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Folding Disulfide-Containing Proteins Faster with an Aromatic Thiol

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Abstract: The traditional method for in vitro folding of disulfide-containing proteins is slow and involves a redox buffer of glutathione and glutathione disulfide. To increase the folding rate and to gain insight into the folding process, we replaced glutathione, an aliphatic thiol, with a commercially available aromatic thiol, 4-mercaptobenzeneacetate (**1**). Aromatic thiol **1** was selected due to its enhanced nucleophilicity and its enhanced leaving-group ability relative to glutathione at pH 7.7. To demonstrate the advantages of **1**, the folding of reduced and scrambled RNase A at pH 7.0 and 7.7 in the presence of **1** and glutathione was investigated. For each set of folding conditions, the optimum concentration of each thiol was initially determined and then the folding rates in the presence of each thiol were measured concurrently. In all cases examined, the folding rate enhancement with the aromatic thiol was 5− 6-fold. Furthermore, under similar conditions folding rates were almost identical with either reduced or scrambled RNase A. In addition the 5−6-fold folding rate enhancement varied only slightly with pH, 7.0 vs 7.7.

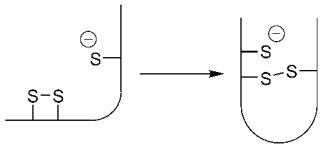
Introduction

Almost all pharmaceutically relevant proteins and many extracellular proteins contain disulfide bonds. To obtain these proteins in biologically active form, it is often necessary to employ in vitro folding of inclusion bodies or synthetic peptides.[1−4] A significant challenge for the in vitro folding process is the rapid formation of the correct disulfide bonds between cysteine residues. Disulfide bonds are the only covalent bonds formed during in vitro folding and their formation is usually the rate-determining step. Formation of native disulfide bonds involves thiol−disulfide interchange reactions. These interchange reactions occur within the protein itself (Scheme 1) or between the protein and the redox buffer (Scheme 2), which is a mixture of small-molecule thiols (RSH) and disulfides (RSSR). In vitro, the small molecule thiol is usually dithiothreitol (DTT, pK_a = 9.2, 10.1), mercaptoethanol (ME, pK_a = 9.6), or a cysteine derivative such as glutathione (pK_a = 8.7). Surprisingly, very few other thiols have been investigated as redox buffers.

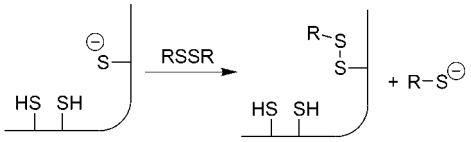
In vivo, the thiol−disulfide interchange reaction is catalyzed by protein disulfide isomerase (PDI). Each active site of PDI contains two cysteine thiol groups in a CXXC motif (two cysteines separated by two amino acids);[5] one thiol is solvent-exposed and the other is buried.[6] The solvent-exposed thiol has a low pK_a value, 6.7, and is exceptionally reactive with disulfides compared to small molecule aliphatic thiols, such as cysteine derivatives.[7,8] However, the use of PDI for in vitro folding is usually prohibitive as it is expensive, requires the use of approximately stoichiometric amounts relative to the protein of interest, and requires an additional protein purification step after catalysis.[9] The dithiol nature of PDI has recently been modeled with a small molecule, which was shown to increase the yield of active protein but not the rate constant for folding.[10]

To increase the rate of in vitro folding of disulfide-containing proteins, we envisioned utilizing a small-molecule thiol with a thiol pK_a value similar to that of the solvent-exposed thiol in PDI. Furthermore, our rationally designed thiol should have enhanced reactivity toward disulfides at pH 7 relative to

Scheme 1



Scheme 2



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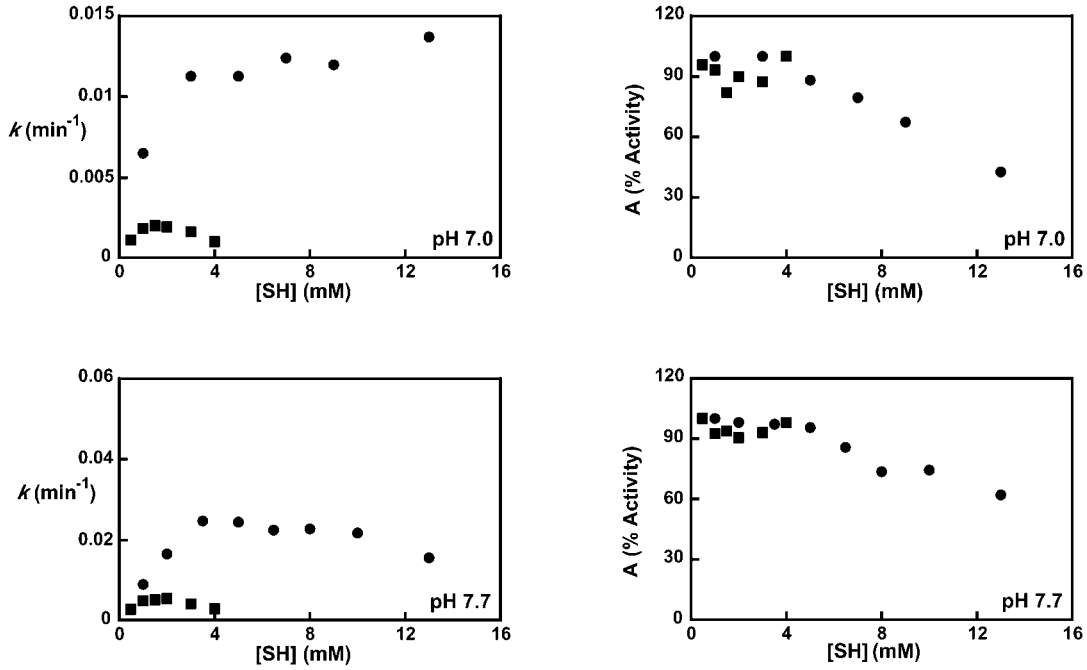

Figure 1. Rate constant, *k*, and maximum percent activity, *A*, for the folding of scrambled RNase A versus varying concentrations of thiol, RSH: glutathione (squares) and **1** (circles). Assays were performed at pH 7.0 or 7.7 (as indicated on the graph) and 25 °C in the presence of 0.2 mM GSSG and 1 mM EDTA. Maximum percent activity, *A*, was normalized to 100% for each compound.

glutathione. To accomplish our goal we prepared aromatic thiols, which despite advantageous properties have never been investigated previously for protein folding. Aromatic thiols have lower pK_a values (pK_a = 3−7) than aliphatic thiols (pK_a = 7−11) and are more reactive in thiol−disulfide interchange reactions than aliphatic thiols with similar pK_a values.[11−14]

Herein, we report the ability of an aromatic thiol to increase the folding rate of reduced and oxidized RNase A relative to glutathione. Initial studies determined the optimum concentration of aromatic thiol and glutathione required for protein folding. The protein folding rates of the two thiols (aromatic thiol and glutathione) at their optimum concentrations were then measured side by side. The effect of pH on the relative and absolute folding rates was then examined.

Results

The selection of the aromatic thiol was based upon its thiol pK_a value and water solubility. The pK_a value of the aromatic thiol should be similar to that of PDI (6.7).[7] Because most protein folding assays are performed in aqueous media at close to neutral pH, the aromatic thiol chosen must be water-soluble at pH 7 in both the thiol and disulfide forms. Compound **1**, 4-mercaptobenzeneacetate (pK_a = 6.6),[14] was selected on the basis of these criteria and was purchased or synthesized following literature procedures.[15,16] The effect of **1** on the rate of protein folding compared to glutathione (pK_a = 8.7) was determined by a variation of the discontinuous assay developed by Konishi and Scheraga.[17,18] In the assay detailed herein, the wavelength of observation was shifted from 286 to 292 nm to lower the background absorbance of aromatic thiol and 2′,3′-cyclic CMP (cCMP). In some cases, reduced RNase A was replaced with scrambled RNase A, an oxidized form of RNase A with a random distribution of four disulfide bonds.[19] A continuous assay was not practical due to the large background absorbance of the aromatic thiol.[9] Protein folding rate constants are illustrated in Figures 1 and 2 and were determined at either pH 7.0 (0.10 M Bis-tris propane acetate) or 7.7 (0.10 M Tris acetate).

To determine the folding rate constant of scrambled RNase A (Figure 1), aliquots were withdrawn at prescribed times from a mixture at 25 °C containing 0.025 mM scrambled RNase A, 0.2 mM glutathione disulfide (GSSG), 1.0 mM EDTA, and varying concentrations of reduced glutathione (GSH) or **1**. The concentration of 0.2 mM glutathione disulfide was chosen because Lyles and Gilbert[9] had shown it to be the optimum concentration for PDI-catalyzed folding of fully reduced RNase A at pH 8. The EDTA was added to suppress metal-catalyzed oxidation of the thiols by oxygen. The aliquots were immediately assayed for RNase A activity by following the hydrolysis of cCMP at pH 5 for 2 min. The solution was lowered to pH 5 and the redox reagents were diluted in an effort to minimize any folding during the 2 min of the assay.[9,20,21] A plot of RNase A activity versus time was fitted to a single exponential defined as percent activity = $A(1 - e^{-kt})$, where percent activity is proportional to the activity of fully folded native RNase A (Figure 3). The constants *A* and *k* represent the maximum

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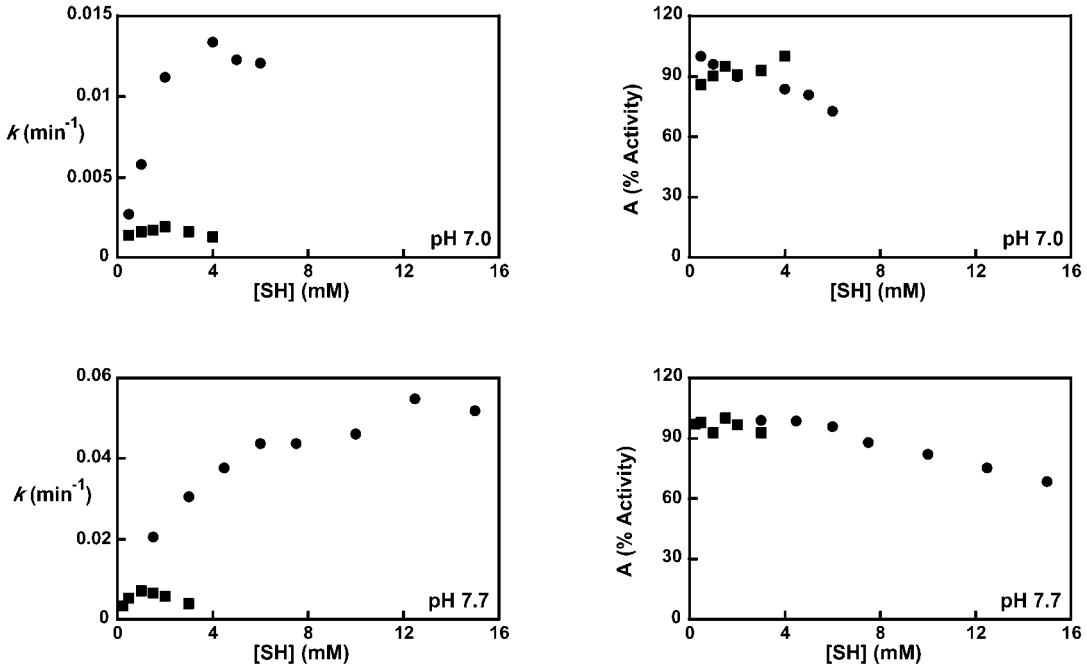
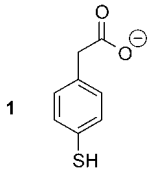


Figure 2. Rate constant, *k*, and maximum percent activity, *A*, for the folding of reduced RNase A versus varying concentrations of thiol, RSH: glutathione (squares) and **1** (circles). Assays were performed at pH 7.0 or 7.7 (as indicated on the graph), and 25 °C in the presence of 0.5 mM GSSG and 1 mM EDTA. Maximum percent activity, *A*, was normalized to 100% for each compound.

percent activity and the folding rate constant, respectively. The percent activity should correspond closely to the percent native protein, as was indicated previously for glutathione-folding studies.[18] Some of the folding intermediates do possess catalytic activity,[22−25] but these intermediates are likely to be poorly populated during the folding process and/or possess low specific activity in the enzymatic assay.[22,26,27]



The same general procedure was used to determine the folding rate constant of reduced RNase A (0.025 mM) (Figure 2) with the exception of increasing the concentration of GSSG to 0.5 mM. Because folding of reduced RNase A to native protein results in the net consumption of 4 equiv of GSSG, a higher initial concentration of GSSG was employed to maintain relative uniformity.

To elucidate the advantage of using aromatic thiols to fold proteins relative to traditional methods, the optimum concentrations of glutathione or **1** for protein folding at pH 7.0 and 7.7 were determined (Figures 1 and 2). The optimum concentration was defined as the concentration of thiol at which the initial rate of protein folding, $A \times k$, was at a maximum. The rates of protein folding at the optimum concentrations of glutathione

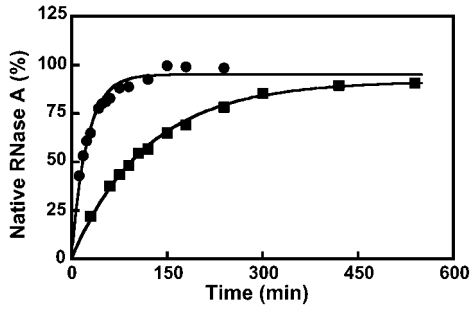


Figure 3. Folding of scrambled RNase A (0.025 mM) at the optimum concentration of glutathione (squares) or **1** (circles). Assays were performed at pH 7.7 and 25 °C in the presence of 0.2 mM GSSG and 1.0 mM EDTA. For the second batch of scrambled RNase A, the optimum concentrations of glutathione and **1** were 2.0 and 6.5 mM, respectively (Table 1).

and **1** were then measured side by side at each pH value (Tables 1−3, Figure 3). In addition, at pH 7.7 the folding rates were measured side by side at a constant thiol concentration (2 mM) which is the optimum concentration of glutathione for folding scrambled RNase A at pH 7.7 (Table 4). The complete process, including determining the optimum concentrations, was repeated for each batch of protein investigated. The results obtained from the folding experiments with glutathione at pH 7.7 were consistent with published values for scrambled (0.010 or 0.006 min^{-1})[10,28] or reduced RNase A (0.007 or 0.02 min^{-1})[9,29] obtained at or near pH 7.7.

To establish the validity of the data, several control experiments were performed. (1) To ensure that the rate increase was independent of the batch of scrambled RNase (Tables 1 and 2), several batches were tested, including one from commercial sources (batch 1). The relative rates of protein folding were always within error of each other, although the absolute rate

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Table 1. Folding of Three Separate Batches of Scrambled RNase A at pH 7.0[a]

additive	concn (mM)	concn (mg/mL)	A (%)	$k \times 10^3$ (min^{-1})	relative rate
			Batch 1		
glutathione	1.0	0.31	105 ± 15	2.1 ± 1.0	1
1	6.0	1.0	97 ± 21	14 ± 2	6.9 ± 2.7
			Batch 2		
glutathione	1.5	0.46	98 ± 19	2.8 ± 0.7	1
1	5.0	0.84	100 ± 15	13.8 ± 0.9	5.2 ± 1.4
			Batch 3		
glutathione	1.5	0.46	117 ± 13	2.0 ± 0.1	1
1	3.0	0.50	107 ± 4	11.2 ± 0.3	5.7 ± 0.3
			Overall Average		
glutathione	1.33	0.41	107 ± 10	2.3 ± 0.4	1
1	4.67	0.78	101 ± 5	13 ± 2	5.8 ± 0.9

[a] For the results from each batch the error corresponds to the 95% confidence limit, $ts/N^{0.5}$, 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 three assays for batch 1 and six assays (three duplicate side by side runs comparing glutathione to **1**) for batches 2 and 3. The error for the overall average is the standard deviation when each batch is treated as a single point.

Table 2. Folding of Scrambled RNase A at pH 7.7[a]

additive	concn (mM)	concn (mg/mL)	A (%)	$k \times 10^3$ (min^{-1})	relative rate
			Batch 2		
glutathione	2.0	0.61	90 ± 18	8.2 ± 0.5	1
1	6.5	1.1	94 ± 15	40 ± 4	4.9 ± 0.4
			Batch 3		
glutathione	2.0	0.61	94 ± 3	5.0 ± 0.6	1
1	5.0	0.84	84 ± 14	25 ± 1	5.0 ± 0.8
			Average		
glutathione	2.0	0.61	92	6.6	1
1	5.8	1.0	89	32	5.0

[a] The error corresponds to the 95% confidence limit, $ts/N^{0.5}$, 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 six assays, three duplicate side by side runs comparing glutathione to **1**. Batch 1, which was obtained from commercial sources, became unavailable from the vendor during the course of this work.

Table 3. Folding of Reduced RNase A at pH 7.0 and 7.7[a]

additive	concn (mM)	concn (mg/mL)	A (%)	$k \times 10^3$ (min^{-1})	relative rate
			pH 7.0		
glutathione	2.0	0.61	114 ± 21	2.0 ± 0.4	1
1	4.0	0.67	98 ± 4	11.7 ± 1.2	5.9 ± 1.3
			pH 7.7		
glutathione	1.0	0.31	101 ± 4	7.5 ± 1.2	1
1	7.5	1.3	93 ± 5	33 ± 4	4.5 ± 0.7

[a] See footnote *a* in Table 2.

constants did vary somewhat. (2) To demonstrate that the enhanced folding rate constant was due to the aromatic thiol group, we measured protein folding rates at the optimum concentration of glutathione (determined in the absence of additives) in the presence of *p*-hydroxyphenylacetate (5 mM, pH 7.7, for reduced and scrambled RNase A, and 12 mM, pH 7.7, for scrambled RNase A). *p*-Hydroxyphenylacetate is an analogue of **1** in which the thiol group is replaced with a hydroxyl group. No appreciable change relative to glutathione alone was observed at either concentration. The folding rate constant, k, decreased less than 15% and the maximum percent activity, A, decreased less than 10%. (3) To confirm that **1** was not stimulating or inhibiting the native enzyme in the cCMP assay, aliquots were removed from a mixture containing native RNase A (0.025 mM), **1** (15 mM), and glutathione disulfide (0.2 mM) at prescribed times and the enzyme's activity was determined with the cCMP assay. No measurable change in activity was observed with time. The activity was the same as that of a 0.025 mM solution of native RNase A. (4) In fitting the data to the equation percent activity $= A(1 - e^{-kt})$, it is assumed that scrambled RNase A itself has little or no activity. To verify this assumption, the activity of a scrambled RNase A solution (0.025 mM) containing no thiols was measured. Essentially no activity was found.

Table 4. Folding of Reduced and Scrambled RNase A at pH 7.7 and 2.0 mM Thiol[a]

additive	concn (mM)	concn (mg/mL)	A (%)	$k \times 10^3$ (min^{-1})	relative rate
			Scrambled RNase A		
glutathione	2.0	0.61	102 ± 5	6.7 ± 1.8	1
1	2.0	0.34	98 ± 4	14 ± 6	2.2 ± 0.4
			Reduced RNase A		
glutathione	2.0	0.61	115 ± 7	7.0 ± 1.3	1
1	2.0	0.34	93 ± 5	18 ± 2	2.6 ± 0.5

[a] See footnote *a* in Table 2. The error was determined from three assays, three side-by-side runs.

Table 5. Folding of SRNase A with Various Disulfides at pH 7.7[a]

disulfide additive	**1** (mM)	disulfide (mM)	A (%)	$k \times 10^3$ (min^{-1})	relative rate
GSSG	4.0	0.2	102 ± 28	26 ± 6	1
2	4.0	0.2	91 ± 32	25 ± 6	1.0 ± 0.1
no disulfide	4.0	0.0	102 ± 28	28 ± 6	1.1 ± 0.1

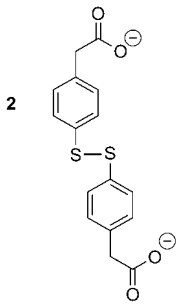
[a] See footnote *a* in Table 2. The error was determined from three assays, three side-by-side runs.

Table 6. Folding of Reduced RNase A with Various Disulfides at pH 7.7[a]

disulfide additive	**1** (mM)	disulfide (mM)	A (%)	$k \times 10^3$ (min^{-1})	relative rate
GSSG	4.0	0.5	113 ± 9	26 ± 11	0.8 ± 0.2
GSSG	4.0	0.2	112 ± 20	33 ± 5	1
2	4.0	0.2	104 ± 22	33 ± 3	1.0 ± 0.2

[a] See footnote *a* in Table 2. The error was determined from three assays, three side-by-side runs.

To investigate the importance of the disulfide on protein folding rates, glutathione disulfide was replaced with aromatic disulfide **2**, the oxidized form of **1**. Protein folding rates were determined in the presence of 0.2, 0.5, and 1.0 mM aromatic disulfide. The optimum concentration of aromatic disulfide was 0.2 mM. At higher aromatic disulfide concentrations the A value (maximum percent refolded) decreased. When 0.2 mM aromatic disulfide was employed, the optimum concentration of aromatic thiol was 4 mM. The folding of RNase A in the presence of glutathione disulfide or aromatic disulfide **2** was then measured side by side (Tables 5 and 6). The concentration of **1** in these experiments was 4.0 mM, as it is close to the optimal value for each disulfide. The folding of scrambled RNase A in the absence of disulfide was also investigated. Scrambled RNase A, unlike reduced RNase A, does not require oxidation to form native RNase A. No significant difference in k or A was detected for any of these variations in disulfide.



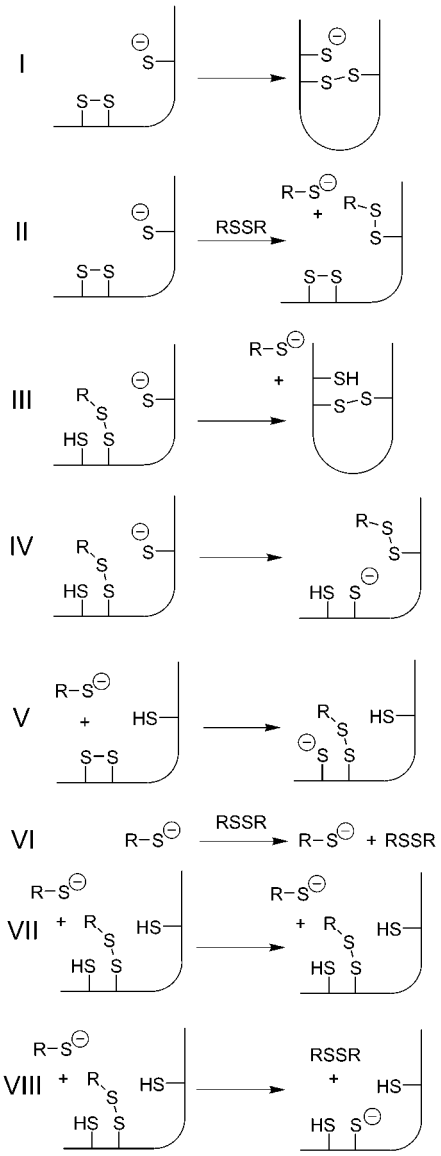
Discussion

Explanation of the enhanced folding rate constants achieved with aromatic thiols requires consideration of the reactions that take place during protein folding. During the in vitro folding of disulfide containing proteins, eight variations of the thiol−disulfide interchange reaction can occur (Scheme 3). The first four reactions in Scheme 3 involve the protein thiolate acting as the nucleophile. The last four reactions involve the small-molecule thiolate acting as the nucleophile. These thiolates can nucleophilically attack a protein disulfide (reactions I and V), a small-molecule disulfide (reactions II and VI), or a mixed disulfide between the small-molecule thiol and a protein thiol (reactions III, IV, VII, and VIII). As detailed below, aromatic thiols are better nucleophiles, better leaving groups, and better central thiols than glutathione at pH 7.0 or 7.7.[12,14] Therefore, the observed rate constants for reactions II−VIII are expected to be greater with aromatic thiol than with glutathione. We confine our comparison to glutathione, but very similar comparisons can be made with either mercaptoethanol or DTT.

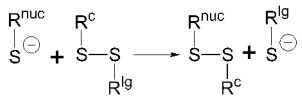
The inherent nucleophilicity of aromatic thiolate **1** and glutathione thiolate is approximately the same, but at pH 7.0 or 7.7 the bulk of the glutathione ($pK_a = 8.7$) is in the unreactive thiol form while the bulk of aromatic thiol ($pK_a = 6.6$) is in the reactive thiolate form.[14] Therefore, at pH 7.0 or 7.7 compound **1** will be a better nucleophile than glutathione.[14] Prior work has shown that, at pH 7.0, the observed rate constant for reaction with a small-molecule disulfide, 2-pyridyldithioethanol (2-PDE), is 32 times greater for **1** than for glutathione.[14] At pH 7.7, the observed rate constant is expected to be 9 times greater for **1** than glutathione. The expected observed rate constant at pH 7.7, $k^{obsd}(7.7)$, is obtained from the observed rate constant at pH 7.0, $k^{obsd}(7.0)$, and the proportion of the thiol in the reactive thiolate form, $[1/(1 + 10^{pK_a - pH}]$, at pH 7.0 and 7.7. Thus, at both pH 7.0 and 7.7 compound **1** is expected to react faster with disulfides than glutathione.

The leaving-group ability of thiols is inversely correlated to the pK_a of the thiol, and **1** is thus a better leaving group than glutathione because **1** has a lower pK_a value.[12,14] The relative leaving group ability of the two thiolates can be qualitatively compared. Equation 1 represents a formula developed to predict the rate of thiol−disulfide interchange reactions.[12] In eq 1, k is the rate constant of the reaction, pK_a^{nuc} is the pK_a of the conjugate acid (thiol) of the nucleophile (thiolate), pK_a^{c} is the pK_a of the conjugate acid (thiol) of the central sulfur, and pK_a^{lg} is the pK_a of the conjugate acid (thiol) of the leaving group (thiolate); see Scheme 4. The rate constant k can be converted to k^{obsd} by taking into account the proportion of the nucleophile in the reactive thiolate form, eq 2. In eq 2, pK_a corresponds to the pK_a of the nucleophile (pK_a^{nuc}) and pH corresponds to the pH of the solution. If the nucleophile is an aromatic thiol, then the constant in eq 1 is increased by 0.9 from 6.3 to 7.2 to account for the greater nucleophilicity of aromatic thiols relative to aliphatic thiols of similar thiol pK_a values.[12] From eq 1, rate constants with compound **1** as the leaving group are calculated to be 17 times greater than those with glutathione as the leaving group, all else being equal.

Scheme 3



Scheme 4



$$\log k = 6.3 + 0.59pK_a^{nuc} - 0.40pK_a^{c} - 0.59pK_a^{lg} \quad (1)$$

$$\log k^{obsd} = \log k - \log(1 + 10^{pK_a - pH}) \quad (2)$$

The reaction rates of thiol−dusulfide interchange reactions are also inversely correlated with the pK_a of the central thiol (R^c, Scheme 4).[12] Again according to eq 1, the rate constant of thiol−disulfide interchange reactions in which compound **1** corresponding to the central sulfur should be approximately 7

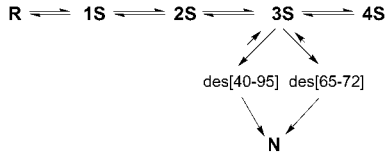
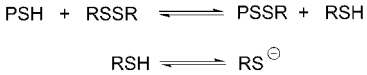


Figure 4. Folding pathway of RNase A in the presence of DTT proposed by Scheraga et al.[32−34] The folding pathway involves reduced RNase A (R); RNase A with one (1S), two (2S), three (3S), or four diuslfide (4S) bonds; native RNase A (N); and RNase A with three native disulfide bonds but lacking the disulfide bond between amino acids 40 and 90 (des[40−95]) or between amino acids 65 and 72 (des[65−72]. A minor pathway between the 2S species and the two des species exists but is not shown.

times faster than those with glutathione, all else being equal. The rate constant for reaction I in Scheme 3, which does not involve the small-molecule thiol, should be unaltered by the choice of aromatic thiol or glutathione.

On the basis of the above analysis, aromatic thiol **1** is predicted to increase the observed rate constants for seven of the eight thiol−disulfide interchange reactions (reactions II−VIII) relative to glutathione. Thus, aromatic thiol **1** might be expected to enhance the folding rate of disulfide-containing proteins considerably relative to glutathione at pH 7.0 or 7.7. However, increasing the folding rate of disulfide-containing proteins is much more complex than increasing the rates of these eight reactions in isolation.[9,30,31] For example, increasing the concentration of glutathione beyond a certain point only decreases the folding rate of RNase A, although it should increase the rate of many of the eight reactions.[9] This result can be understood on the basis of the mechanism of protein folding. The formation of native RNase A, which contains four correctly matched disulfide bonds, from reduced RNase A in the presence of a redox buffer has been well studied.[27,32−34] The work has concentrated on the folding pathway in the presence of glutathione or DTT, a less efficient catalyst for folding RNase A. In the presence of oxidized DTT, reduced RNase A is rapidly converted into a mixture containing many different protein species such as reduced RNase A (R) and RNase A with one (1S), two (2S), three (3S), or four (4S) disulfide bonds (Figure 4).[26,27,32−34] The species within the mixture reach a quasi-equilibrium state (preequilibrium mixture). The 3S species, excluding des[40−95] and des[65−72], are then transformed via rate-determining steps to des[40−95] and des[65−72], which are native RNase A lacking either the disulfide bond between amino acids 40 and 95 or between amino acids 65 and 72, respectively. The two des species are then converted to native RNase A. The folding pathway of RNase A in the presence of glutathione, a monothiol, is similar to that with DTT, a dithiol. A preequilibrium mixture is formed and then via rate-determining steps native RNase A is formed. However, the rate-determining steps may be different with monothiols. Also, the preequilibrium mixture probably will contain a greater proportion of mixed disulfides between protein and redox buffer. Mixed disulfides between proteins and DTT usually have a fleeting existence due to an intramolecular displacement by the second thiol of DTT.

Scheme 5



The portion of species in the preequilibrium mixture that can be directly and rapidly converted to native RNase A is critical for the overall rate of protein folding. We will refer to these species as productive intermediates. For the folding of RNase A with DTT, the relative concentration of the 3S species, excluding des[40−95] and des[65−72], within the preequilibrium mixture is crucial. For the folding of RNase A with DTT or monothiols, such as glutathione, the relative portion of productive intermediates will vary with the composition of the redox buffer. If the concentration of the small molecule thiol (RSH) is higher than optimal, then the portion of productive intermediates is reduced, presumably by inhibiting the formation of disulfide bonds. As a result, at high glutathione concentrations folding rates of RNase A decrease even though the rates of many of the reactions involved actually increase. Therefore, increasing the rate of the eight thiol−disulfide interchange reactions in Scheme 3 is likely very desirable but may not result in an overall increased rate of protein folding due to changes in the composition of the preequilibrium mixture.

The composition of the preequilibrium mixture will be dependent on the concentration of the redox buffer thiol in the protonated SH form. The equilibrium constant for the formation of mixed disulfide with the protein is shown in eq 3, where PSSR is the mixed disulfide between protein and small-molecule thiol, RSH is the small-molecule thiol, RSSR is the small-molecule disulfide, and PSH is a free thiol group on the protein. The redox potential is proportional to $\ln([RSH]^2/[RSSR])$. In both cases it is the concentration of the small-molecule thiol in the protonated form that is important (Scheme 5). Thus, in comparing results at the optimum concentrations of **1** and glutathione, we report below both the total concentration of thiol and the concentration of thiol in the protonated form. The concentration of the thiol in the protonated form will be a function of the total thiol concentration, the thiol pK_a value, and the pH of the solution.

$$K_{(\text{mixed disulfide})} = [\text{PSSR}][\text{RSH}]/([\text{RSSR}][\text{PSH}]) \qquad (3)$$

On the basis of the preequilibrium analysis it is predicted that protein folding rates should increase with the concentration of small molecule thiol, reach a plateau, and then decrease. As expected, the protein folding rate constants of both reduced and scrambled RNase A as a function of glutathione concentration increase to an optimum concentration and then decrease (Figures 1 and 2). The optimum concentration was between 1 and 2 mM, similar to what had been observed previously at pH 8 with reduced RNase A.[9] With the aromatic thiol the rate constants increased and then remained uniform as the concentration of thiol was increased (Figures 1 and 2). However, the initial rate of protein folding, $A \times k$, in the presence of an aromatic thiol increased, plateaued, and then decreased. On the basis of the initial rate of protein folding, the optimum concentrations of aromatic thiol were approximately 4.5 (pH 7.0) and 6.5 mM (pH 7.7).

Comparing the optimum concentrations of thiols in terms of the concentration of protonated (SH) form results in the optimum glutathione concentration being greater than that of the aromatic

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thiol. As mentioned previously (Scheme 5), the concentration of thiol in the protonated form is important in establishing equilibria. At pH 7.0, the aromatic thiol ($pK_a = 6.6$) exists mainly in the deprotonated thiolate form (72%), while glutathione ($pK_a = 8.7$) exists almost exclusively (97%) in the protonated form. Thus at pH 7.0, when the concentration of the protonated form is compared, the optimum concentrations of aromatic thiol (1.3 mM SH form) and glutathione (1−2 mM) are very similar. At pH 7.7, the bulk of the aromatic thiol is in the thiolate form (93%) but glutathione still exists almost exclusively in the protonated form (91%). The optimum concentration of aromatic thiol in the protonated form (0.5 mM = 6.5 mM × 0.07) is slightly less than that of glutathione (0.9−1.8 mM). However, results obtained at high aromatic thiol concentrations are affected by the decrease in the maximum percent refolded, *A*, which may result in a lowering of the optimum aromatic thiol concentration.

At higher concentrations of **1** the folding rate constant was relatively uniform up to 15 mM (1.1 mM protonated form) but the maximum percent folding, *A*, decreased. A decrease in RNase A activity was not observed when native RNase A was incubated with the refolding mixture, suggesting that the decrease in activity is taking place during refolding. The most likely cause is aggregation of the RNase A folding intermediates at high aromatic thiol or aromatic disulfide concentrations. Aggregation is supported by the fact that a white precipitate is observed in protein folding mixtures containing high concentrations of aromatic thiol but not those containing glutathione at its optimal concentration.

Once the optimum concentrations of glutathione and aromatic thiol for protein folding were determined, the relative folding rates were measured side by side. Aromatic thiol **1** significantly enhances the folding rate for both reduced and scrambled RNase A at pH 7.0 and 7.7 relative to glutathione. At both pH values the rate enhancement is between a factor of 5 and 6 (Tables 1−3). Surprisingly, there is very little variation in the rate enhancement with pH. For comparable sets of experiments, the folding rate constant at pH 7.7 is almost 3 times that at pH 7.0. Explanations for the increase in folding rate constant with pH include an increase in the concentration of protein thiolate or small-molecule thiolate, a change in the conformation of the protein folding intermediates, or a change in the equilibrium between the folding intermediates. An increase in thiolate concentration would be expected to increase the reaction rate, as the thiolate form of the thiol is the active species in thiol−disulfide interchange reactions (Scheme 3). Since the concentration of glutathione thiolate increases more dramatically (360%) than aromatic thiolate (29%) with the change in pH (7.0 to 7.7), the folding rate constant with glutathione might be expected to increase considerably more than that with aromatic thiol. However, our experiments showed this is not the case. Therefore, an increase in the concentration of small-molecule thiolate is not the lone reason for the increased rate of protein folding with pH. Furthermore, these results suggest that it is not the enhanced nucleophilicity of the aromatic thiol relative to glutathione that is most important. As previously outlined, the enhanced nucleophilicity of aromatic thiols relative to glutathione changes dramatically with pH (32-fold at pH 7.0 and 9-fold at pH 7.7). However, the enhanced folding rates (6-fold at pH 7.0 and 5-fold at pH 7.7) are relatively independent of pH. The folding rate enhancement of aromatic thiols is likely the result of pH-independent effects such as the enhanced leaving-group ability of aromatic thiols or the enhanced reactivity of disulfides in which an aromatic group is attached to the central thiol (Rc, Scheme 4).

Having increased the folding rate constant of proteins by replacing glutathione with an aromatic thiol, we investigated replacing glutathione disulfide with an aromatic disulfide **2** (Table 6). The replacement of glutathione disulfide with **2** in the protein folding reaction has little to no effect. Therefore, we recommend the use of glutathione disulfide since it is commercially available. The lack of an effect is likely the result of glutathione disulfide being rapidly converted to aromatic disulfide in the presence of aromatic thiol (4.0 mM). A lower limit can be placed on the reaction rate. At 30 °C the rate constant for glycol dimercaptoacetate, which is generally less reactive than the aromatic thiol, reacting with glutathione disulfide is 500 M^{-1} min^{-1}.[11−14] Assuming a similar rate constant for the aromatic thiol (4.0 mM) reacting with glutathione disulfide (much less than 4.0 mM), the half-life of glutathione disulfide would be 20 s, in the absence of equilibrium effects. The initially formed mixed disulfide between glutathione and aromatic thiol is most likely more reactive than glutathione disulfide itself.[12] Thus, even in reactions initially containing glutathione disulfide, the concentration of aromatic disulfide very quickly becomes significant.[35]

Combining all the data together, we found that for a similar set of conditions the folding rate constants of reduced and scrambled RNase A are almost identical. The similarity in rates can be explained on the basis of a similarity of preequilibrium mixtures. Since oxidation of reduced RNase A occurs much more rapidly than formation of the native form, reduced RNase A very quickly reaches its preequilibrium state.[9,26,29] Presumably, scrambled RNase A rapidly reaches a similar preequilibrium state under comparable redox conditions. The folding rate constants from these two similar preequilibrium states are alike.

In conclusion, we have demonstrated a simple method to dramatically increase the rate of in vitro folding of disulfide-containing proteins relative to traditional methods. Improved folding rates are observed by replacing glutathione with an aromatic thiol. Aromatic thiols were originally selected because of their enhanced nucleophilicity and enhanced leaving-group ability relative to glutathione at pH 7.7. With aromatic thiol **1** the folding rate enhancement is 5−6-fold at either pH 7.0 or 7.7, even though the protein folding rates increase by a factor of approximately 3 with the increase in pH. The relative invariance of the folding rate enhancement with pH suggests that pH-dependent effects such as the concentrations of thiol in the deprotonated thiolate form and the enhanced nucleophilicity of the aromatic thiol are not dominant factors in enhancing the protein folding rate. The enhanced folding rate was not increased further by replacing glutathione disulfide, the traditional reagent, with aromatic disulfide **2**. Under similar conditions, folding rate constants and enhanced folding rates were nearly the same for both reduced and scrambled RNase A.

Experimental Section

General Information. NMR spectra were recorded at 300 MHz (^1H) and at 75 MHz (^{13}C) on a Bruker spectrometer. Chemical shifts were internally referenced to the solvent. Thin-layer chromatography (TLC)

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was conducted on Aldrich general-purpose silica gel on polyester plates with UV indicator. All reagents purchased were used without purification. UV−vis spectra were recorded on a Cary 1 UV−visible spectrophotometer. All proteins were purchased from Sigma unless otherwise noted. Compound **1** was obtained from Toronto Research Corporation or synthesized.[15,16] E & R Microanalytical Laboratory Inc. performed elemental analysis.

Reduced RNase A (0.27 mM)[18,36] and scrambled RNase A (0.27 mM),[19,37] which is RNase A with a random distribution of disulfide bonds, were stored at −5 °C in an aqueous solution (0.6% AcOH and 1 mM EDTA). All buffers were prepared by the addition of base to a 0.6% solution of AcOH. Tris, [tris(hydroxymethyl)aminomethane], was used in the preparation of all pH 7.7 buffers. Bis-tris, propane [1,3-bis[tris(hydroxymethyl)methylamino]propane], was used in the preparation of all pH 7.0 buffers. All pH 7.0 and 7.7 buffers contained 1 mM EDTA. Solutions were deoxygenated by bubbling Ar through the solution for 30 min.

Calibration of Assay for Native RNase A.[17,18] A stock solution of native RNase A (≈0.25 mM) was prepared by dissolving native protein in buffer. Protein concentration was determined from the absorbance at 275 nm ($\epsilon_{275} = 9300$ for native RNase A).[18] From the stock solution of RNase A, solutions varying in concentration from 0.5 to 25 μM were prepared. Aliquots of these solutions, varying in volume from 20 to 50 μL, were diluted to 1 mL in a cuvette by the addition of 0.1 M Tris−acetate buffer (pH 5.0). To the cuvette, 10 μL of a cytidine 2′,3′-cyclic monophosphate (cCMP) (20 mg/mL) solution was added. After the solution was mixed, the change in absorbance at 292 nm was measured over the course of 2 min. The rate of change in absorbance with time was determined by a linear least-squares fit (initial rate analysis). These values were plotted vs native protein concentration. The rate was found to vary linearly ($y = mx + b$) with respect to protein concentration when the concentration of active protein inside the cuvette was between 5×10^{-8} M and 5×10^{-7} M.

Protein Folding.[17,18] Refolding experiments for reduced and scrambled RNase A were conducted at 25 °C. Protein aliquots were adjusted to the pH of the refolding experiment by the addition of 0.1 M base: Tris for pH 7.7, Bis-tris propane for pH 7.0. Solutions of 5 mM glutathione disulfide (GSSG), 10 mM glutathione (GSH), and 20 mM aromatic thiol (ArSH-**1**) were prepared individually in deoxygenated buffer and deoxygenated for use in the refolding reactions. The refolding reaction was initiated by the addition of the pH-adjusted scrambled or reduced RNase A. The refolding reaction (total volume 500 μL) contained protein (25 μM), GSSG (0.5 mM for reduced RNase A and 0.2 mM for sRNase A), thiol (GSH or ArSH-**1**), and buffer. The protein was allowed to refold until no further increase in activity was observed (up to 2 days). The concentration of active protein was determined by the assay for native RNase A.

Assay for Native RNase A. An aliquot (20−50 μL) of the refolding reaction was diluted to 1 mL in a cuvette by the addition of pH 5.0 buffer such that the final concentration of active protein was between 5×10^{-7} and 5×10^{-8} M. The pH 5.0 buffer was prepared from the same base that was used in the refolding mixture. To the cuvette was added 10 μL of a 20 mg/mL cCMP solution. The rate of the change in absorbance at 292 nm was used to determine concentration of native RNase A; see calibration of assay.

Refolding Rate. The refolding rate was determined by plotting the concentration of active protein versus time. The concentration of active protein was expressed as percent refolded, where percent refolded = ([active protein]/(2.5×10^{-5} M)) × 100. The plot was fit by least squares to percent refolded $= A(1 - e^{-kt})$, where A is maximum percent activity achieved, k is the refolding rate, and t is time in minutes.

4,4′-Dithiobisbenzeneacetic acid, Disulfide 2. An aqueous solution (125 mL) of **1** (1.00 g, 6.0 mmol) and Na_2CO_3 (3.05 g, 29 mmol) was vigorously stirred under air until the solution tested negative for thiols with Ellman's reagent (36 h). The solution was then acidified to pH 1 with concentrated HCl and filtered. The solid was washed with water and then dried in a lyophilizer to yield 0.894 g (90% yield) of disulfide. ^{1}H NMR (300 MHz, CD_3OD) δ 7.44 (d, $J = 8.4$ Hz, 4H), 7.24 (d, $J = 8.5$ Hz, 4H), 3.58 (s, 4H); ^{13}C NMR (75 MHz, CD_3OD) δ 175.33, 136.80, 135.87, 131.47, 129.26, 41.44. Anal. Calcd for $C_{14}H_{14}O_2S_2$: C, 57.47; H, 4.22; S, 19.18. Found: C, 57.42; H, 4.19; S, 19.12.

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