Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry 16 (2008) 2579–2590
Bioorganic & Medicinal Chemistry

Rate enhancement of the oxidative folding of lysozyme by the use of aromatic thiol containing redox buffers

Minakshi C. Gurbhele-Tupkar, Lissette R. Perez, Yenia Silva and Watson J. Lees*

Department of Chemistry and Biochemistry, Florida International University, 11200 SW 8th Street, Miami, FL 33199, USA

Received 26 September 2007; revised 13 November 2007; accepted 16 November 2007
Available online 22 November 2007

Abstract—Almost all therapeutic proteins and most extracellular proteins contain disulfide bonds. The production of these proteins in bacteria or in vitro is challenging due to the need to form the correctly matched disulfide bonds during folding. One important parameter for efficient in vitro folding is the composition of the redox buffer, a mixture of a small molecule thiol and small molecule disulfide. The effects of different redox buffers on protein folding, however, have received limited attention. The oxidative folding of denatured reduced lysozyme was followed in the presence of redox buffers containing varying concentrations of five different aromatic thiols or the traditional aliphatic thiol glutathione (GSH). Aromatic thiols eliminated the lag phase at low disulfide concentrations, increased the folding rate constant up to 11-fold, and improved the yield of active protein relative to GSH. The yield of active protein was similar for four of the five aromatic thiols and for glutathione at pH 7 as well as for glutathione at pH 8.2. At pH 6 the positively charged aromatic thiol provided a higher yield than the negatively charged thiols.

1. Introduction

The folding of proteins in vitro has served as a method of producing therapeutic proteins. When proteins, especially those containing disulfides, are overexpressed in bacteria they can aggregate/precipitate inside the cell forming inclusion bodies.[1–5] These inclusion bodies, which contain inactive protein, are then isolated and resolubilized using a denaturant such as guanidine hydrochloride or urea. The resulting denatured reduced protein is then folded in vitro. The in vitro folding step is usually the lowest yielding. Many therapeutic proteins, almost all of which contain disulfide bonds, are produced using a variation of this procedure. For disulfide containing proteins the common model systems are bovine pancreatic trypsin inhibitor (BPTI), ribonuclease A (RNase A), and lysozyme.

Several methods have been developed to improve the in vitro folding of disulfide containing proteins. The key parameters for the folding of proteins are the pH of the solution, the composition of the redox buffer, and the presence of folding aids.[6] In addition, the presence of biological catalysts such as protein disulfide isomerase or molecular chaperones can have a dramatic effect on folding but these tend to be expensive and have low catalytic activity.[7] Folding aids, which are proposed to alter the interactions between or within proteins, retard protein aggregation/precipitation. Common folding aids include urea, guanidine hydrochloride, arginine, and glycerol.[5,8–14] These aids, however, with limited exception,[10,11] increase the yield of active protein but decrease the in vitro folding rate constants of model disulfide containing proteins such as lysozyme. Folding aids also tend to be protein specific and thus have to be optimized for each protein.[2]

The composition of the redox buffer is very important for the successful folding of disulfide containing proteins. The redox buffer is usually composed of a mixture of a small molecule disulfide and/or a small molecule thiol. The traditional mixture contains glutathione disulfide and glutathione, although other small molecule aliphatic thiols and their corresponding disulfides have been used, for example, mercaptoethanol, thioglycolic acid, cysteine, cystamine, and dithiothreitol (DTT).[1,2,15] The redox buffer both oxidizes protein thiols to form protein disulfide bonds and helps rearrange mismatched disulfide bonds to form native disulfide bonds. With redox buffers containing small molecule disulfides and/or thiols, the oxidation and rearrangement occurs via

Keywords: Protein folding; Lysozyme; Aromatic thiol; Redox buffer.
* Corresponding author. Tel.: +1 305 348 3993; fax: +1 305 348 3772; e-mail: leeswj@fiu.edu

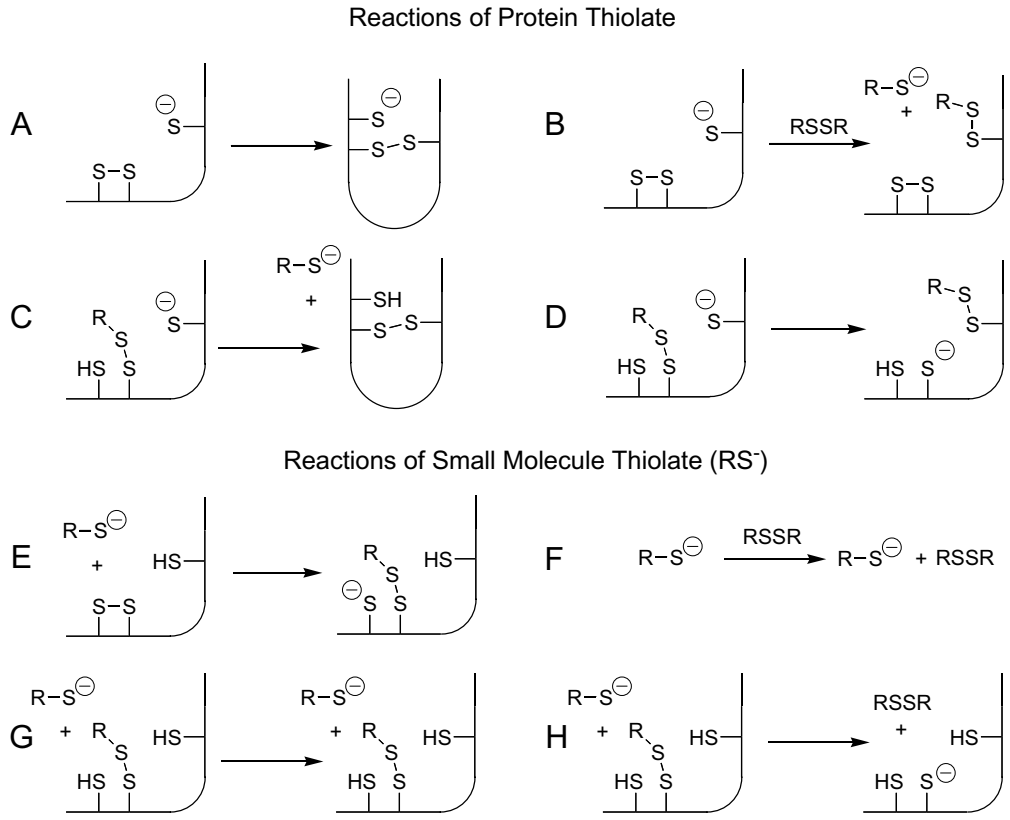
0968-0896/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2007.11.047

the thiol-disulfide interchange reaction. Mechanistically, redox buffers and folding aids are thus different as folding aids affect interactions within or between proteins and redox buffers enhance the thiol-disulfide interchange reactions.

Several new small molecule thiol redox buffers have recently been introduced. These compounds can be subdivided into two classes; dithiols and aromatic thiols.[16] Redox buffers that contain a small molecule aliphatic dithiol or peptide dithiol increase the yield of active protein but do not increase the rate constant of protein folding more than 3-fold relative to glutathione.[16–20] Dithiols are proposed to improve protein folding by shortening the lifetime of kinetically stable mixed disulfides formed between the protein and the small molecule thiol. With dithiols the mixed disulfide can be displaced intramolecularly by the second thiol on the dithiol instead of intermolecularly, as is the case for monothiols. Intramolecular displacement is proposed to be more rapid than intermolecular displacement.

Aromatic thiols can increase the rate of almost all of the thiol-disulfide interchange reactions that occur during protein folding under standard conditions, relative to aliphatic thiols such as glutathione, Scheme 1. Any reaction in which the aromatic thiolate, the deprotonated thiol form, acts as a leaving group will be enhanced (reactions B, C, F, and H) and any reaction in which the aromatic thiolate acts as a nucleophile may be enhanced (reactions E–H). Aromatic thiolates are better leaving groups than aliphatic thiolates, such as glutathione, due to their lower thiol pK_a values. In addition, aromatic thiolates are better nucleophiles than aliphatic thiolates with similar thiol pK_a values.[21] Only intramolecular reactions between a protein thiol and protein disulfide (reaction A) and reactions that are rate limited by protein conformational changes will be unaffected by switching to an aromatic thiol from glutathione.[22] As a result of these factors aromatic thiols increase the oxidative folding rate of RNase A by up to 25-fold relative to glutathione depending on the pH.[7,22–25] In order to examine the generality of the rate enhancement, we decided to examine the folding of lysozyme.

The folding of denatured reduced lysozyme into its active form has been extensively studied under a wide variety of conditions and its folding pathway is relatively well characterized.[10,14,26–31] However, unlike RNase A, the yield of active protein is sensitive to the conditions used. At relatively low protein concentrations, 0.2 mg/mL, with low concentrations of folding aids, the yield of active protein can be 20%.[10] At higher concentrations of folding aids the yield increases substantially, approaching 100% under certain circumstances.[10] Therefore, lysozyme is an ideal protein for testing folding aids and protein folding technology. Although redox buffers are known to affect protein folding, only a limited number of studies have investigated the effects of different redox buffers on folding and they have focused



Scheme 1. Thiol-disulfide interchange reactions that occur during protein folding with a small molecule thiol (RSH) and a small molecule disulfide (RSSR).

exclusively on the yield of active protein.[15,32–34] With the standard redox buffer, glutathione and glutathione disulfide, the optimal conditions have traditionally been reported as a ratio of [GSH]/[GSSG] and the results have varied from 10:1 to 1:1.[27,30,32,35]

Herein, the standard redox buffer for the folding of denatured/reduced lysozyme, glutathione and glutathione disulfide, is optimized at five different glutathione disulfide concentrations. The protein folding rates obtained with the optimized standard redox buffer are then compared with those obtained using redox buffers containing one of five different aromatic thiols. A systematic study of the effects of a series of redox buffers on the folding of lysozyme has not been undertaken previously, even though redox buffers and pH have large effects on protein folding.[6] Aromatic thiols are also known to significantly enhance the folding of scrambled RNase A under a variety of conditions.[24] The data obtained with lysozyme are discussed with respect to the results obtained with RNase A, and general rules for the folding of disulfide containing proteins efficiently in vitro with aromatic thiols are presented.

2. Results

The folding of reduced lysozyme was undertaken in the presence of the traditional redox buffer, glutathione and glutathione disulfide, and redox buffers containing five different aromatic thiols. Initially, the concentrations of glutathione and glutathione disulfide were optimized for protein folding. The glutathione was then replaced with an aromatic thiol and again the thiol concentration was varied. The effect of pH on folding was also investigated.

The folding of reduced lysozyme was followed using the recovery of enzymatic activity. Denatured reduced lysozyme (10 mg/mL), from which the dithioerythreitol (DTE) had been removed by gel permeation chromatography,[36] was rapidly diluted 100-fold into renaturation buffer containing 0.1 M buffer (Bis–Tris propane for pH 7.0 or Bis–Tris for pH 6.0), 1 mM EDTA, 0.5 M guanidine hydrochloride (GdnHCl), and redox buffer.[37] The EDTA was added to prevent metal catalyzed air oxidation of the thiols.[32] The common additive GdnHCl was included to minimize protein aggregation during folding.[8,37] The final concentration of lysozyme was 0.1 mg/mL or 7 μM. Aliquots were removed from the folding reaction at specific times and the enzymatic activity determined by adding the aliquots to a suspension of *M. lysodeikticus*.[32,37,38] The decrease in light scattering was then monitored for 2 min at 450 nm. The change in light scattered per minute is proportional to enzymatic activity.

2.1. Folding with glutathione

The folding of reduced lysozyme was performed in redox buffers composed of various concentrations of GSH and GSSG at pH 7. Five GSSG concentrations were selected to encompass the range of optimal values previously reported, 0.06, 0.2, 0.5, 2.0, and 5.0 mM. At each GSSG concentration 1, 2, 4, 7, and 10 mM GSH were examined, Figure 1. Lower concentrations of GSH were examined at 0.06 and 0.2 mM GSSG and higher concentrations of GSH were examined at 5 mM GSSG.

The optimum concentration of GSH at each GSSG concentration was determined either by inspection or by fitting the curve to an exponential function. At 0.06 and 0.2 mM GSSG a noticeable lag occurred before the appearance of significant enzymatic activity, as indicated in Figure 1 for 0.2 mM GSSG. This lag period made fitting the folding curve to a single exponential function inappropriate.[30,37,39] The optimum GSH concentration was thus determined by inspection. At 0.5, 2, and 5 mM GSSG the lag period had diminished and

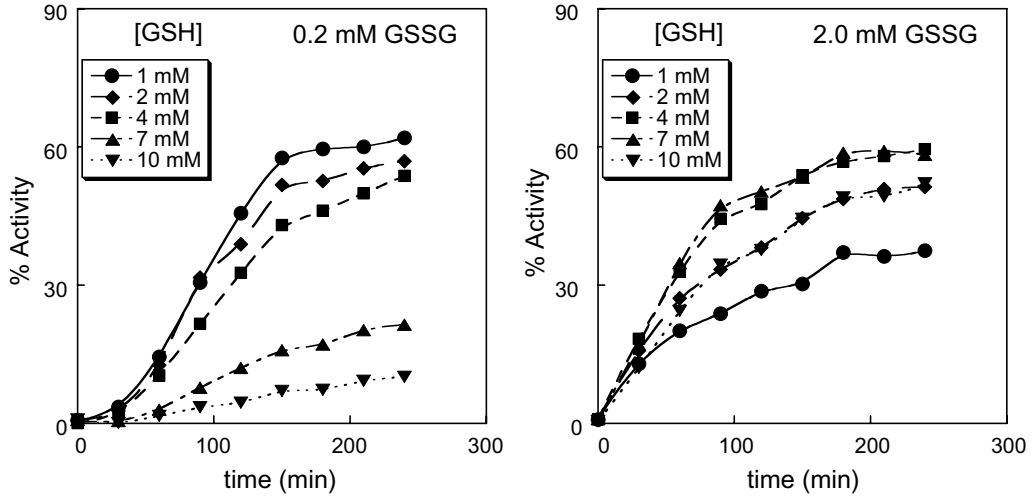


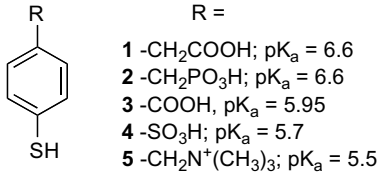
Figure 1. Recovery of enzymatic activity during the folding of denatured reduced lysozyme (7 μM) in the presence of 0.2 or 2 mM GSSG and various GSH concentrations. Assays were performed at pH 7.0 and 25 °C in the presence of 1 mM EDTA and 0.5 M GdnHCl. The curves for 2 mM GSSG were fit to an exponential, $y = A(1 - e^{-kt})$, where A is maximal activity, k is the folding rate constant, and t is time.

Table 1. Folding of reduced lysozyme at optimum conditions using a redox buffer of GSH and GSSG at pH 7

GSSG concd (mM)	Optimum GSH concd[a] (mM)	Maximal % activity[b]	Time to 90% of maximal activity[c] (min)	GSH/GSSG ratio
0.06	1	[d]	[d]	17:1
0.2	1	60	190	5:1
0.5	2	60	180	4:1
2.0	7	65	150	3.5: 1
5.0	10	65	180	2:1

[a] For 0.06 and 0.2 mM GSSG, the optimum concentration was determined by inspection. For 0.5 mM GSSG and higher, the optimum concentration was determined by the value of Ak.
[b] Maximal activity was estimated from the activity at long time points, between 240 and 300 min. The average standard deviation of three independent measurements performed on separate days was 15%.
[c] Due to significant differences in lag periods, the time to 90% completion was used, vide supra. The average standard deviation of three independent measurements performed on separate days was 25 min.
[d] Maximal activity was not reached within 300 min.

the folding curve was fit to a single exponential. The exponential function used was $y = A(1 - e^{-kt})$ where A is the maximal enzymatic activity achieved, k is the apparent folding rate constant, and t is time. The concentration of GSH that provided the greatest value of $A * k$, the initial rate of protein folding, was defined as optimal, Table 1. Other GSH concentrations did, however, provide $A * k$ values similar to those obtained at the optimum concentration. The following were all within 20% of the optimal: 0.5 mM GSSG, 1–4 mM GSH; 2.0 mM GSSG, 4–7 mM GSH; 5.0 mM GSSG, 4–10 mM GSH. The optimum concentrations also exhibited the highest maximal activities or within 5% of the highest for a given GSSG concentration. The folding experiment was then repeated two additional times at the optimum GSH concentrations, Table 1.



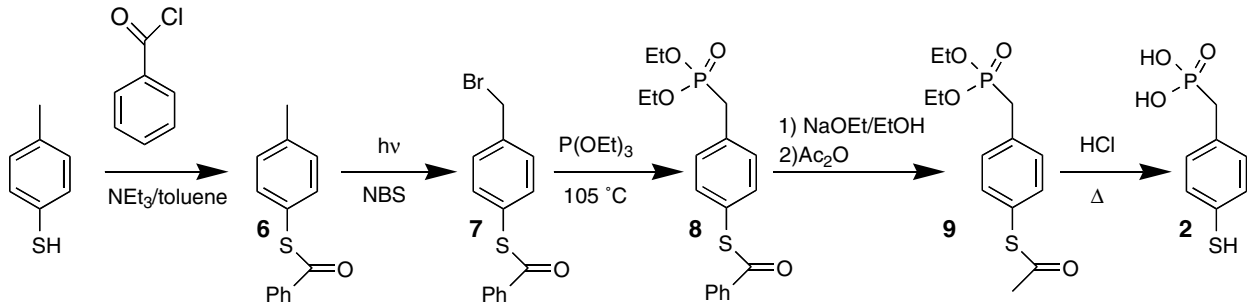
Scheme 2. Aromatic thiols.

2.2. Folding with aromatic thiols

A series of aromatic thiols was selected to improve the folding rate and yield of lysozyme, Scheme 2. The thiols were chosen for their solubility in buffer containing 0.5 M GdnHCl at pH 6–8 and to provide a range of thiol pK_a values. Thiols **1**, **3**, and **4** were used previously to fold RNase A but thiols **2** and **5** have not been used previously for protein folding experiments. *p*-Mercaptobenzyl alcohol previously used with RNase A was not adequately soluble in 0.5 M GdnHCl at pH 6. Aromatic thiols **2** and **5** were selected because at pH 7 they would be soluble in water, the former would be negatively charged and the latter would be positively charged. Furthermore, their thiol pK_a values were predicted to differ substantially.

Aromatic thiols **1**, **3**–**5** were purchased or prepared as previously reported.[23,40,41] Aromatic thiol **2** was synthesized in five steps from *p*-mercaptotoluene, Scheme 3. The brominated intermediate is also used in the preparation of the quaternary ammonium salt. The thiol protecting group was changed midway through the synthesis to prevent benzoic acid precipitating out of solution along with the product in the final step.

The folding of reduced lysozyme was undertaken in the presence of various concentrations of each aromatic thiol and 0.2 mM GSSG. The concentration of 0.2 mM GSSG was selected for several reasons. Lysozyme folds rapidly in the presence of 0.2 mM GSSG and 1 mM GSH. Our previous results obtained with RNase A used 0.2 mM GSSG. Also, we expected the lag phase to disappear in the presence of 0.2 mM GSSG and an aromatic thiol, due to the greater reactivity of aromatic thiols and disulfides, formed by the rapid reaction of GSSG with the aromatic thiol before the lysozyme is added.[21,24] The equilibrium concentrations can be calculated using a set of simultaneous equations.[21,24] All the aromatic thiols were assayed at 1, 2, 4, 6, and 8 mM. Aromatic thiols **2**, **4**, and **5**, which were more soluble, were assayed at 8, 10, 15, and 20 mM. The folding curves were again fit to an exponential function, $y = A(1 - e^{-kt})$ where A is the maximal enzymatic activity achieved, k is the apparent folding rate constant, and t is time, Figures 2 and 3. Even though the folding of lysozyme is complex with many different reactions, a simple exponential fit appears to fit the data quite well. The value of $A * k$ corresponds to the initial rate of protein folding. In each case, with the exception of thiol **2**,



Scheme 3. Preparation of aromatic thiol **2**.

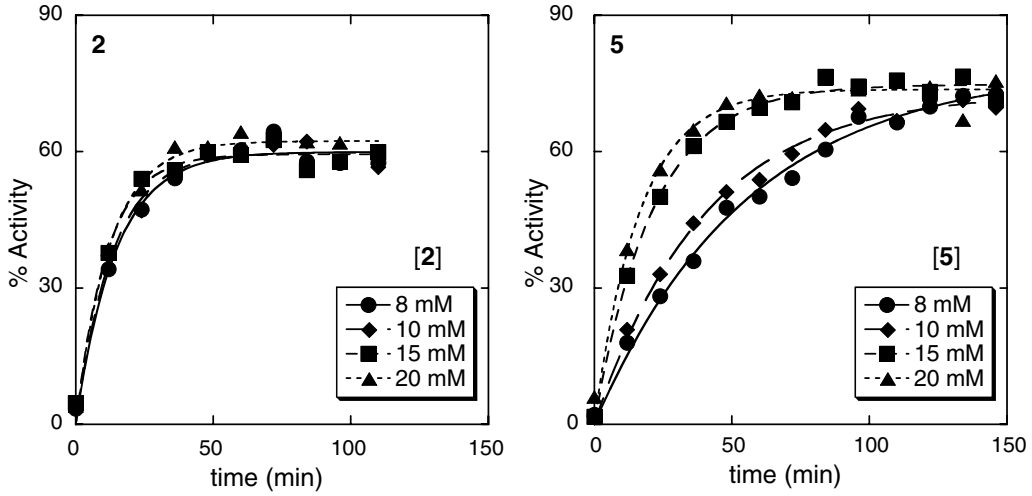


Figure 2. Recovery of enzymatic activity during the folding of denatured reduced lysozyme (7 μM) at pH 7 in the presence of various concentrations of aromatic thiol **2** or **5**. Assays were performed at 25 °C in the presence of 0.2 mM GSSG, 1 mM EDTA, and 0.5 M GdnHCl. The 0 min time point was taken immediately after mixing the denatured reduced protein with the refolding mixture. The curves were fit to an exponential function $y = A(1 - e^{-kt})$, where A is maximal activity, k is the folding rate constant, and t is time.

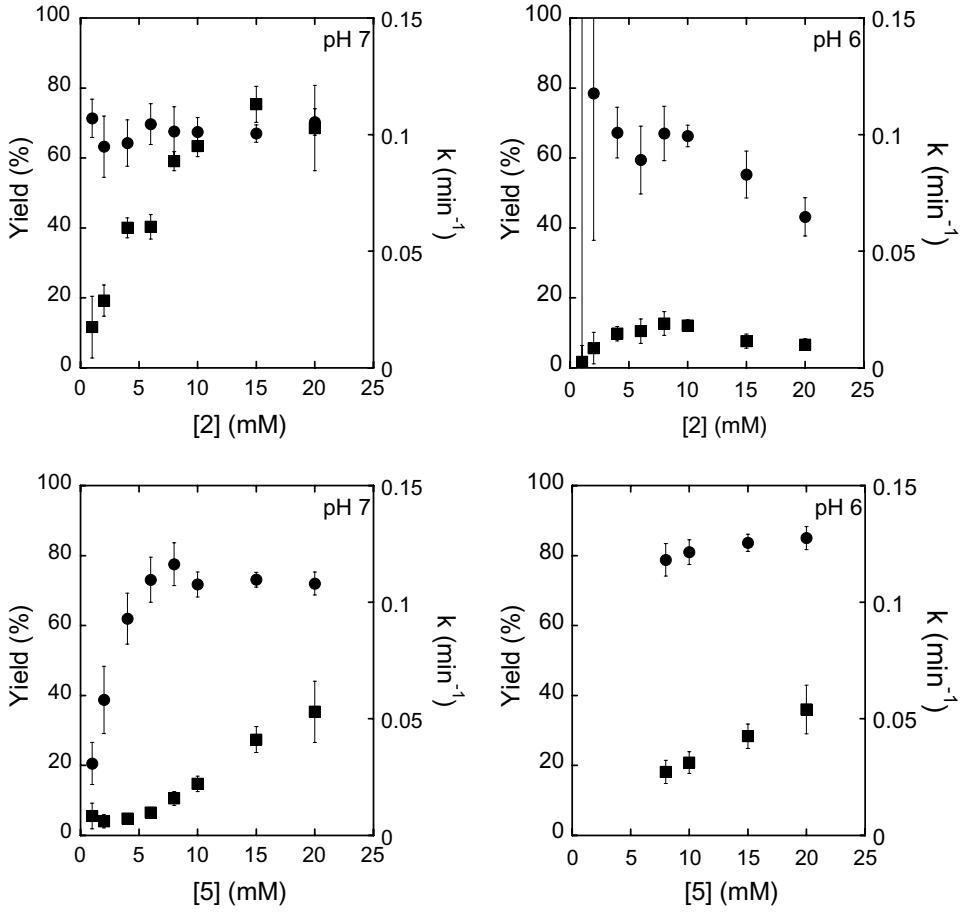


Figure 3. A or maximum activity (circles) and k (squares) values as a function of thiol concentration. Values were normalized to those in Tables 2 and 3, and the error is twice the curve fitting error for each point. At high thiol concentrations (20 mM **5**, 15 mM **2** at pH 7, and 20 mM **5**, 8 mM **2** at pH 6) the approximate experimental error was 10% in A and 15% in k.

the greatest $A * k$ value was obtained at the highest thiol concentration examined. For thiol **2**, the greatest $A * k$ value occurred at 15 mM. The folding experiments were then repeated multiple times at the best concentrations for each aromatic thiol, Table 2. The standard deviations were approximately 10% in A, 15% in k, and 20% in $A * k$. Similar errors were obtained with ribonuclease A.[24] The results with lysozyme were also

Table 2. Refolding kinetics of lysozyme with various aromatic thiols (ArSH) and glutathione at pH 7.0 and glutathione at pH 8.2

ArSH	pK_a	Thiol concd (mM)	Maximal % activity, A	k (min^{-1})
			pH 7[a]	
1	6.6	8	45 ± 5 (7)	0.125 ± 0.014 (0.021)
2	6.6	15	67 ± 5 (7)	0.113 ± 0.013 (0.019)
3	5.95	8	70 ± 3 (5)	0.064 ± 0.005 (0.008)
4	5.70	20	62 ± 5 (6)	0.105 ± 0.009 (0.014)
5	5.5	20	72 ± 6 (6)	0.053 ± 0.006 (0.007)
GSH[b]	8.7	7	64 ± (2)	0.014 ± (0.002)
			pH 8.2[c]	
GSH	8.7	2	70 ± (10)	0.095 ± (0.011)

[a] The error was determined from at least seven assays with each assay being performed independently on separate days. The error is the standard deviation of the mean at 95% confidence using the Student's t test, $=ts/n^{0.5}$. The error in parentheses is the standard deviation. The GSSG concentration was 0.2 mM unless specified otherwise.

[b] The GSH concentration was 7 mM and the GSSG concentration was 2 mM and is based on three independent measurements. The lag period under these conditions is small.

[c] The GSH concentration was 2 mM and the GSSG concentration was 0.4 mM and is based on five independent measurements.

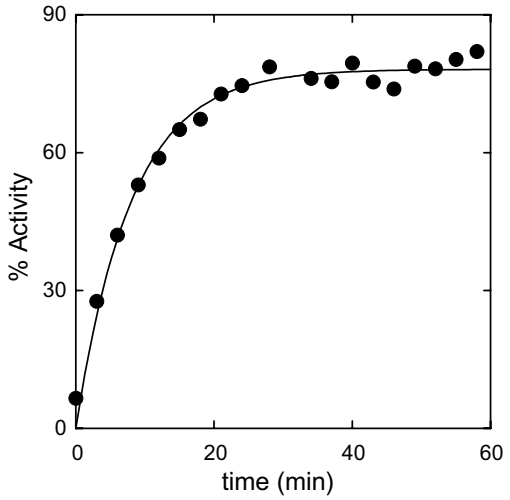


Figure 5. The folding of denatured reduced lysozyme (7 μM) in the presence of 50 mM thiol **5** and 0.2 mM GSSG. Assays were performed at pH 7.0 and 25 °C in the presence of 1 mM EDTA and 0.5 M GdnHCl.

independent of the disulfide used with the aromatic thiols, the commercially available GSSG or aromatic disulfide (ArSSAr), as is the case with ribonuclease A.[22] For thiols **1** and **5**, the 0.2 mM GSSG was replaced with 0.2 mM ArSSAr. The improved folding rates in the presence of aromatic thiols relative to glutathione can be seen in Figure 4.

Aromatic thiol **5** maintains a high maximal activity, A, but has a low protein folding rate constant. However, it is also clear from Figure 2 that as the concentration of **5** is increased the folding rate constant increases. To examine what happens at much higher concentrations of **5**, the folding of lysozyme in the presence of 50 and 100 mM of **5** and 0.2 mM GSSG was investigated, Figure 5. The folding rates and maximum activity were 0.122 min^{-1} and 78%, and 0.153 min^{-1} and 80%, respectively, for 50 and 100 mM of **5**. The results are the average of two experiments performed on separate days. To ensure that the enhanced rate was due to the presence of the thiol group and not due to the high concentration of a quaternary ammonium salt, a control experiment was carried out. Folding was performed with 1 mM GSH and 0.2 mM GSSG in the presence of 50 and 100 mM benzyltrimethyl ammonium bromide, the non-thiol containing analog of **5**. The control experiment showed a slight decrease in the folding rate and activity as the concentration of benzyltrimethyl ammonium bromide was increased from 0 to 50 to 100 mM; from 2 to 4 h the activity decreased by 10% and 20%, respectively.

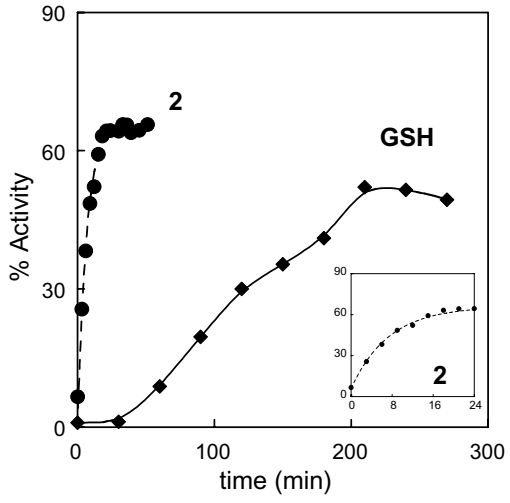


Figure 4. The folding of denatured reduced lysozyme (7 μM) in the presence of 15 mM aromatic thiol **2** (circles) and 1 mM GSH (diamonds). The insert corresponds to the early time points for aromatic thiol **2**. Assays were performed at pH 7.0 and 25 °C in the presence of 0.2 mM GSSG, 1 mM EDTA, and 0.5 M GdnHCl. The 0 min time point was taken immediately after mixing the reduced denatured protein with the refolding mixture. The insert was fit to an exponential function $y = B + A(1 - e^{-kt})$, see text.

The folding of reduced lysozyme at pH 6 in the presence of the aromatic thiols and 0.2 mM GSSG was also examined. Aromatic thiols **1**–**4** were assayed at 1, 2, 4, 6, and 8 mM. The aromatic thiols **2**, **4**, and **5** were then assayed at 8, 10, 15, and 20 mM. Aromatic thiol **5** was only assayed at high concentrations as the best concentration was expected to be greater than 8 mM. The optimum concentrations were 8 mM for **1**–**3** and 15 mM for **4**, Figure 6. The best concentration for **5** was again the highest concentration tested, 20 mM. As is the case at pH 7, we expect the optimal concentration for **5** to be higher still, Figure 5. The folding experiments were then repeated several times at the best concentrations for each aromatic thiol, Table 3.

3. Discussion

The optimum glutathione and glutathione disulfide concentrations for the folding of lysozyme were reinvesti-

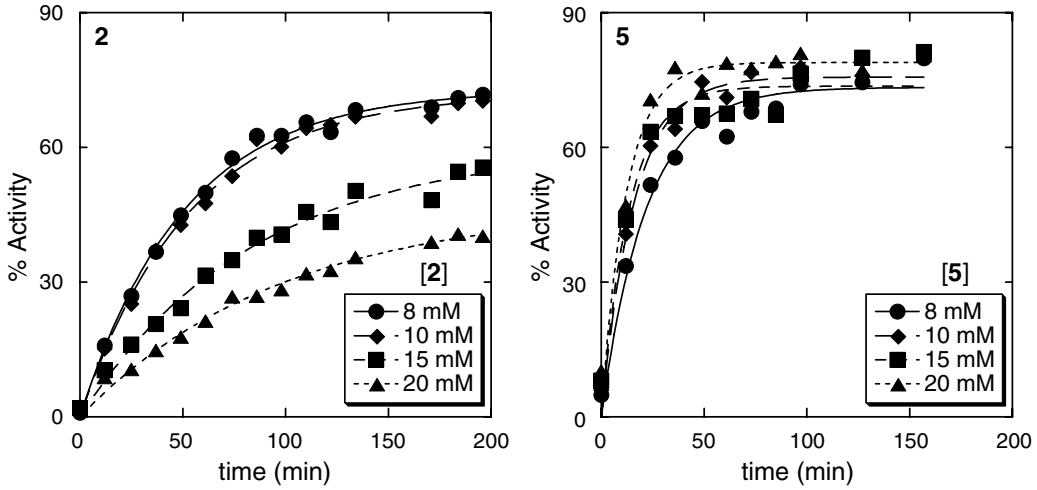


Figure 6. Recovery of enzymatic activity during the folding of denatured reduced lysozyme (7 μM) at pH 6 in the presence of various concentrations of aromatic thiol **2** or **5**. Assays were performed at 25 °C in the presence of 0.2 mM GSSG, 1 mM EDTA, and 0.5 M GdnHCl. The 0 min time point was taken immediately after mixing the denatured reduced protein with the refolding mixture. The curves were fit to an exponential function $y = A(1 - e^{-kt})$, where A is maximal activity, k is the folding rate constant, and t is time.

Table 3. Refolding kinetics of lysozyme with various aromatic thiols (ArSH) at pH 6

ArSH	pK_a	Thiol concd (mM)	Maximal % activity, A[a]	k (min^{-1})
1	6.6	8	16 ± (1)	0.092 ± (0.006)
2	6.6	8	67 ± (19)	0.019 ± (0.0014)
3	5.95	8	60 ± (3)	0.061 ± (0.005)
4	5.7	15	50 ± (7)	0.089 ± (0.015)
5	5.5	20	85 ± (4)	0.054 ± (0.006)

[a] The error was determined from at least four assays with each assay being performed on separate days. The error is the standard deviation. The solution contained 0.2 mM GSSG, 1 mM EDTA, and 0.5 M GdnHCl.

gated. The optimum [GSH]/[GSSG] ratio and GSSG concentration have been reported previously to be 10:1 and 0.5 mM GSSG, 5:1 and 0.4 mM GSSG, and between 2:1 and 1:1 and 4 mM GSSG, respectively.[27,30,32,35] Given the range of optimum ratios and concentrations, the optimal conditions for the folding of denatured reduced lysozyme were reexamined. For GSSG concentrations of 0.2, 0.5, 2, and 5 mM at optimal GSH concentration, the maximal activity and the time taken to achieve 90% of the maximal activity were similar, Table 1. The major difference between the GSSG concentrations was that the lag period decreased as the GSSG concentration increased, Figure 1, suggesting that the lag period may result from the slow oxidation of protein thiols at low GSSG concentrations.[30,37,39] Due to the decrease in the lag period, the time taken to achieve half-maximal activity was decreased at higher GSSG concentrations. Optimal folding might be expected to occur at a constant [GSH]/[GSSG] ratio or at a constant redox potential, constant [GSH]^2/[GSSG] ratio. The results, including those in Figure 1, suggest that neither option is valid, but not conclusively, as the optimal conditions are quite broad. The [GSH]/[GSSG] ratio decreased with increasing [GSSG] as has been reported for RNase A by Gilbert et al.[42–44] and is close to the 10:1 to 5:1 ratio generally recommended for protein folding.[5,45] On the other hand, the [GSH]^2/[GSSG] ratio increased with increasing [GSSG] concentration,[45] 5–20 mM. The absolute value of [GSH]^2/[GSSG] is similar to that obtained for RNase A, 5–20 mM,[22,42] and to what is generally recommended for protein folding, 5–30 mM.[5,45] In summary, the redox buffer has a broad optimum but the [GSH]/[GSSG] ratio should be selected with consideration of the GSSG concentration. It is probable that there is no fixed optimal ratio of [GSH]/[GSSG].

The maximal activity, A, achieved at each glutathione disulfide concentration and optimal glutathione concentration varied only within experimental error, Table 1. A previous report demonstrated that at higher GSSG concentrations (2–5 mM), the folding rate showed a limited correlation with yield, increasing the GSSG concentrations at pH 8 resulted in slightly slower folding rates and higher yields.[27] The report provided the relative rate constants for partitioning between productive folding to native protein, k_2, and non-productive folding, k_3, instead of maximal activity, A, but the two are mathematically interchangeable.[27,35,46,47] At significantly below optimal GSH concentration, a lower maximal activity and lower folding rate is observed, Figure 1, with 2 mM GSSG.

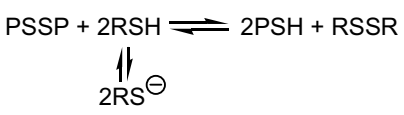
The folding of lysozyme in the presence of a series of aromatic thiols was investigated and shown to vary with the thiol pK_a value and the thiol concentration. Initially, the thiol concentration was varied up to 8 or 20 mM depending on the solubility of the thiol, Table 2. Since no lag period was observed, probably due to the greater reactivity of aromatic disulfides relative to GSSG, the folding curves were fit to an exponential function.[21] The aromatic disulfide is produced by the rapid equilibration of aromatic thiol and GSSG before the lysozyme is added, the concentrations of which can be calculated using a series of simultaneous equations.[21,24]

Replacing GSSG with aromatic disulfide in the presence of aromatic thiols does not affect the folding of lysozyme within experimental error as was shown previously for RNase A. GSSG is more practical as it is commercially available. For thiols with high pK_a values (**2**), increasing the thiol concentration above 10 mM did not significantly change the folding rate constant; for thiols with low pK_a values (**5**), increasing the thiol concentration above 10 mM significantly increased the folding rate, Figure 2. Ultimately, thiols with lower pK_a values achieved higher or equivalent folding rates if the thiol concentration was increased substantially, Figure 5.

Aromatic thiols significantly enhanced the folding rates of lysozyme relative to glutathione. The enhanced rate constant is proposed to be due to the enhanced leaving group ability of aromatic thiols relative to glutathione and their enhanced nucleophilicity relative to aliphatic thiols with similar thiol pK_a values.[21] With lysozyme, the greatest folding rate constant obtained with glutathione and glutathione disulfide at pH 7 was 0.014 min^{-1}. The optimum folding rate for aromatic thiol **2** was 9 times that of glutathione and the folding rate for **5** was 11 times that of glutathione (7 μM lysozyme, 0.5 M guanidine hydrochloride, pH 7, 25 °C). In comparison, under very similar conditions (6 μM lysozyme, 1 M urea, pH 7.4, 25 °C) the in vivo catalyst of thiol-disulfide interchange reactions during protein folding, protein disulfide isomerase (PDI), enhanced the folding rate constant of lysozyme approximately 15-fold, 3:1 ratio of lysozyme to PDI, relative to glutathione under the same conditions.[48] In the absence of urea (10 μM lysozyme, pH 7, 37 °C) the folding rate constant was enhanced 7-fold by PDI, 2:1 ratio lysozyme to PDI, but at 1 μM lysozyme the rate enhancement was 60-fold, 2:1 ratio of lysozyme to PDI.[49] Even at pH 6, aromatic thiols can fold lysozyme faster than glutathione does at pH 7 or at a comparable rate to glutathione at pH 8.2. Similarly, with RNase A the folding rate constants in the presence of aromatic thiols under optimal conditions at pH 7 were 7–12 times those of glutathione and the rate constants at pH 6 with aromatic thiols were greater than those of glutathione at pH 7.7.[24] In summary, aromatic thiols can be used to significantly enhance the folding rates of lysozyme at a range of pH values.

The optimal or best thiol concentrations for the folding of lysozyme are similar but not identical for glutathione and aromatic thiols when the concentration of thiol in the neutral RSH form is taken into account, as opposed to the thiolate form (RS^-). With 0.2 mM GSSG, the optimal GSH concentration was 1 mM (pK_a = 8.7; 1 mM RSH), the optimal concentration of aromatic thiol **2** was 15 mM (pK_a = 6.6; 4 mM RSH and 11 mM thiolate), and the optimal concentration of aromatic thiol **5** was at least 100 mM (pK_a = 5.5; 3 mM RSH and 97 mM thiolate). For lysozyme at pH 6 the optimal concentration of the aromatic thiols in the RSH form was 4–6 mM. Thus, the optimal concentrations of aromatic thiols in the RSH form were similar at pH 6 and 7, 4–6 mM, but the optimal concentration for glutathione was less at pH 7, 1 mM. A fixed concentration of RSH also corresponds to a fixed [RSH]/[RSSR] ratio and a fixed $[RSH]^2$/[RSSR] ratio. For RNase A at pH 6 and 7 the optimal concentration of thiol in the RSH form was the same for glutathione and the aromatic thiols, 1–2 mM.[24] Overall, the concentration of thiol in the RSH form is important for folding lysozyme.

Several factors affect the folding rates of disulfide containing proteins with aromatic thiols including the concentration of thiol in the neutral RSH form, the concentration of the thiolate anion, and the reactivity of the thiolate anion.[23,24] For a specific aromatic thiol the relative concentrations of thiol in the neutral RSH form and in the thiolate form vary only with the pH as the thiol pK_a is fixed. On the other hand, with a series of aromatic thiols with different thiol pK_a values the relative concentrations will vary with the thiol pK_a values at a fixed pH. With lysozyme and RNase A, the concentration of thiol in the neutral RSH form affects the equilibrium between the small molecule thiol and the protein disulfide, Scheme 4. If the concentration of RSH in the neutral form is too high, then the environment is too reducing and the formation of disulfide bonds is difficult. If the concentration of RSH in the neutral form is too low, then the environment is too oxidizing and the formation of disulfide bonds is facile but their rearrangement is slow, as there are no free protein thiols. At low pH, where most of the thiols are in the neutral form, the equilibrium between aromatic thiols and glutathione is close to unity.[23,24] Thus, the concentration of thiol in the neutral RSH form affects the equilibria that occur during protein folding. Partially as a result of these factors both glutathione and aromatic thiols have optimal concentrations beyond which the addition of more thiol leads only to poorer folding, Figures 1 and 6. The concentration of thiol in the thiolate form is also important, as thiolate is the reactive species in the thiol-disulfide interchange reaction, Scheme 1; the greater the thiolate concentration the faster the reaction. The reactivity of the thiolate is linked to the thiol pK_a; the lower the aromatic thiol pK_a, the less reactive the thiolate, although aromatic thiolates are more reactive than aliphatic thiolates if the pK_a of the conjugate acids (thiols) are the same.[21] With RNase A, optimal conditions occurred when the concentration of thiol in the neutral RSH form was 1–2 mM.[23,24] For lysozyme the value was 4–6 mM. However, the absolute rate under optimal conditions was determined by the thiolate concentration and its reactivity, which varied with the thiol pK_a value. With lysozyme at pH 6 under optimal conditions, compound **2** (pK_a = 6.6) has a much smaller concentration of thiolate (6.3 mM neutral RSH and 1.7 mM thiolate) than compound **4** (pK_a = 5.7, 5 mM neutral RSH, 10 mM thiolate), although the concentration of RSH in the neutral form is similar for both. The greater thiolate con-



Scheme 4. Reaction of small molecule thiol (RSH) with protein disulfide (PSSP) to form small molecule disulfide (RSSR) and protein thiol (PSH).

centration results in greater folding rates with **4** than **2** under optimal conditions even though the thiolate of **2** is inherently more reactive than that of **4**.

The maximal activity or yield of active protein obtained with aromatic thiols under optimal conditions at pH 6 and 7 varied with the aromatic thiol and in some cases was greater than that obtained with glutathione at pH 7 or 8.2. Interestingly, the maximal activity did not show a strong dependence on the concentration of glutathione, Figure 1, unless the glutathione concentration was changed from optimal by a factor of 3 or more.[30] At pH 7 with aromatic thiols, the maximal activity was also relatively unaffected by the thiol concentration, although in one case the maximal activity decreased as the thiol concentration increased, **1**. No correlation was observed between the folding rate constant and maximal activity.[37,47,49] At pH 6, the same general trend in maximal activity was observed with aromatic thiol **1** giving the lowest maximal activity followed by **4** then **2** and **3**, and finally **5**. With RNase A the folding yields under optimal conditions were also relatively independent of the aromatic thiol, although thiols **2** and **5** were not studied. The maximal activity obtained for lysozyme with positively charged **5** at pH 6 was the highest we obtained at any pH value or with any thiol, Table 3. All the other thiols will be negatively charged at pH 6 or 7. A previous report demonstrated that acetylating or carbamoylating the lysine residues on lysozyme reduced the net positive charge on the protein and decreased the folding yield in a systematic manner.[15] In addition, the same report has shown that a positively charged disulfide, cystamine, was better at folding lysozyme than a negatively charged disulfide, dithiodiglycolic acid, at the same concentration.[15] The authors proposed that the introduction of additional positive charges derived from the oxidizing agent, as a result of the formation of mixed disulfides between the protein and the oxidizing agent, suppressed aggregation during the early stages of folding. The pI of hen egg white lysozyme is approximately 11 so the protein has a net positive charge at both pH 6 and 7. Therefore, we conclude that the net charge of the thiol may be important for the folding yield of proteins with aromatic thiols.

Summarizing, the folding rate constant of denatured reduced lysozyme was increased 10-fold at pH 7 by the addition of aromatic thiols instead of glutathione to the redox buffer. The rate enhancement is similar to that reported for PDI in vitro at approximately 10 μM lysozyme and a 3 or 2:1 ratio of lysozyme to PDI. The use of aromatic thiols also eliminated the lag period observed at low glutathione disulfide concentrations. The optimal concentration of aromatic thiol occurred when the concentration of thiol in the neutral RSH form, as opposed to the thiolate form, was 4–6 mM. The maximal activity is similar for four of the five aromatic thiols at pH 7 and for glutathione at pH 7 and 8.2, Table 2. The maximal activity for the positively charged aromatic thiol at pH 6 and 7 was greater than that of the negatively charged thiols, glutathione included, suggesting that there is a charge effect for the folding of lysozyme with aromatic thiols, Figure 5 and Table 3.

4. Conclusions

In conclusion, the folding of lysozyme and ribonuclease A follows the same general trends, which suggest that aromatic thiols should enhance the folding rates of many, if not almost all, disulfide containing proteins under a variety of conditions. The rate enhancement expected from replacing glutathione with an aromatic thiol at pH 7 is approximately 10-fold, which increases as the pH decreases. The optimal aromatic thiol varies with the pH of the solution. An aromatic thiol with a thiol pK_a approximately one unit lower than the pH is ideal. The optimal aromatic thiol concentration also varies with the aromatic thiol, solution pH, and protein but a concentration of 1–6 mM in the neutral RSH form appears to be general in the presence of 0.2 mM disulfide.

5. Experimental

5.1. General information

Hen egg white lysozyme (>23,000 U/mg) was purchased from Roche Applied Science and used without purification. Reduced lysozyme was prepared according to the procedure of Goldberg et al.[50] Biochemicals were purchased from Sigma. The *p*-mercaptobenzoic acid, *p*-mercaptophenylacetic acid, and the reagents used to synthesize *p*-mercaptophosphonic acid were purchased from Aldrich. The *p*-mercaptobenzenesulfonic acid,[23,40] *p*-mercaptobenzyl trimethyl ammonium bromide,[41] 4,4′-dithiobis(benzeneacetic acid),[22] and 4,4′-dithiobis(benzenesulfonic acid)[23,40] were synthesized as described previously. The synthetic aromatic thiols and disulfides were greater than 97% pure by proton NMR. The 4,4′-dithiobis(benzyl trimethyl ammonium bromide) was synthesized by stirring the corresponding thiol in water for several months in the dark to produce a mixture containing 90% disulfide and 10% thiol. ^{13}C NMR spectra were referenced to 77.00 ppm ($CDCl_3$) and 49.00 ppm (CD_3OD). ^1H NMR spectra were referenced to TMS at 0.00 ppm. Elemental analyses were performed by Complete Analysis Inc., Parsippany, NJ.

5.2. Enzymatic assays

5.2.1. Preparation of denatured reduced lysozyme.[50,51] Lysozyme (20 mg/mL) in 0.1 M Tris–HCl, pH 8.6, containing 6 M GdnHCl and 0.15 M dithioerythreitol was incubated for 2 h at room temperature. The solution was then acidified to pH 3 by the addition of 0.1 M acetic acid and applied to a column packed with Sephadex™ G-25 fine, which had been pre-equilibrated with 0.1 M acetic acid solution. The fractions containing reduced lysozyme, as determined by Ellman's reagent (5,5′-dithiol-bis(2-nitrobenzoic acid)), were collected and added together in a 50 mL centrifuge tube. The fractions containing DTE, which eluted from the column after the protein, were discarded. The concentration of the reduced protein was determined from its absorbance at 280 nm using a UV spectrophotometer ($\varepsilon_{280\ nm} = 2.37\ mL\ mg^{-1}\ cm^{-1}$).[32,52] The reduced lysozyme displayed no enzymatic activity.

The reduced lysozyme was then divided into portions containing 5 mg of protein and each portion was placed in a 15 mL centrifuge tube. Each portion was lyophilized and stored at −20 °C.

5.2.2. Calibration of assay for native lysozyme. A stock solution of native lysozyme (1 mg/mL) was prepared by dissolving the native protein in 0.1 M Bis–Tris propane, pH 7.0, with 1 mM EDTA. The protein concentration (mg/mL) was determined spectrophotometrically by measuring the absorbance at 280 nm ($\varepsilon_{280\ nm}$ = 2.63 mL mg^{-1} cm^{-1}).[32,53] From the stock solution of lysozyme, solutions varying in concentration from 0.002 mg/mL to 0.075 mg/mL were prepared. The activity of each solution was then determined using the enzymatic assay (vide infra). The activity was then plotted versus the native protein concentration. The rate was found to vary linearly with respect to protein concentration.

5.2.3. Assay for lysozyme activity.[32,37,38] In all experiments the lysozyme activity was measured by mixing 20 μL aliquots of native or renatured lysozyme solution (0.1 mg/mL) with 0.980 μL of a *M. lysodeikticus* solution (0.25 mg/mL) in 66 mM monobasic potassium phosphate, pH 6.2, equilibrated at 25 °C. The samples were mixed by repeatedly inverting the cuvette for 15 s. After the solution was mixed, the light scattered at 450 nm was monitored for 2 min. The slope of the line was used to determine the lytic activity of lysozyme.

5.2.4. Refolding of denatured reduced lysozyme.[37,50] The lyophilized reduced lysozyme was dissolved at 10 mg/mL in 6 M GdnHCl, 0.1 M acetic acid, pH 2.5 ($\varepsilon_{280\ nm}$ = 2.37 mL mg^{-1} cm^{-1}).[32,52] The pH of the protein solution was then adjusted to the pH of the folding experiments by addition of 0.1 M base, Bis–Tris propane for pH 7.0 or Bis–Tris for pH 6.0. The pH of stock solutions containing the thiol or disulfide was also adjusted to the appropriate pH prior to use with base. Refolding of reduced lysozyme was achieved in a deoxygenated renaturation buffer (0.1 M Bis–Tris propane–HCl, pH 7.0, 1 mM EDTA, 0.5 M GdnHCl, or 0.1 M Bis–Tris–HCl, pH 6.0, 1 mM EDTA, 0.5 M GdnHCl, and various concentrations of GSH or aromatic thiol and GSSG or aromatic disulfide). Ten microliters of reduced lysozyme solution (10 mg/mL) was added to 990 μL of renaturation buffer in a 1.5 mL Eppendorf tube (100-fold dilution). To ensure reproducible mixing conditions, the renaturation buffer was added to an Eppendorf tube, the protein was deposited as a droplet on the wall of the tube above the buffer meniscus, and mixing was achieved by vigorous agitation with a Vortex mixer for 15 s. Subsequently, aliquots were removed at specific times and assayed for enzymatic activity. All the refolding experiments were conducted at 25 °C. In general, we did not observe any turbidity after several hours of folding, consistent with the literature.[27] The exceptions occurred when the aromatic thiol concentration was close to saturation.

5.2.5. Control experiments. To exclude the possibility of a direct effect of the aromatic thiol on the cell wall of *M. lysodeikticus*, a series of control experiments was performed along with the regular experiments. Varying concentrations of aromatic thiol, 20 μL, in the absence of lysozyme solution were added to 980 μL of *M. lysodeikticus* solution and the change in light scattering at 450 nm was monitored. To test if the aromatic thiol was altering the enzymatic activity, a native lysozyme solution was prepared, 10 mg/mL, and diluted 100-fold with renaturation buffer containing varying concentrations of aromatic thiol (10 μL of native lysozyme solution added to 990 μL of specific renaturation buffer). Then, 20 μL aliquots were assayed with 980 μL of *M. lysodeikticus* solution. For comparison, the activity of lysozyme in the absence of the aromatic thiols was determined. Native lysozyme was diluted with Bis–Tris buffer containing no aromatic thiols or oxidized disulfides (10 μL of native lysozyme solution and 990 μL of buffer). Then, 20 μL aliquots were assayed with 980 μL of *M. lysodeikticus* solution.

5.3. Synthesis

5.3.1. *S*-(4-Methylphenyl)benzenecarbothioic acid (6).[41,54] *p*-Toluenethiol (25.83 g, 0.208 mol) and benzoyl chloride (29.60 g, 0.211 mol) were added to toluene (1 L). The solution was cooled to 0 °C and triethylamine (27.0 g, 0.267 mol) was added slowly over 1 h. After stirring for an additional hour, the mixture was filtered and the solid was washed with toluene. The filtrate was then extracted with water (500 mL), dried ($MgSO_4$), filtered, and concentrated in vacuo. The crude solid was recrystallized from ethanol to provide 33.38 g of product, 70% yield.

5.3.2. *S*-(4-Bromomethyl)phenyl benzenecarbothioic acid (7).[41] *S*-(4-Methylphenyl)benzenecarbothioic acid (19.08 g, 84 mmol) and *N*-bromosuccinimide (15.0 g, 84 mmol) were added to benzene (125 mL) that had been deoxygenated by bubbling Ar through it for 15 min. The mixture was then irradiated with a 250 W GE heat lamp, which provided sufficient energy to reflux the solvent. After refluxing for 30 min, the solution was cooled to 0 °C, filtered, concentrated in vacuo, and partitioned between 600 mL CH_2Cl_2 and 300 mL H_2O. The water layer was washed with 150 mL CH_2Cl_2. The combined organics were dried ($MgSO_4$), filtered, and concentrated in vacuo. The crude solid was recrystallized from hexanes to provide 17.21 g of product (approx. 64% yield), as a 24:1 mixture of *S*-(4-bromomethyl)phenyl benzenecarbothioic acid and *S*-(4-dibromomethyl)phenyl benzenecarbothioic acid.

5.3.3. Diethyl (4-benzoylthiophenyl)methylphosphonate (8).[55] A 24:1 mixture of *S*-(4-bromomethyl)phenyl benzenecarbothioic acid and *S*-(4-dibromomethyl)phenyl benzenecarbothioic acid (6.06 g, 19.7 mmol), and triethylphosphite (8.54 g, 51.5 mmol) were stirred at 105 °C under a flow of Ar for 90 min. The reaction mixture was cooled and the excess triethylphosphite was distilled off under reduced pressure. The residue was purified by silica gel flash chromatography (1:1 to 1:8 hexane/ethyl acetate). The resulting solid was then recrystallized from CH_2Cl_2/hexanes to provide 5.03 g of product, 70% yield. ^1H NMR ($CDCl_3$, 300 MHz) δ 8.02 (d, J = 7.2 Hz, 2 H), 7.61 (t, J = 7.2 Hz, 1 H),

7.51–7.45 (m, 4H), 7.40 (dd, J = 8.3, 2.2 Hz, 2H), 4.05 (quintet, J = 7.3 Hz, 4H), 3.20 (d, J = 21.8 Hz, 2H), 1.27 (t, J = 7.0 Hz, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 190.1, 136.7, 135.3, 133.8, 133.6 (d, J = 9 Hz), 130.8 (d, J = 6 Hz), 128.9, 127.6, 126.0, 62.4 (d, J = 6 Hz), 33.8 (d, J = 38 Hz), 16.5 (d, J = 5 Hz); Anal. calcd for $C_{18}H_{21}O_4PS$: C, 59.33; H, 5.81. Found C, 59.54; H, 5.76.

5.3.4. Diethyl (4-acetylthiophenyl)methylphosphonate (9). Sodium hydride (1.57 g of 60 wt%, 39.2 mmol) was added to ethanol (240 mL) and the fizzing solution was deoxygenated by bubbling Ar through it for 45 min. Diethyl (4-benzoylthiophenyl)methylphosphonate (4.19 g, 11.5 mmol) was then added. After 25 min the reaction was quenched by the addition of acetic anhydride (11.02 g, 108 mmol). No thiol groups were detected by Ellman's reagent. The solution was concentrated in vacuo and the slurry was partitioned between 300 mL CH_2Cl_2 and 150 mL satd aq sodium bicarbonate. The aqueous layer was washed with 150 mL CH_2Cl_2. The combined organics were dried ($MgSO_4$), filtered, and concentrated in vacuo. The crude material was purified by flash chromatography (1:1 hexanes/ethyl acetate to neat ethyl acetate) to provide 3.34 g of an oil, 96% yield. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.36 (s, 4 H), 4.02 (quintet, J = 7.3 Hz, 4H), 3.17 (d, J = 21.8 Hz, 2H), 2.41 (s, 3H), 1.25 (t, J = 7.1 Hz, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 193.9, 134.6, 133.4 (d, J = 9 Hz), 130.7 (d, J = 6 Hz), 126.5, 62.3 (d, J = 7 Hz), 33.7 (d, J = 38 Hz), 30.2, 16,4 (d, J = 5 Hz); Anal. calcd for $C_{13}H_{19}O_4PS$: C, 51.65; H, 6.33. Found C, 51.43; H, 6.29.

5.3.5. (4-Thiophenyl)methylphosphonic acid (2).[55] A mixture of diethyl (4-acetylthiophenyl)methylphosphonate (3.12 g, 10.3 mmol) and concd HCl (500 mL) was heated to reflux for 5 h. The oil bath was at 130 °C. The reaction mixture was concentrated in vacuo to approximately 100 mL, warmed, filtered, and cooled to 0 °C. The mixture was filtered, and the solid washed with water before being lyophilized to provide 1.75 g of product, 83% yield. ^{1}H NMR (CD_3OD, 300 MHz) δ 7.23–7.14 (m, 4 H), 3.04 (d, J = 21.6 Hz, 2H); ^{13}C NMR (CD_3OD, 75 MHz) δ 131.7 (d, J = 6 Hz), 131.5, 131.1 (d, J = 4 Hz), 130.2 (d, J = 3 Hz), 35.4 (d, J = 35 Hz); Anal. calcd for $C_7H_9O_3PS$: C, 41.18; H, 4.44. Found C, 41.10; H, 4.36.

5.4. Determination of thiol pK_a values[56,57]

The thiol pK_a of the aromatic thiols was determined in buffers with and without 0.5 M GdnHCl. The error is 0.1 U and comparison with literature values for some of the compounds has been published previously.[21] The addition of 0.5 M GdnHCl increased the value by 0.1 for the positively charged aromatic thiol and decreased the value by 0.1 for the negatively charged aromatic thiols, all within experimental error.

Acknowledgments

This work was supported in part by the National Science Foundation under Grant No. CHE-0342167 to W.J.L. M.T.-G. was supported in part by a Student Summer Research Award funded by the Biomedical Research Initiative NIH/NIGMS R25 GM061347. L.P. was supported by an undergraduate fellowship NIH/NIGMS R25 GM061347. Y.S. was supported by an undergraduate fellowship NIH/NIGMS T34 GM008771.

References and notes

1. De Bernardez Clark, E. *Curr. Opin. Biotechnol.* **1998**, *9*, 157.
2. De Bernardez Clark, E. *Curr. Opin. Biotechnol.* **2001**, *12*, 202.
3. Lange, C.; Rudolph, R. *Protein Folding Handbook* **2005**, *5*, 1245.
4. Lilie, H.; Schwarz, E.; Rudolph, R. *Curr. Opin. Biotechnol.* **1998**, *9*, 497.
5. Rudolph, R.; Lilie, H. *FASEB J.* **1996**, *10*, 49.
6. Willis, M. S.; Hogan, J. K.; Prabhakar, P.; Liu, X.; Tsai, K.; Wei, Y.; Fox, T. *Protein Sci.* **2005**, *14*, 1818.
7. Gough, J. D.; Lees, W. J. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 777.
8. Orsini, G.; Goldberg, M. E. *J. Biol. Chem.* **1978**, *253*, 3453.
9. Expert-Bezancon, N.; Rabilloud, T.; Vuillard, L.; Goldberg, M. E. *Biophys. Chem.* **2003**, *100*, 469.
10. Dong, X.-Y.; Huang, Y.; Sun, Y. *J. Biotechnol.* **2004**, *114*, 135.
11. Maeda, Y.; Yamada, H.; Ueda, T.; Imoto, T. *Protein Eng.* **1996**, *9*, 461.
12. Rariy, R. V.; Klibanov, A. M. *Proc. Natl. Acad. Sci.* **1997**, *94*, 13520.
13. Arakawa, T.; Tsumoto, K. *Biochem. Biophys. Res. Commun.* **2003**, *304*, 148.
14. Reddy, K. R. C.; Lilie, H.; Rudolph, R.; Lange, C. *Protein Sci.* **2005**, *14*, 929.
15. Maeda, Y.; Ueda, T.; Yamada, H.; Imoto, T. *Protein Eng.* **1994**, *7*, 1249.
16. Kersteen, E. A.; Raines, R. T. *Antioxid. Redox Signal.* **2003**, *5*, 413.
17. Woycechowsky, K. J.; Wittrup, K. D.; Raines, R. T. *Chem. Biol.* **1999**, *6*, 871.
18. Woycechowsky, K. J.; Raines, R. T. *Biochemistry* **2003**, *42*, 5387.
19. Cabrele, C.; Fiori, S.; Pegoraro, S.; Moroder, L. *Chem. Biol.* **2002**, *9*, 731.
20. Cabrele, C.; Cattani-Scholz, A.; Renner, C.; Behrendt, R.; Oesterhelt, D.; Moroder, L. *Eur. J. Org. Chem.* **2002**, 2144.
21. DeCollo, T. V.; Lees, W. J. *J. Org. Chem.* **2001**, *66*, 4244.
22. Gough, J. D.; Williams, R. H., Jr.; Donofrio, A. E.; Lees, W. J. *J. Am. Chem. Soc.* **2002**, *124*, 3885.
23. Gough, J. D.; Gargano, J. M.; Donofrio, A. E.; Lees, W. J. *Biochemistry* **2003**, *42*, 11787.
24. Gough, J. D.; Lees, W. J. *J. Biotechnol.* **2005**, *115*, 279.
25. Gough, J. D.; Barrett, E. J.; Silva, Y.; Lees, W. J. *J. Biotechnol.* **2006**, *125*, 39.
26. Yoshii, H.; Furuta, T.; Yonehara, T.; Ito, D.; Linko, Y.-Y.; Linko, P. *Biosci. Biotechnol. Biochem.* **2000**, *64*, 1159.
27. De Bernardez Clark, E.; Hevehan, D.; Szela, S.; Maachupalli-Reddy, J. *Biotechnol. Prog.* **1998**, *14*, 47.
28. Ueda, T.; Nagata, M.; Imoto, T. *J. Biochem.* **2001**, *130*, 491.
29. Yasuda, M.; Murakami, Y.; Sowa, A.; Ogino, H.; Ishikawa, H. *Biotechnol. Prog.* **1998**, *14*, 601.

30. van den Berg, B.; Chung, E. W.; Robinson, C. V.; Dobson, C. M. *J. Mol. Biol.* **1999**, *290*, 781.
31. Guez, V.; Roux, P.; Navon, A.; Goldberg, M. E. *Protein Sci.* **2002**, *11*, 1136.
32. Saxena, V. P.; Wetlaufer, D. B. *Biochemistry* **1970**, *9*, 5015.
33. Yoshii, H.; Furuta, T.; Yonehara, T.; Ito, D.; Linko, P. *J. Chem. Eng. Jpn.* **2001**, *34*, 211.
34. Raman, B.; Ramakrishna, T.; Rao, C. M. *J. Biol. Chem.* **1996**, *271*, 17067.
35. Hevehan, D. L.; De Bernardez Clark, E. *Biotechnol. Bioeng.* **1997**, *54*, 221.
36. Lanckriet, H.; Middelberg, A. P. J. *J. Chromatogr. A* **2004**, *1022*, 103.
37. Roux, P.; Delepierre, M.; Goldberg, M. E.; Chaffotte, A. F. *J. Biol. Chem.* **1997**, *272*, 24843.
38. Jolles, P. *Method Enzymol.* **1962**, *5*, 137.
39. Anderson, W. L.; Wetlaufer, D. B. *J. Biol. Chem.* **1976**, *251*, 3147.
40. Kawai, H.; Sakamoto, F.; Taguchi, M.; Kitamura, M.; Sotomura, M.; Tsukamoto, G. *Chem. Pharm. Bull.* **1991**, *39*, 1422.
41. Moss, R. A.; Dix, F. M. *J. Org. Chem.* **1981**, *46*, 3029.
42. Lyles, M. M.; Gilbert, H. F. *Biochemistry* **1991**, *30*, 613.
43. Konishi, Y.; Ooi, T.; Scheraga, H. A. *Biochemistry* **1982**, *21*, 4734.
44. Ahmed, A. K.; Schaffer, S. W.; Wetlaufer, D. B. *J. Biol. Chem.* **1975**, *250*, 8477.
45. Wetlaufer, D. B.; Branca, P. A.; Chen, G. X. *Protein Eng.* **1987**, *1*, 141.
46. Kiefhaber, T.; Rudolph, R.; Kohler, H. H.; Buchner, J. *Bio/Technology* **1991**, *9*, 825.
47. Buswell, A. M.; Middelberg, A. P. J. *Biotechnol. Bioeng.* **2003**, *83*, 567.
48. Van den Berg, B.; Chung, E. W.; Robinson, C. V.; Mateo, P. L.; Dobson, C. M. *EMBO J.* **1999**, *18*, 4794.
49. Puig, A.; Gilbert, H. F. *J. Biol. Chem.* **1994**, *269*, 7764.
50. Goldberg, M. E.; Rudolph, R.; Jaenicke, R. *Biochemistry* **1991**, *30*, 2790.
51. Perraudin, J. P.; Torchia, T. E.; Wetlaufer, D. B. *J. Biol. Chem.* **1983**, *258*, 11834.
52. Wetlaufer, D. B.; Johnson, E. R.; Clauss, L. M. In *Lysozyme*; Osserman, E. F., Canfield, R. E., Beychok, S., Eds.; Academic Press: New York and London, 1974; p 269.
53. Sophianopoulos, A. J.; Rhodes, C. K.; Holcomb, D. N.; Van Holde, K. E. *J. Biol. Chem.* **1962**, *237*, 1107.
54. Aitken, R. A.; Drysdale, M. J.; Ryan, B. M. *J. Chem. Soc., Perkin Trans. 1* **1998**, 3345.
55. Coogan, M. P.; Harger, M. J. P. *J. Chem. Soc., Perkin Trans. 2* **1994**, 2101.
56. Benesch, R. E.; Benesch, R. *J. Am. Chem. Soc.* **1955**, *77*, 5877.
57. Lees, W. J.; Singh, R.; Whitesides, G. M. *J. Org. Chem.* **1991**, *56*, 7328.