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Increased catalytic activity of protein disulfide isomerase using aromatic thiol based redox buffers

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Abstract—PDI is an enzyme that acts as a chaperone, shufflase, and oxidase during the folding of disulfide-containing proteins. The ability of aromatic thiols to increase the activity of PDI-catalyzed protein folding over that of the standard thiol glutathione (GSH) was measured. 4–Mercaptobenzoic acid (ArSH) increased the activity of PDI by a factor of three.

1. Introduction

Although the production of disulfide-containing proteins via recombinant means is routine, several problems still exist.[1] In many cases the proteins aggregate during in vivo folding to form inactive inclusion bodies instead of soluble, active protein.[2–5] The yield-limiting step in obtaining active protein from inclusion bodies is typically the in vitro formation of the correct disulfide bonds. Active protein is obtained by placing resolubilized inclusion bodies in the presence of a redox buffer. Redox buffers, which facilitate the formation of the correct disulfide bonds, typically consist of a small molecule thiol, a small molecule disulfide and, sometimes, denaturants. Unfortunately, in some cases the redox buffers alone can be ineffective.[1,3] To further aid in obtaining active protein, an enzyme, which increases the rate of protein folding is added to the redox buffer.[2,4–10] The most commonly used and best characterized enzyme[11–13] is protein disulfide isomerase (PDI).[2,4–10] However, PDI is expensive and has low catalytic activity.

PDI is a 57 kDa protein and a member of the thioredoxin superfamily of proteins.[14,15] PDI facilitates both in vivo and in vitro folding of disulfide-containing proteins.[14,16–25] The two active sites of PDI each consist of a Cys-Gly-His-Cys motif;[26] one cysteine thiol is solvent-exposed (N-terminal) and the other is buried (C-terminal).[27] The in vitro mechanism of PDI functions through an escape pathway (Fig. 1a): the N-terminal cysteine thiolate nucleophilically attacks a disulfide bond to form a mixed disulfide, then the C-terminal cysteine thiolate attacks the mixed disulfide bond thereby releasing the protein and leaving the active site of PDI in its (oxidized) disulfide form. Site-directed mutagenesis of the cysteine residues confirms that both cysteine residues are necessary for full catalytic activity.[19,28]

Thiols, as a component of the redox buffer, are known to affect the rates of thiol–disulfide interchange reactions involved in protein folding.[29–31] The redox buffer thiols can act as nucleophile, center thiol, or a leaving group (Fig. 1b).[29] Increasing the reactivity of these reactions can increase the overall rate of protein folding. Similarly, in the presence of PDI, the overall rate of protein folding can be increased due to the redox buffer interacting with PDI and the protein being folded.

In vitro protein folding with PDI is accomplished in the presence of a small molecule redox buffer, typically glutathione (a naturally occurring tri-peptide)–glutathione disulfide (GSH–GSSG). A limited number of other redox buffers have been investigated and were found to be less efficient. Optimum conditions for folding a protein in vitro in the presence and absence of PDI using GSH–GSSG were determined by Gilbert and found to be the same.[32,33] GSH–GSSG is also the most

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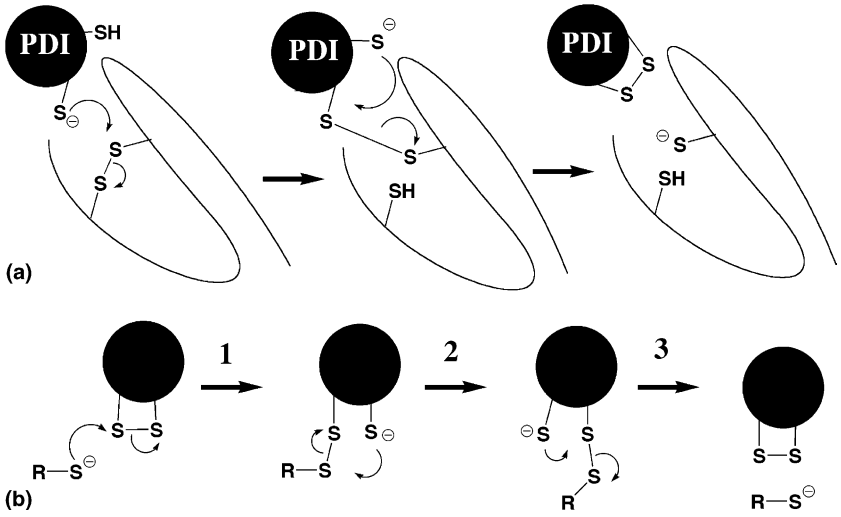


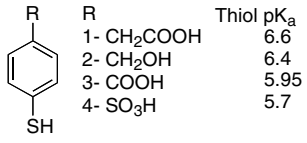
Figure 1. (a) PDI escape pathway as described in the text. (b) Three examples of protein-redox buffer thiol–disulfide interchange reactions, where the redox buffer thiol (R–S) is acting as a 1—nucleophile, 2—a center thiol, and 3—a leaving group.

commonly used redox buffer for folding disulfide-containing proteins in the absence of PDI. Recently, the initial folding velocity of ribonuclease A (RNase A) in the presence of GSH–GSSG without PDI was significantly improved by replacing GSH with an aromatic thiol.[29,30] Thus, aromatic thiols might increase the velocity of protein folding in the presence of PDI. An increase in the activity of PDI would be useful for the production of recombinant disulfide-containing proteins. The ability of aromatic thiols to increase the activity of PDI is investigated herein.

2. Methods and results

A series of *p*-substituted aromatic thiols **1**–**4** (Scheme 1) was tested for its ability to enhance the catalytic activity of protein disulfide isomerase (PDI). The thiols were selected because they increased the velocity of folding scrambled RNase A to native RNase A in the absence of PDI relative to glutathione (GSH).[29,30] Scrambled RNase A (sRNase A), the standard substrate for measuring PDI activity, is fully oxidized RNase A with a relatively random distribution of non-native disulfide bonds. The velocity of sRNase A folding at pH 7.0 was measured at various concentrations of PDI in the presence of each of the thiols.

Protein folding reactions were initiated by simultaneous addition of a concentrated sRNase A solution and PDI (PDI rapidly equilibrates with the redox buffer in situ;[17]) they were performed on a scale of 500 μL at 25 °C, at pH 7.0 (Bis–Tris–propane/AcOH buffer, 10^{-3} M EDTA). All folding reactions contained 25 μM protein, 1 mM EDTA, 0.5–1.5 μM PDI, varying concentrations of each of the four thiols or GSH, and 0.2 mM disulfide (GSSG, or aromatic disulfide). Protein folding was followed by measuring the enzymatic activity using the discontinuous assay developed by Konishi and Scheraga,[34,35] which was modified as previously described.[29,30] The concentration of sRNase A, 0.3 mg/mL, is typical for in vitro protein folding experiments and allows comparison with previously collected data.[1–3] Gilbert observed that the optimal redox buffer conditions (concentrations of GSH and GSSG) for folding RNase A with and without PDI entailed using 0.2 mM glutathione disulfide (GSSG).[32] Optimal redox buffer conditions for folding RNase A using aromatic thiols, in the absence of PDI, are also at 0.2 mM disulfide (aromatic disulfide or GSSG).[29,30] Side-by-side comparison of the PDI-catalyzed folding of sRNase A, using thiol **1** and GSSG or the aromatic disulfide of **1**, revealed that no observable velocity enhancement is gained by using an aromatic disulfide. Therefore, the commercially available GSSG was used as the disulfide in all subsequent folding experiments.



Scheme 1. *para*–Substituted aromatic thiols **1**–**4**.

To maximize the initial folding velocity, V_0, of RNase A the thiol concentration was varied.[29,30] The optimal thiol concentration in the presence of 0.5 μM PDI was similar to that observed in the absence of PDI (Fig. 2a).[29,30] Paralleling experiments performed in the absence of PDI, velocities were measured side-by-side in the presence of 0.5 μM PDI under optimal conditions (Table 1).[29] A significant increase in the initial velocity was observed in the presence of PDI for thiols **1**–**3** and GSH. No significant increase in the initial velocity was observed for thiol **4**, and thus it was not investigated further.

For all the thiols, the optimal folding conditions occurred at approximately the same concentration of protonated thiol, $[RSH]_{prot}$, and not total thiol, (protonated thiol, RSH, plus thiolate, RS^-). Since the concen-

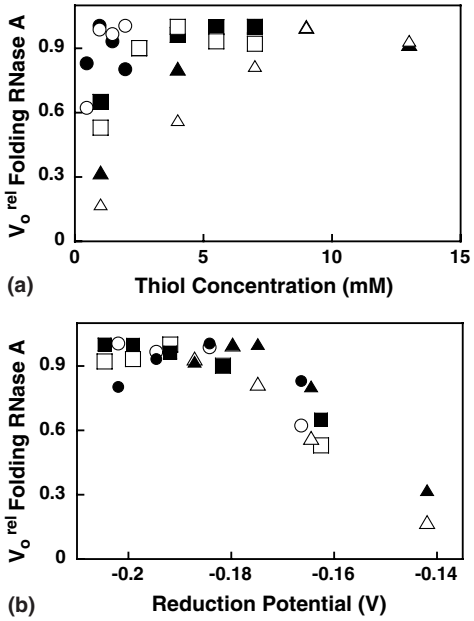


Figure 2. Relative initial velocities, V_0^{rel}, for the folding of scrambled RNase A versus thiol concentration and reduction potential. The maximum value for each compound with and without PDI was normalized to 1. Closed symbols are with 0.5 μM PDI and open symbols are without PDI: glutathione (circles), **1** (squares), and **3** (triangles). Disulfide concentration was held constant (0.2 mM) and the thiol concentrations were varied. Absolute V_0 values are in Table 1.

tration of the small molecule disulfide was fixed, optimal folding conditions also occurred at about the same reduction potential, $\log([RSH]^2_{prot}/[RSSR])$, and at about the same $[RSH]_{prot}$ to[RSSR] ratio. In the case of the reduction potential we assume an equilibrium constant of close to 1 between one monothiol and the disulfide of another monothiol at low pH where almost all of the thiol groups are protonated. The K_{eq} value between GSSG and **1** and **4** for the formation of GSH and aromatic disulfide are 1.0 ± 0.2 and 0.67 ± 0.02, respectively, at low pH.[29] For thiol **3** the K_{eq} is approximately 0.6. The initial folding velocity as a function of reduction potential is shown in Figure 2b.

To further characterize the effects of **1**–**3**, the concentration of PDI was varied. The initial velocities of RNase A folding at the optimal reduction potential with PDI (0.5, 1.0, and 1.5 μM PDI) were measured side-by-side for thiols **1**–**3** and GSH (Fig. 3). The turnover frequency of PDI (Δ folding velocity/Δ [PDI]) was determined from

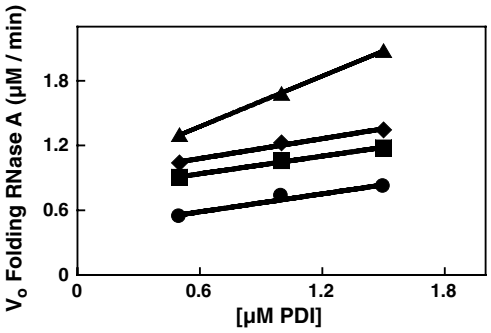


Figure 3. Initial velocity V_0, for the folding of scrambled RNase A using glutathione (○) and aromatic thiols **1**–**3** (**1**–■, **2**–◆, **3**–▲) versus various concentrations of PDI.

the slope of the velocity versus [PDI] curve. The slope at low PDI concentrations is observed to be linear as reported previously for redox buffers composed of GSH–GSSG.[28,36] The turnover frequencies for PDI using glutathione and aromatic thiols **1** and **2** as redox buffers were similar and comparable to those previously measured.[28,36] Aromatic thiol **3** increased the turnover frequency by a factor of three.

3. Discussion

The catalytic ability of PDI to fold sRNase A to native RNase A is well documented. Typically, in vitro, a redox buffer composed of a small molecule thiol and a small molecule disulfide is also added to the reaction mixture (glutathione–glutathione disulfide, cysteamine-cystamine, or redDTT–oxDTT).[32,33,37] The concentration and molecular structure of the small molecule redox buffer components affect the catalytic (oxoreductase) activity of PDI. The effect is a result of the small molecule's involvement in the underlying thiol–disulfide interchange reactions.

In the absence of PDI, the thiol–disulfide interchange reactions involved in protein folding appear to be somewhat similar to those involving small molecules;[29,30,38] in the presence of PDI this is not the case. A kinetic analysis of the rates involved in the protein disulfide bond formation of a model one-disulfide-protein[16] in the presence and absence of PDI performed by Creighton confirmed this. Without PDI, the relative reaction rates in the presence of cysteamine ($HSCH_2CH_2NH_2$) and

Table 1. Folding of scrambled RNase A with and without 0.5 μM protein disulfide isomerase (PDI) at pH 7.0

Additive	[ArSH] (mM)	V_0 (μM min^{-1}) (no PDI)[a]	V_0 (μM min^{-1}) (0.5 μM PDI)[a]	Turnover frequency $\Delta V_0/\Delta$ [PDI][b]
1	4.0	0.48 ± 0.10	0.73 ± 0.04	0.27 ± 0.02
2	5.3	0.58 ± 0.10	0.85 ± 0.04	0.31 ± 0.03
3	13	0.63 ± 0.11	1.0 ± 0.09	0.78 ± 0.01
4	22	0.73 ± 0.07	0.73 ± 0.05	Na[c]
GSH	1.0	0.06 ± 0.005	0.55 ± 0.03	0.28 ± 0.06

[a] The error corresponds to the 95% confidence interval, $ts/N^{0.5}$ where N is the number of data points, s is the standard deviation, and t is from the t-test table. The error was determined from four side-by-side runs comparing all five thiols.
[b] Standard error.
[c] Value not determined.

GSH were found to be similar to those predicted based only on the thiol pK_a values.[39] The predicted relative rates were based on measurement of thiol–disulfide interchange reactions involving only small molecules. In the presence of PDI, the predicted and measured relative reaction rates were no longer similar. The observed differences were attributed to undetermined small-molecule-protein interactions at or near one of the four active-site cysteine residues of PDI.

In the absence of PDI, a series of structurally diverse *para*-substituted aromatic thiols increased the velocity of RNase A folding by a factor of 4–20 times over that observed in the presence of GSH.[29,30] The velocities were comparable to the expected relative velocities based on the thiol pK_a values and the measurement of thiol–disulfide interchange reactions involving only small molecules.[39] Therefore, it was important to determine if aromatic thiols would enhance the PDI-catalyzed folding of RNase A, especially since they significantly enhance the folding velocity of RNase A in the absence of PDI.

The folding rate of sRNase A with GSH and thiols **1**–**3** was increased by the addition of 0.5 μM PDI (Table 1). The greatest folding rates in the presence of PDI were obtained with aromatic thiols. The largest relative increase was with GSH, in large part due to its low rate in the absence of PDI. The velocity with thiol **4** did not change significantly upon addition of PDI.

The effect a redox buffer has on PDI can be measured by the turnover frequency (Δ folding velocity/Δ [PDI]). The turnover frequency has the advantage that it measures the catalytic activity of PDI and corrects for the large differences in rates observed in the absence of PDI. The turnover frequencies of thiols **1** and **2** are the same as that of GSH. This observation suggests that the manner in which PDI acts in the presence of thiols **1** and **2** and GSH is similar (Fig. 3). Thiol **3** increases the turnover frequency of PDI by a factor of three. Due to the contrasting results with thiols **1**–**4**, it can be concluded that the thiol pK_a value does not correlate with folding rates in the presence of PDI as it does in the absence of PDI. The enhanced folding velocities are therefore attributed to undetermined aromatic thiol–protein interactions.

PDI is the most effective in vitro protein catalyst for folding disulfide-containing proteins obtained from inclusion bodies, although it is not without drawbacks. In addition to providing enhanced oxidative properties as an oxidoreductase, PDI is thought to provide chaperone activity that aids in exposing kinetically trapped disulfide bonds.[11,37,40–43] By exposing these kinetically trapped intermediates, PDI assists the overall production of active protein by allowing thiol–disulfide interchange reactions to occur. The drawbacks of PDI are that it requires the use of nearly stoichiometric amounts relative to the protein of interest, and is expensive.

Using aromatic thiol **3** with PDI would be advantageous for folding disulfide-containing proteins obtained from inclusion bodies. With the increased turnover frequency in the presence of **3**, a smaller amount of enzyme would be required. The increased velocity could also result in an increase in the yield of active protein. It is expected that 1/3rd the typical amount of PDI would be needed using the commercially available thiol **3**.

In summary, PDI increases the folding velocity of sRNase A to native RNase A in the presence of aromatic thiols **1**–**3**. The optimal reduction potential for folding RNase A without PDI is the same as with PDI. In the presence of PDI, the thiol pK_a values did not correlate with the observed rate enhancement, as is the case in the absence of PDI. The best aromatic thiol, **3**, increases the turnover frequency of PDI relative to GSH by a factor of three and is commercially available. Compound **3** could be used to decrease the amount of PDI needed to fold proteins, and to increase the yield of active protein obtained from inclusion bodies.

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