the catalytic efficiency of the enzyme.

Experimental Procedures

Materials. Glutathione disulfide (GSSG), glutathione (GSH), glutathione reductase (yeast, type III), cytidine 2′,3′-cyclic monophosphate (cCMP), NADPH (type III), and ribonuclease A from bovine pancrease (type III A) were purchased from Sigma. Dithiothreitol (DTT) was obtained from Boerhringer Mannheim. Protein disulfide isomerase was purified from bovine liver according to the method of Hillson et al. (1984). The purified protein was >95% homogeneous on SDS–polyacrylamide 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, 0.2 μmol of GSSG formed min^{-1} (mg of protein)$^{-1}$, was 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). GSSG concentrations were determined from the decrease in absorbance at 340 nm due to the oxidation of NADPH (ϵ = 6.23 mM^{-1} cm^{-1}) in the presence of glutathione reductase. The concentrations of cCMP and RNase A were determined spectrophotometrically at a wavelength of 296 nm for cCMP (ϵ = 0.19 mM^{-1} cm^{-1}) and 277.5 nm for RNase (ϵ = 9.8 mM^{-1} cm^{-1} for native RNase and 9.3 mM^{-1} cm^{-1} for reduced RNase) (Schaffer et al., 1975). Spectrophotometric measurements were performed with a Beckman DU70 spectrophotometer equipped with a thermostated cell compartment maintained at 25.0 °C.

Reduction of RNase. Reduced, denatured RNase was generated by incubating 5 mg of native enzyme overnight in 1 mL of 0.1 M Tris–acetate (pH 8.0) that was 2 mM in EDTA, 6 M in guanidine hydrochloride, and 0.14 M in DTT (Creighton, 1977). Immediately before use, the reduced, denatured enzyme was separated from excess DTT and guanidine hydrochloride by centrifugal gel filtration employing Bio-Gel P4 equilibrated with 0.1% acetic acid. The number of thiol groups per reduced RNase molecule was determined prior to each experiment by using the method of Ellman (1959). Typically, 1 mM reduced RNase contained 7.8 ± 1.5 mM SH groups.

Assay for RNase Oxidative Folding. The oxidative renaturation of ribonuclease activity was measured spectrophotometrically in the presence of cCMP as an RNase substrate. Tris–acetate buffer (pH 8.0), 4.5 mM cCMP, GSH and GSSG (at concentrations to yield a redox buffer of chosen composition), and 1.4 μM PDI were equilibrated at 25 °C. The assay was initiated by the addition of reduced, denatured RNase. The hydrolysis of cCMP resulting from the gain in ribonuclease activity was recorded continuously as an increase in absorbance at 296 nm. The uncatalyzed reaction (−PDI) was monitored in a parallel sample under identical redox conditions. The concentration of active RNase at any time (E_t, in μM) was calculated at each time from the first derivative of the absorbance versus time data (v_t, in μM/min) after correcting for the time-dependent depletion of cCMP during the assay and for the competitive inhibition of RNase by the hydrolysis product (CMP) using eq 1, where k_{cat} is the

$$E_t = v_t/\{k_{cat}[\mathrm{cCMP}]_t/([\mathrm{cCMP}]_t + K_{mc}(1 + [\mathrm{CMP}]_t/K_i))\} \quad (1)$$

turnover number for fully active RNase [14.3 ± 1.1 μmol of cCMP min^{-1} (μmol of RNase)$^{-1}$], K_{mc} is the K_m for cCMP under these conditions (8.0 ± 0.5 mM), and K_i is the inhibition constant for CMP (2.1 ± 0.4 mM). The kinetic constants k_{cat},

K_{mc}, and K_i that are substituted into eq 1 were determined independently in preliminary experiments under the same assay conditions. At any time t, E_t is the concentration of active RNase (μM), $[cCMP]_t$ is the instantaneous concentration of cCMP, and $[CMP]_t$ is the instantaneous concentration of CMP. The concentration of cCMP and CMP can be obtained from the initial concentration of cCMP, the observed absorbance, and the extinction coefficients of cCMP (ϵ = 0.19 mM^{-1} cm^{-1}) and CMP (ϵ = 0.38 mM^{-1} cm^{-1}) at 296 nm, pH 8.0.

Under most conditions, the reaction displays a reasonably linear, steady-state velocity, which in some cases is preceded by a short lag or burst in the regain of RNase activity. Lags are common observations in the uncatalyzed regeneration of RNase A and have been attributed to the prerequisite formation of RNase redox isomers that can be converted to the native protein (Schaffer, 1975). Steady-state rate measurements were made in the approximately linear region of the assay. Under some conditions, particularly near the optimum velocity, the linear region was reasonably small, and the velocity was measured immediately after any lag or burst. In these cases, the slope was determined by eye, since conventional least-squares fits or extrapolation of the velocity to zero time could not accommodate the lags and bursts in a reliable fashion.

All PDI-catalyzed reactions were corrected for the uncatalyzed rate of oxidative folding observed in a parallel control in the absence of PDI. Control experiments established that there was negligible (<5–10%) oxidation of GSH to GSSG during the 30–60-min assays. Replicate assays performed over the course of the experiment (over 300 individual assays) show an average deviation of 11%, although somewhat larger errors are observed at the extreme rates (both very low and high). Considering the presence of lags and bursts and the strong dependence on redox buffer composition, this is reasonable precision. The assay is linear with PDI concentration between 0.3 and 2 μM.

Direct comparison of the traditional, discontinuous assay with the continuous method gives identical results. This establishes the validity of the assay and confirms that the presence of cCMP or CMP during refolding does not affect the folding of RNase (Schaffer et al., 1975). Garel (1977) used the increase in slope without considering substrate depletion or product inhibition to follow the very early generation of an active RNase that has 0.01% the activity of the native enzyme.

Results

The addition of reduced, denatured RNase to a glutathione redox buffer containing PDI and the RNase substrate, cCMP, results in an increase in the activity of RNase A, which can be observed as a continual increase in the rate of cCMP hydrolysis (Figure 1A). The concentration of active RNase can be calculated at any time from the instantaneous slope of the absorbance versus time trace after considering the change in cCMP concentration and the formation of the RNase inhibitor, CMP (see the Experimental Procedures section for details) (Figure 1B). The activity observed in this continuous assay is comparable to that measured by withdrawing aliquots and determining RNase activity in a separate assay (Figure 1B). The PDI-catalyzed reaction is accompanied by an uncatalyzed reaction that is generally 1–5 times slower.

In the absence of a glutathione redox buffer and with catalytic concentrations of PDI, there is no substantial gain in RNase activity during the 30–60-min assay period. Over 60 min, the measured thiol concentration (mostly GSH) was constant within experimental error (±10%). Thus, under the conditions of these experiments there is no substantial contribution of oxygen-dependent oxidation of the protein, nor is there substantial autoxidation of GSH to GSSG (Wetlaufer et al., 1987).

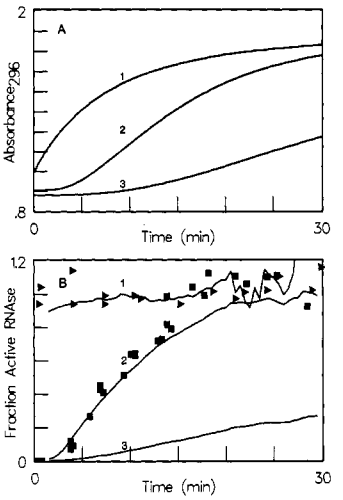

FIGURE 1: Assay of the oxidative folding of RNase. (A) Time course for the absorbance change at 296 nm produced by RNase-catalyzed hydrolysis of cCMP at pH 8.0 (0.1 M Tris–acetate buffer), 25 °C. Curve 1: After the addition of 8.4 μM native RNase A in the presence of 0.2 mM GSSG and 1.0 mM GSH. Curve 2: After the addition of 8.4 μM fully reduced RNase A in the presence of 1.4 μM PDI, 0.2 mM GSSG, and 1.0 mM GSH. Curve 3: After the addition of 8.4 μM fully reduced RNase A in the presence of 0.2 mM GSSG and 1.0 mM GSH without the addition of PDI. (B) The individual solid curves (1, 2, and 3) show the activity of RNase calculated from the instantaneous slopes of the corresponding curves shown in panel A according to the method described under Experimental Procedures (eq 1). The solid symbols show the RNase activity measured in parallel incubations of RNase with 1 mM GSH and 0.2 mM GSSG in the absence of cCMP assayed by withdrawing periodic aliquots and determining the RNase A activity at pH 5.0 with cCMP as the substrate: (■) fraction of activity regained from fully reduced RNase A (8.4 μM) in the presence of PDI (1.4 μM); (▶) fraction of activity observed with native RNase (8.4 μM). The solid curves are not theoretical fits of the data represented by the symbols but represent the activity determined in parallel samples by the continuous assay of RNase activity.

The PDI-catalyzed, oxidative folding of RNase displays saturation kinetics with respect to the concentration of RNase with a K_m of 8.0 ± 1.5 μM (data not shown). The k_{cat} at saturating RNase is 0.46 ± 0.05 μmol of RNase formed min^{-1} (μmol of PDI)$^{-1}$. With an RNase concentration of 8.3 μM, the velocity is linear with PDI concentration between 0.3 and 2 μM.

The catalyzed and uncatalyzed renaturation of RNase A were examined as a function of the redox buffer composition over a wide range of GSH and GSSG concentrations (Figure 2). The dependence of the velocity of active RNase formation on the GSH and GSSG concentration is complex. The velocity of both the catalyzed and uncatalyzed reaction shows a distinct optimum with respect to the GSH and GSSG concentration. In the presence of PDI, RNase is renatured most efficiently at [GSH] = 1 mM and [GSSG] = 0.2 mM; the uncatalyzed reaction displays a similar optimum for GSH and GSSG concentrations. Lower as well as higher GSH and GSSG concentrations support slower rates of oxidative folding. With increasing GSSG concentrations, the optimum GSH concentration increases and the optimum rate falls. For the uncatalyzed reaction, the results are in reasonable agreement with previous observations using fixed time or discontinuous assays of RNase activity (Saxena & Wetlaufer, 1970; Konishi et al., 1983).

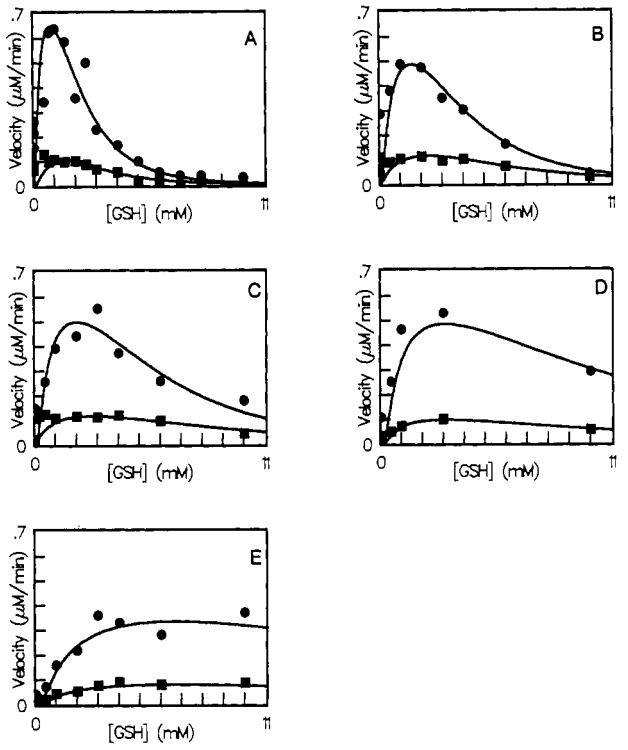

FIGURE 2: Effects of GSH and GSSG concentrations on the steady-state rate of RNase renaturation. All assays were performed at pH 8.0 (0.1 M Tris–acetate, 2 mM EDTA), 25 °C, and were initiated by the addition of fully reduced RNase (8.4 μM). Most of the points shown represent the average of duplicate or triplicate determinations. Approximately 300 independent rate measurements are represented. In each panel the solid circles represent the velocity in the presence of 1.4 μM PDI and the solid squares represent the velocity in the absence of PDI. Each panel represents a different, fixed concentration of GSSG: panel A (0.2 mM), panel B (0.5 mM), panel C (1 mM), panel D (2 mM), and panel E (4 mM).

Figure 3 shows the effect of GSSG concentration on the uncatalyzed and catalyzed oxidative regeneration of native RNase at a constant, optimum, GSH concentration of 1.0 mM. This figure corresponds to a perpendicular slice through the GSH optimum shown in Figure 2A. Clearly, under the conditions imposed, a narrow range of low GSSG concentration (0.01–0.2 mM) sharply enhances the rate of PDI-catalyzed RNase folding with the maximum rate occurring at 0.2 mM; concentrations of GSSG exceeding this optimum inhibit the reaction. Note that, under these conditions (8.3 μM RNase), 0.033 mM GSSG is stoichiometrically required to regenerate full RNase activity.

Discussion

PDI displays saturation kinetics with respect to the concentration of reduced RNase. The K_m observed (8 μM) is somewhat higher than but comparable to the value of about 2 μM reported by Lambert and Freedman (1983) for the PDI-catalyzed renaturation of randomly oxidized, denatured RNase in the presence of 10 μM DTT (no added oxidant); however, they noted that the reaction did not give a good fit to hyperbolic kinetics. The V_{max} of 0.43 μmol of RNase folded min^{-1} (μmol of PDI)$^{-1}$ ([GSH] = 1.5 mM, [GSSG] = 3.4 mM) is also comparable to the turnover number of approximately 1 min^{-1} for the GSH-dependent rearrangement of scrambled RNase (Lambert & Freedman, 1983).

At pH 8.0, the second-order rate constant for a simple, intermolecular thiol/disulfide exchange involving thiols with a normal pK_a of 8.6 is in the vicinity of 150 M^{-1} min^{-1} (Gilbert, 1990). This may be compared to the k_{cat}/K_m for the PDI-catalyzed reaction (5.4×10^4 M^{-1} min^{-1}), which describes the

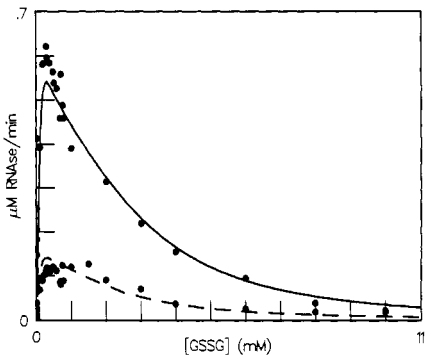

FIGURE 3: Effect of varying GSSG concentration on the PDI-catalyzed and uncatalyzed oxidative folding of reduced RNase at a fixed GSH concentration of 1.0 mM. All assays were performed at pH 8.0 (0.1 M Tris–acetate, 2 mM EDTA) and were initiated by the addition of 8.4 μM fully reduced RNase (solid curve) in the presence of 1.4 μM PDI and (broken curve) in the absence of PDI.

second-order reaction of PDI with RNase. This is a rather modest rate acceleration of about 400-fold, in keeping with earlier observations of the PDI-catalyzed reduction of the disulfide-containing peptide CYIQNC ($k_{cat}/K_m = 1.2 \times 10^5$ M^{-1} min^{-1}) (Gilbert, 1989). In actuality, this represents a lower limit on the observed rate acceleration since the actual substrate for the rate-determining step of the reaction of PDI with RNase under second-order conditions may require a thiol/disulfide redox isomer of RNase that is present as only a small fraction of the total RNase.

The k_{cat} of approximately 0.4 min^{-1} is also reasonably slow. This low efficiency of catalysis and the recent observations that PDI may perform a myriad of very diverse functions in the endoplasmic reticulum–Golgi complex have been the cause for speculation that PDI is not involved in the folding process in vivo. Bulleid and Freedman (1988) have shown that depletion of PDI significantly reduces the ability of microsomes to oxidatively fold γ-gliadin in a in vitro translation system. This observation coupled with the in vitro catalytic activity and the observation that high PDI levels are specifically associated with tissues that secrete large amounts of disulfide-containing proteins argues that disulfide bond formation and rearrangement is at least one of the likely in vivo functions of this protein (Lambert & Freedman, 1985; Bulleid & Freedman, 1988; Freedman, 1987, 1989).

Both in the presence and in the absence of PDI, the dependence of the rate of RNase regeneration on the GSH and GSSG concentrations of the folding redox buffer is complex (Figures 2 and 3). Increasing GSH and GSSG concentrations initially increases the rate, but higher concentrations of either are inhibitory. A distinct optimum concentration of GSH and GSSG is required to support the oxidative folding of RNase. For the uncatalyzed reaction, the establishment of a proper redox environment of the glutathione redox buffer is also required to support optimal oxidative folding rates. Too high a GSH concentration has been suggested to inhibit disulfide bond formation in the intermediates leading to native RNase, and too high a GSSG concentration has been suggested to promote the formation of nonproductive mixed disulfides between glutathione and RNase (Saxena & Wetlaufer, 1970; Konishi et al., 1982).

For a protein containing eight cysteine residues such as RNase, a bewildering array of intra- and intermolecular disulfide redox isomers exists. For RNase A, 7193 species with different numbers and arrangements of intra- and intermolecular disulfides could be formed (Scheraga et al., 1984), any of which could be a potential substrate for PDI and only one

of which represents the native, four-disulfide structure. During the oxidative folding of RNase in the absence of PDI, Creighton (1979) trapped a number of these redox isomers by alkylation during the folding reaction initiated with 0.2 mM GSSG (no added GSH). Intermediates with one to four disulfide bonds were observed to form rapidly (1–16 min) followed by a slower (hours) formation of the native molecule. Konishi et al. (1981) made similar observations using a wide range of glutathione redox buffers by fractionating the population of RNase redox isomers by glutathione mixed disulfide content.

In analogy with the folding pathway elucidated by his detailed studies of the oxidative folding of BPTI, Creighton (1978, 1979, 1988) has proposed that the uncatalyzed RNase oxidative folding proceeds by one predominant pathway in which the rate-limiting step in the overall folding process is the rearrangement of a three-disulfide-containing intermediate to the native species; a single transition state defining a single folding pathway is proposed to account for the observed kinetics of the generation of native RNase. In contrast, Konishi et al. (1982) propose that an equilibrium distribution of multiple RNase redox isomers is established rather rapidly and that correctly folded RNase is generated by up to six, multiple, parallel pathways. This difference has proved controversial (Creighton, 1988; Scheraga et al., 1987), yet either mechanism is capable of generating the complex dependence of the velocity on the redox buffer.

While a considerable literature and some controversy surrounds the mechanism of RNase oxidative folding in the absence of PDI, there have been few studies that address the effects of a glutathione redox buffer on the PDI-catalyzed, oxidative folding of reduced RNase. A priori, there is no reason to suspect that changes in the composition of the reox buffer will effect the PDI-catalyzed reaction and the uncatalyzed reaction in the same way. Two factors may contribute to the effects of GSH and GSSG concentrations on the observed velocity of the PDI-catalyzed reaction. In analogy with the uncatalyzed reaction, the glutathione redox buffer will undoubtedly influence the availability of the proper RNase redox isomer or isomers that are the substrate(s) for PDI. In addition, changes in the redox buffer may also influence the redox state and catalytic effectiveness of PDI itself.

At the concentration of PDI employed (1.4 μM), the velocity of the PDI-catalyzed reaction is a constant 4-fold faster than that of the uncatalyzed reaction over a wide range of GSH and GSSG concentrations, even though the individual rates change by more than 10-fold. However, under more oxidizing conditions (lower GSH and higher GSSG), PDI becomes a less effective catalyst, and the rate acceleration diminishes significantly (Figure 4).

Under conditions where the rate acceleration is constant, changes in the composition of the redox buffer must affect the catalyzed and uncatalyzed reactions by the same factor. The simplest interpretation is that the change in rate can be ascribed to changes in the distribution of RNase redox isomers that have the same effect on the rates of both the catalyzed and uncatalyzed reactions. With this interpretation, the same RNase redox isomer(s) would be involved in the rate-determining step(s) for both the catalyzed and uncatalyzed reactions, and PDI would accelerate the rate-determining step(s) without altering the distribution of RNase redox isomers. If multiple, parallel pathways actually contribute significantly to the folding, then PDI must catalyze them all with nearly the same effectiveness. Using DTT disulfide as the oxidant under conditions where RNase forms only two of the four

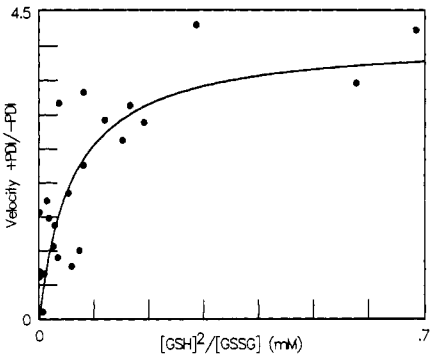

FIGURE 4: Ratio of the PDI-catalyzed velocity to the uncatalyzed velocity as a function of the composition of the glutathione redox buffer. The rate ratio (with 8.3 μM reduced RNase, 1.4 μM PDI, pH 8.0, 25 °C) is displayed as a function of the quantity $[GSH]^2/[GSSG]$. High $[GSH]^2/[GSSG]$ provides a more reducing redox environment. The rate enhancement at high $[GSH]^2/[GSSG]$ is a constant factor of 4.1 ± 0.8 between a $[GSH]^2/[GSSG]$ of 0.7 and 10 mM (data not shown). The solid curve is a hyperbola determined by a nonlinear least-squares fit of the entire data set (approximately 100 rate ratios). The curve is drawn with a limiting rate enhancement at a high $[GSH]^2/[GSSG]$ of 4.1 ± 0.8 and a K_{ox} of 0.06 ± 0.01 mM (eq 3).

disulfide bonds, Creighton et al. (1980) found that PDI increases the rate of disulfide bond formation but does not significantly alter the composition of the RNase redox isomers, in keeping with this interpretation.

The decrease in the catalytic effectiveness of PDI observed only under more oxidizing conditions requires that the rate of the PDI-catalyzed reaction changes more than that of the uncatalyzed reaction. This is consistent with a dithiol/disulfide transition of PDI itself in which the dithiol form is more active than the disulfide. A plot of the ratio of the rate of the PDI-catalyzed reaction to that of the uncatalyzed reaction is displayed as a function of the quantity $[GSH]^2/[GSSG]$ in Figure 4. Plots of the rate enhancement against [GSH]/[GSSG], [GSH], [GSSG], or $[GSH]^2$ do not show any such consistent trend.

For a dithiol/disulfide transition in a glutathione redox buffer, the fraction of enzyme in the dithiol form will be a hyperbolic function of $[GSH]^2/[GSSG]$ (Gilbert, 1990) (eq 3); the value of $[GSH]^2/[GSSG]$ where half of the protein

(2)

$$\frac{[E_{(SH)2}]}{[E_{tot}]} = \frac{[GSH]^2/[GSSG]}{K_{ox} + [GSH]^2/[GSSG]} \quad (3)$$

is reduced is equal to the thiol/disulfide exchange equilibrium constant (K_{ox}, eq 3). As the redox buffer becomes more oxidizing (lower $[GSH]^2/[GSSG]$), the catalytic effectiveness of PDI decreases. While there is scatter in the data as a result of the combination of error in the two measured velocities, Figure 4 suggests that the presence of a reduced (dithiol) form of PDI (present only at higher $[GSH]^2/[GSSG]$) is associated with a higher catalytic efficiency. The K_{ox} (0.06 mM) for the thiol/disulfide exchange with glutathione is low compared to that of other proteins, including thioredoxin (K_{ox} = 2 M) (Gilbert, 1990).

The requirement of a thiol form of PDI for activity has been suggested by chemical modification experiments. PDI is inactivated by alkylating agents only after reduction of the enzyme, and a logical role exists for an essential thiol in catalyzing the thiol/disulfide exchanges required for rearrangement of disulfide bonds (Saxena & Wetlaufer, 1970; Creighton et al., 1980; Freedman et al., 1988). These ob-

servations suggest that the rate-limiting step of the PDI-catalyzed folding of reduced RNase is a rearrangement or reduction involving a PDI thiol or dithiol rather than an oxidative process requiring a PDI disulfide. Although the chemistry of thiol/disulfide exchange suggests that a covalent disulfide between RNase and PDI is a logical intermediate in the catalytic reaction (Creighton et al., 1980), no evidence for such an intermediate has been reported.

Generally, the uncatalyzed renaturation of small cysteine-containing proteins that require the formation of disulfide bonds is considerably slower than the renaturation of small proteins without disulfide bonds or the same protein with disulfide bonds intact (Creighton, 1986; Baldwin, 1989). While disulfide bond formation may guide the folding process (or vice versa), disulfide bond formation imposes kinetic barriers on the renaturation process. The renaturation of RNase with its disulfides intact exhibits biphasic renaturation kinetics as a consequence of a required trans/cis isomerization of proline peptidyl bonds (Brandts et al., 1975; Cook et al., 1979). Normally, proline isomerization is considerably faster than the uncatalyzed, oxidative folding of reduced RNase, even under optimum conditions. However, the turnover number of the PDI-catalyzed oxidative folding approaches 0.5–1 min^{-1} under optimum concentrations of GSH and GSSG. This is comparable to the observed rate constant for the slow proline isomerization observed at pH 9 (0.6 min^{-1}) (Udgaonkar & Baldwin, 1988) and pH 6.2 (0.4 min^{-1}) (Schmid, 1986). Thus, it is possible that, under optimum conditions, the PDI-catalyzed oxidative renaturation of RNase may be at least partially limited by proline isomerization. Using a 5-fold lower concentration of PDI and a somewhat nonoptimal glutathione redox buffer (4 mM GSH, 0.4 mM GSSG), Lang and Schmid (1988) found that the enzyme peptidyl propyl isomerase, which catalyzes the cis/trans proline isomerization in proteins, does not affect the velocity of the PDI-catalyzed oxidative folding of RNase.

PDI catalyzes a variety of thiol/disulfide exchange processes involving a number of different protein and nonprotein thiols and disulfides. The lack of PDI specificity and the lack of a general consensus sequence that directs the formation of specific disulfide bonds in proteins (Thornton, 1981) would suggest that PDI is not designed to facilitate a specific, rate-limiting thiol/disulfide exchange reaction common to all proteins with disulfide bonds but rather that the enzyme, by some mechanism, is designed to provide a modest, nonspecific rate enhancement to thiol/disulfide exchange reactions in general. PDI may make up for its low catalytic efficiently by the exceptionally high local concentration of the enzyme that is present in the lumen of the endoplasmic reticulum. Estimates from the data of Hillson et al. (1984) show that PDI is present in bovine liver at a level of 0.81 g/kg of tissue, corresponding to a concentration of about 20 μM PDI if the enzyme were uniformly distributed throughout all cellular compartments. However, the local concentration in the lumen of the endoplasmic reticulum must be much higher, approaching the millimolar range. At these high local PDI concentrations, high catalytic efficiency is not a prerequisite for efficient formation and rearrangement of disulfide bonds during the synthesis, processing, and export of extracellular proteins.

How PDI gains access to thiols or disulfides that may be sterically shielded by the folded protein is unknown. There is no evidence that the protein actively participates in any unfolding process; however, normal thermal breathing motions, particularly in incorrectly folded proteins, may be sufficiently fast to allow PDI to sample a reasonably large number of conformations for the presence of a thiol or disulfide suitable for exchange.

Registry No. PDI, 37318-49-3; RNase, 9001-99-4; GSH, 70-18-8; GSSG, 27025-41-8.

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Catalysis of the Oxidative Folding of Ribonuclease A by Protein Disulfide Isomerase: Pre-Steady-State Kinetics and the Utilization of the Oxidizing Equivalents of the Isomerase†

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ABSTRACT: At low concentrations of a glutathione redox buffer, the protein disulfide isomerase (PDI) catalyzed oxidative renaturation of reduced ribonuclease A exhibits a rapid but incomplete activation of ribonuclease, which precedes the steady-state reaction. This behavior can be attributed to a GSSG-dependent partitioning of the substrate, reduced ribonuclease, between two classes of thiol/disulfide redox forms, those that can be converted to active ribonuclease at low concentrations of GSH and those that cannot. With catalytic concentrations of PDI and near stoichiometric concentrations of glutathione disulfide, approximately 4 equiv (2 equiv of ribonuclease disulfide) of GSH are formed very rapdily followed by a slower formation of GSH, which corresponds to an additional 2 disulfide bond equiv. The rapid formation of RNase disulfide bonds and the subsequent rearrangement of incorrect disulfide isomers to active RNase are both catalyzed by PDI. In the absence of GSSG or other oxidants, disulfide bond equivalents of PDI can be used to form disulfide bonds in RNase in a stoichiometric reaction. In the absence of a glutathione redox buffer, the rate of reduced ribonuclease regeneration increases markedly with increasing PDI concentrations below the equivalence point; however, PDI in excess over stoichiometric concentrations inhibits RNase regeneration.

The formation of disulfide bonds in extracellular, eukaryotic proteins requires both a source of oxidizing equivalents and a catalyst for the process. Cysteine-containing proteins are synthesized and cotranslationally inserted into the lumen of the endoplasmic reticulum in the reduced (SH) form. Disulfide bond formation occurs during or soon after translation, significantly preceding the appearance of the mature protein at the cell surface (Bergman & Kuehl, 1979; Peters & Davidson, 1982). Disulfide bond formation and rearrangement in in vitro oxidative folding systems is significantly slower than the in vivo process, so the need for some in vivo catalyst has been recognized for some time (Goldberger et al., 1963).

Protein disulfide isomerase (PDI),[1] an abundant protein of the endoplasmic reticulum, catalyses the oxidative folding of proteins in vitro (Lambert & Freedman, 1985) and most likely in vivo (Bulleid & Freedman, 1988). In vitro, oxidizing equivalents may be provided by a variety of disulfides (Hillson et al., 1984; Morin & Dixon, 1985; Varandani et al., 1975), but in vivo, the source of oxidizing equivalents is unknown. The enzyme was initially isolated by its ability to catalyze the oxidative renaturation of proteins that contain disulfide bonds (Goldberger et al., 1963). PDI has been implicated in the oxidative folding process although recently a number of other, very diverse functions have been suggested for the protein (Cheng et al., 1987; Obata et al., 1988; Koivu et al., 1987; Geetha-Habib et al., 1988; Wetterau et al., 1990).

In a glutathione redox buffer, PDI functions catalytically—multiple turnovers regenerate native RNase from the reduced form at the expense of oxidizing equivalents from GSSG (Lyles & Gilbert, 1991). However, PDI is a very abundant protein (Hillson & Freedman, 1984). If the enzyme were uniformly distributed in the cell, the intracellular concentration would be about 20 μM (Lyles & Gilbert, 1991), comparable to the total cellular concentration of glutathione disulfide (Gilbert, 1990). The local concentration of PDI in the lumen of the endoplasmic reticulum would, naturally, have to be much higher, likely approaching the millimolar range. Because of its potentially high, local concentration in vivo, the

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[1] Abbreviations: PDI, protein disulfide isomerase; RNase, bovine pancreatic ribonuclease A; GSH, glutathione; GSSG, glutathione disulfide; DTT, dithiothreitol; cCMP, cytidine 2',3'-cyclic monophosphate.

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