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Oxidative folding of lysozyme with aromatic dithiols, and aliphatic and aromatic monothiols

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ABSTRACT

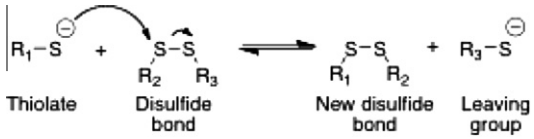
In vitro protein folding of disulfide containing proteins is aided by the addition of a redox buffer, which is composed of a small molecule disulfide and/or a small molecule thiol. In this study, we examined redox buffers containing asymmetric dithiols **1–5**, which possess an aromatic and aliphatic thiol, and symmetric dithiols **6** and **7,** which possess two aromatic thiols, for their ability to fold reduced lysozyme at pH 7.0 and 8.0. Most in vivo protein folding catalysts are dithiols. When compared to glutathione and glutathione disulfide, the standard redox buffer, dithiols **1–5** improved the protein folding rates but not the yields. However, dithiols **6** and **7**, and the corresponding monothiol **8** increased the folding rates 8–17 times and improved the yields 15–42% at 1 mg/mL lysozyme. Moreover, aromatic dithiol **6** increased the in vitro folding yield as compared to the corresponding aromatic monothiol **8**. Therefore, aromatic dithiols should be useful for protein folding, especially at high protein concentrations.

1. Introduction

Most pharmaceutically relevant proteins and many extracellular proteins contain disulfide bonds.[1,2] When disulfide-containing proteins are overexpressed in bacteria, inclusion bodies, which are insoluble protein aggregates, are commonly produced.[3] To obtain protein in its biologically active form, it is necessary to resolubilize the protein and then fold it in vitro. For in vitro protein folding, a significant challenge is the rapid formation of the correct disulfide bonds between cysteine residues.[4–6]

To enhance the in vitro folding rate of disulfide-containing proteins, protein catalysts and a limited number of small molecule thiols and selenols have been used.[7–14] Protein catalysts, for example, protein disulfide isomerase (PDI), which is used in vivo, can increase in vitro protein folding rates significantly. However, the use of PDI for in vitro folding is impractical because of its high cost and low catalytic activity.[8] Therefore, small molecule aliphatic thiols, such as glutathione (GSH) (pK_a = 8.7), cysteine (pK_a = 8.7), β-mercaptoethanol (pK_a = 9.6), and dithiothreitol (DTT) (pK_a = 9.2) are usually employed for in vitro protein folding.[9,12] A small molecule thiol is generally used in combination with a small molecule disulfide to form a redox buffer, for example oxidized glutathione (GSSG) and reduced glutathione (GSH). Small molecule thiols are less expensive than PDI and can easily be removed from the native protein. However, folding rates with small molecule thiols are slow, especially at pH values below 7.5, as very little (less than 3%) of the aliphatic thiol (pK_a approx. 9) is in the reactive thiolate form. The mechanism of disulfide formation involves the nucleophilic attack of a thiolate anion (R_1S^-) on a disulfide bond (R_2SSR_3), displacing one sulfur (R_3S) of the disulfide bond and forming a new disulfide bond (R_1SSR_2), Scheme 1. R_3S^- acts as the leaving group, and R_2S acts as the center sulfur.[9] Recently, more efficient thiol based redox buffers containing aliphatic dithiols, synthetic peptides, or aromatic monothiols have been employed.[10,15–19]

Aliphatic dithiols, for example, (±)-*trans*-1,2-bis(2-mercaptoacetamido)cyclohexane (BMC), and synthetic peptides with two cysteine residues in close proximity, for example, Cys-Gly-Cys (CGC), can significantly increase the overall yield of folded protein.[6] Raines et al. illustrated that BMC increased the folding yield of native ribonuclease A (RNase A) twofold relative to its monothiol analog *N*-methylmercaptoacetamide (NMA).[10] The increase in yield was proposed to be due to the presence of a second thiol group in BMC versus a single thiol group in NMA.[10] Furthermore, Raines et al. showed that addition of BMC to *Saccharomyces cerevisiae* growth medium increased the heterologous production of *Schizosaccharomyces pombe* acid phosphatase.[10] Raines et al. also demonstrated that the dithiol containing peptide CGC could



Scheme 1. Mechanism for thiol/disulfide interchange reaction.

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increase the in vitro folding yield of ribonuclease A threefold relative to the standard thiol glutathione.[20] The increase in yield with dithiols was proposed to be due to an 'escape' mechanism, Figure 1.[10,21] When dithiols or monothiols react with a protein disulfide bond, a mixed disulfide bond is produced. With dithiols, the second thiol group acts as a clock and provides a set amount of time for the protein to rearrange. If the protein does not rearrange, the second thiol of the dithiol makes an intramolecular attack and breaks the mixed disulfide bond. In the case of monothiols, there is no second thiol to act as a clock, and the mixed disulfide can become kinetically trapped, and thus the overall yield of native protein decreases. Although BMC and synthetic peptides increased the yield, they did not change the folding rate constant significantly.[10]

In our previous studies, we have shown that aromatic monothiols (pK_a 5.5–6.6) significantly increased the folding rate and to a lesser extent the overall yield of native protein compared to standard aliphatic thiols (pK_a 8.5–10). With aromatic monothiols, the folding rates of RNase A were 10–23 times faster at pH 6.0, 7–12 times faster at pH 7.0, and 5–8 times faster at pH 7.70 than with glutathione.[11,17,22] Additionally, it was also demonstrated that at a high protein concentration (1 mg/mL of lysozyme), aromatic monothiols increased the folding rate up to 11 times and improved the yield up to 40% at pH 7.0, and the rate increased up to 7 times with an up to 25% improvement in yield at pH 8.0 in comparison with glutathione.[19] Aromatic thiols improve the folding rate because they are better leaving groups and more nucleophilic at pH 7.0 than aliphatic thiols, Scheme 1.[23]

Besides varying the redox buffer, dilution of the sample can be used to improve the in vitro folding of disulfide-containing proteins. Generally, protein folding yields decrease with increasing protein concentration.[24–26] Therefore, folding at low protein concentrations is advantageous (e.g., <0.1 mg/mL), however, large volumes of refolding solution are needed to perform the refolding process, making it impractical after a point depending on the scale.

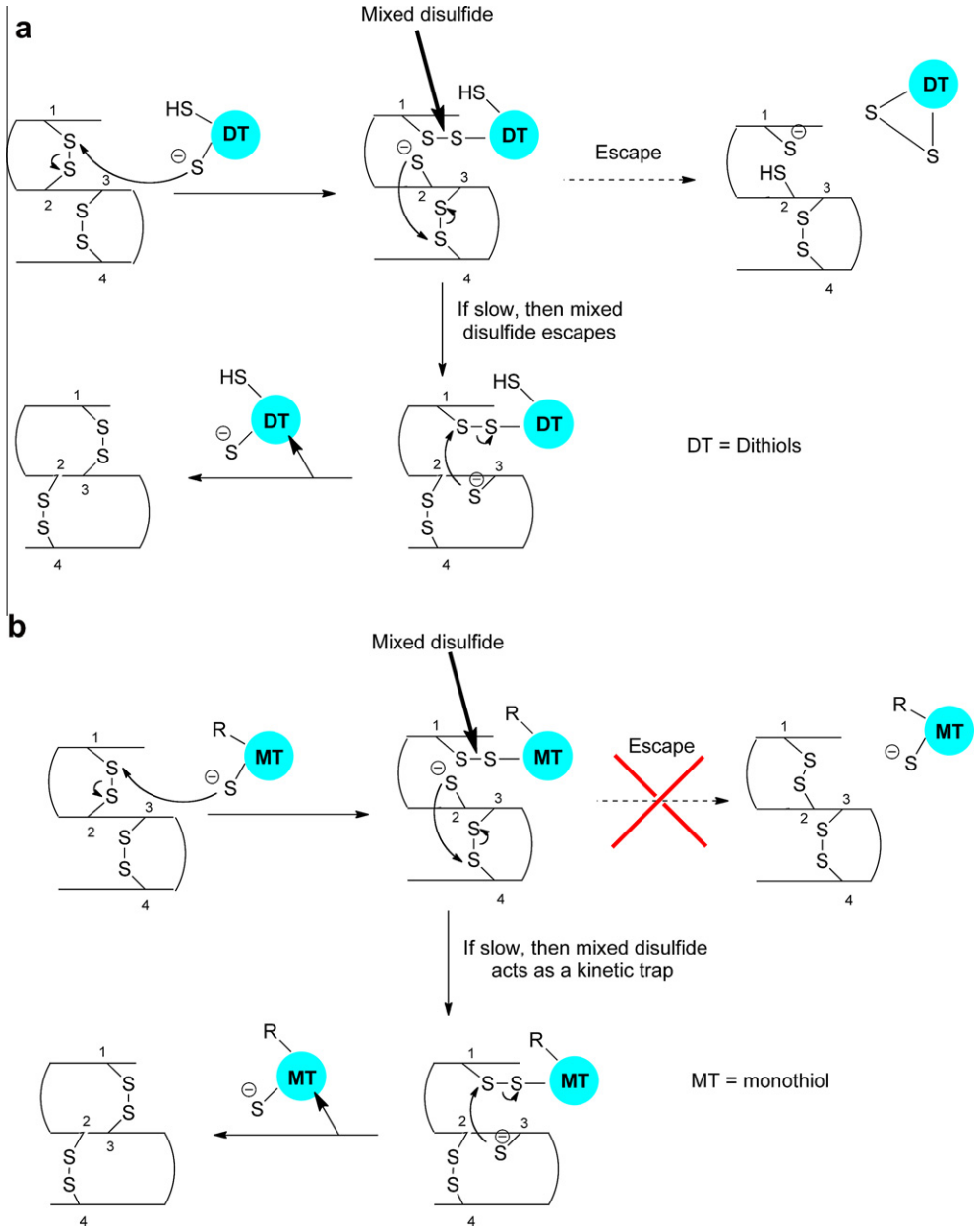
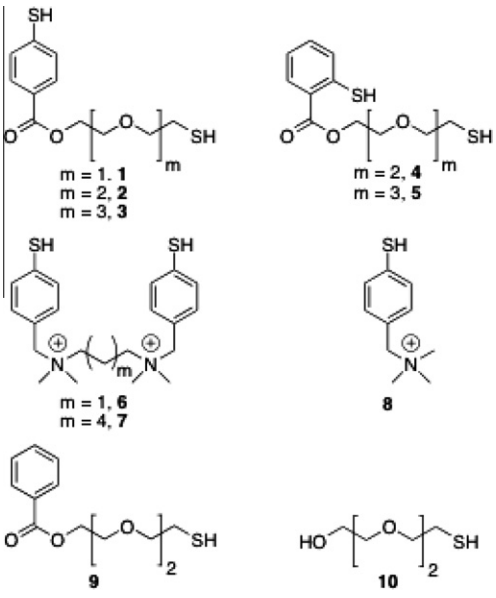


Figure 1. (a) Thiol/disulfide interchange reaction with dithiols, such as PDI, showing escape mechanism.[8,10] (b) Thiol/disulfide interchange reaction with monothiols showing kinetic trap, as a mixed disulfide between monothiol and protein substrate.

Another efficient strategy to increase the folding yield is the addition of folding aids. The most commonly used folding aids are guanidine hydrochloride, urea, arginine, and glycerol.[27–33] Other folding aids include detergent and surfactants, polyethylene glycol, molecular chaperones, artificial chaperones, and several simple compounds, such as acetone, acetamide, sarcosine, and thiourea.[30,34–39] Folding aids are proposed to increase the refolding yield by interfering with the intermolecular hydrophobic interactions that lead to protein aggregation during in vitro protein folding. Aggregation causes reduced overall yields. However, folding aids generally decrease the rate of protein folding.[29–31]

Based on the advantageous properties of aromatic monothiols and aliphatic dithiols, a set of new aromatic dithiols (**1–7**) was designed to examine their effects on protein folding rates and yields. Two series of aromatic dithiols were synthesized, and the ability of each dithiol to enhance the folding of lysozyme was determined. The first series of aromatic dithiols (**1–5**) consisted of one thiol group on an aromatic ring and another thiol group on a polyethylene glycol aliphatic chain, which was either *ortho* or *para* to the aromatic thiol. The second series of aromatic dithiols (**6** and **7**) consisted of a thiol group on each of two aromatic rings connected by a hydrocarbon chain containing quaternary ammonium groups. Aromatic dithiols **6** and **7** were designed on the basis of the previously synthesized quaternary ammonium aromatic monothiol **8.**[18,19] Three aliphatic thiols (**9**, **10**, and the standard thiol glutathione) were used as controls.



2. Results and discussion

2.1. *para*- and *ortho*-Substituted aromatic ethylene glycol dithiols (1–5)

We initially designed asymmetric small molecule dithiols (**1–5**) to mimic PDI, which has two thiol groups, one thiol group more reactive than the other. In addition, upon oxidation PDI forms a 14-membered ring disulfide. In these small molecule dithiols, the more reactive thiol group was modeled as an aromatic thiol and the less reactive thiol group was modeled as an aliphatic thiol; aromatic thiols are more reactive than aliphatic thiols at physiological pH.[23] Additionally, the compounds should be water soluble from pH 6 to 9, the pH range used to fold most proteins in vitro. Therefore, a polyethylene glycol group was used as the side chain to improve water solubility.

2.1.1. Synthesis of *para*- and *ortho*-substituted aromatic ethylene glycol dithiols (1–5)

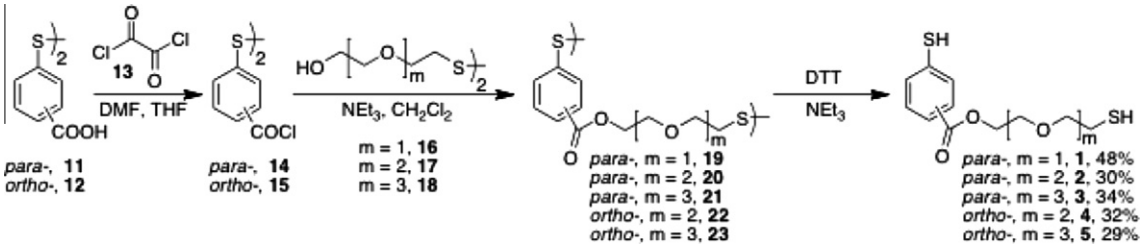
The first step in the synthesis of *para*-substituted aromatic di-, tri-, and tetraethylene glycol dithiols (**1–3**) involved the treatment of the disulfide of 4-mercaptobenzoic acid (**11**) with oxalyl chloride (**13**) and DMF in THF to produce acid chloride **14**, Scheme 2. In the same pot, the acid chloride **14** was treated with di-, tri-, or tetraethylene glycol disulfide (**16**, **17**, or **18**) to form the corresponding polymer (**19**, **20**, or **21**). Di-, tri-, and tetraethylene glycol disulfides (**16–18**) were synthesized by vigorously stirring their respective thiols in water under air for a few days with catalytic base.[40,41] The polymer (**19**, **20**, or **21**) was then reduced with DTT in the presence of Et_3N to give the corresponding *para*-substituted aromatic di-, tri-, or tetraethylene glycol dithiol (**1**, **2**, or **3**), respectively. The *ortho*-substituted aromatic tri- and tetraethylene glycol dithiols (**4** and **5**) were synthesized in the same manner as the *para* analogs, starting from readily available dithiosalicylic acid (**12**). Aromatic monothiol **10** was also synthesized in the same manner using benzoyl chloride as the starting material.

2.1.2. Folding of lysozyme (0.1 mg/mL) with *para*- and *ortho*-dithiols (1–5)

Asymmetric *para*- and *ortho*-substituted aromatic dithiols (**2–5**) were then examined for their ability to fold reduced lysozyme (0.1 mg/mL) at pH 7.0 and 8.0, at 25 °C. Lysozyme was selected as this enzyme has been extensively studied, and its folding pathway has been well characterized.[18,42–46] The folding was carried out in the presence of various concentrations of aromatic dithiols (**2–5**) in combination with either 0.2 or 2.0 mM GSSG. The GSSG concentrations were chosen to match those of previous experiments.[11,16–18] Folding experiments were then compared directly with 7 mM GSH and 2 mM GSSG (standard conditions), as at these concentrations the best combination of folding rate and yield for glutathione was obtained.[18]

The folding of reduced lysozyme was followed using the recovery of enzymatic activity. Reduced lysozyme (10 mg/mL) was diluted 100-fold into renaturation buffer, containing 0.1 M buffer (bis tris propane–HCl for pH 7.0 or Tris–HCl for pH 8.0), 1.0 mM EDTA, 0.5 M guanidine hydrochloride (Gdn HCl), and a redox buffer.[47,48] EDTA and the folding aid Gdn HCl minimize metal catalyzed air oxidation of thiols and protein aggregation during folding, respectively.[27,46,47] The redox buffer was composed of GSH or dithiol, and GSSG. At specific times, aliquots were removed from the folding reaction, and the enzymatic activity was determined by adding the aliquots to a suspension of *Micrococcus lysodeikticus*.[46,47,49] The change in light scattering per minute was proportional to enzymatic activity. The folding of lysozyme in the presence of aromatic dithiols (**2–5**) was compared by fitting enzymatic activity versus time to a single exponential function, $y = A(1 - e^{-kt})$, where A is the maximal enzymatic activity, k is the apparent folding rate constant, and t is time. Although folding is a complex process a single exponential function has historically been used, as it fits the data reasonably well in many cases.

A general decrease in the overall yield of native protein with increasing concentration of aromatic dithiols (**2–5**) was observed in the presence of 0.2 mM GSSG, Table 1 and Figure 2. The *para*-substituted dithiols, **2** and **3**, showed a several fold rate enhancement over GSH (standard conditions) at pH 7.0 but not at pH 8.0 while the *ortho*-substituted dithiols, **4** and **5**, showed little or no rate enhancement. Similar trends were also observed with 2 mM GSSG, except that at pH 7.0 dithiols **4** and **5** showed a general increase in yield with increasing dithiol concentration, although the yield was always at least less than that obtained with GSH



Scheme 2. Synthesis of *para*- and *ortho*-substituted aromatic ethylene glycol dithiols (**1**–**5**).

Table 1
Folding of lysozyme (0.1 mg/mL) with various concentrations of dithiols (**1–5**), and monothiols (**9–11**) in the presence of 0.2 mM GSSG

Compounds[a]	(mM)[a]	*A* (%)	*k* (min^{-1})
pH 7.0			
2	0.5	57	0.025
	3	23	0.020
3	0.5	39	0.038
	3	36	0.045
4	0.5	55	0.013
	2[a]	19	0.001
5	0.5	85	0.008
	3	2	0.010
7 mM GSH/2 mM GSSG		87	0.010
pH 8.0			
1	0.5	53	0.072
	3	9	0.080
2	0.5	63	0.076
	3	16	0.10
3	0.5	73	0.087
	3	43	0.072
4	0.5	58	0.041
	2[a]	62	0.009
5	0.5	76	0.061
	3	27	0.023
9	0.25[a]	68	0.028
	1[a]	2	0.012
10	0.5	72	0.027
	3	68	0.024
7 mM GSH/2 mM GSSG		87	0.05

[a] Solubilities of dithiol **4** at pH 7.0 and monothiol **9** at pH 8.0 were 4 and 2 mM, respectively.

(>15% less at all but one concentration). The faster folding rates of the *para*-substituted dithiols, **2** and **3**, at pH 7.0 relative to the *ortho*-substituted dithiols, **4** and **5**, are likely due in part to less steric hinderance with concomitant lower thiol pK_a values.[16] Aromatic thiols also generally showed a decreased rate enhancement over GSH as the pH was increased from 7.0 to 8.0; the percentage of glutathione in the reactive thiolate form increases fivefold when the pH is increased from 7.0 to 8.0 but for aromatic thiols the increase is at most twofold.

The effect of the stability of the cyclic disulfide and the presence of an aromatic group were further investigated by examining compounds **1**, **9**, and **10**. Compound **1** should be less reducing than **2** and **3** since upon oxidation **1** will form a less stable cyclic disulfide than **2** and **3**.[50] Less reducing environments tend to make it easier to form protein disulfide bonds. However, dithiol **1** at pH 8.0 showed similar trends as the other *para*- and *ortho*-dithiols **2–5**, Table 1. Next, the role of the thiol present on the aliphatic chain was investigated using monothiols **9** and **10** at pH 8.0. Monothiol **9** showed not only a decrease in the yield but also a decrease in the rate compared to the standard conditions, Table 1. Folding results with monothiol **10**, unlike aromatic dithiols, initially showed an increase in yield with increasing concentration followed by a decrease, reaching a maximum at 2 mM, and the maximum was within 3% that of GSH, Figure 2. Thus, monothiol **10** showed similar behavior as the standard thiol, glutathione. The results indicated that the attachment of an aromatic group to an aliphatic thiol decreases the yield of folded protein at higher thiol concentrations.

2.2. Aromatic dithiols 6 and 7 with a quaternary ammonium salt containing linker

A series of symmetrical aromatic dithiols, **6** and **7**, having a thiol group on each of two aromatic rings was prepared. The new series does not contain an aliphatic thiol, as was the case with the initial series of dithiols (**1–5**). Aromatic dithiols **6** and **7** were designed on the basis of a previously synthesized quaternary ammonium aromatic monothiol **8** (thiol pK_a = 5.5), which increased the folding rate and yield of reduced lysozyme.[18,19] The criteria for selecting dithiols **6** and **7** were water solubility, the presence of only aromatic thiols, and *para*-substitution. Additionally, the low thiol pK_a values of **6** and **7** should allow for higher dithiol concentrations without making the solution too reducing; reduction potential is dependant upon the concentration of the neutral RSH form. Forming disulfide bonds becomes slower if the solution is too reducing while higher concentrations of the reactive thiolate leads to faster reactions, which may correspond to faster folding.[18,19] Furthermore, aromatic dithiols **6** and **7** can be compared with aromatic

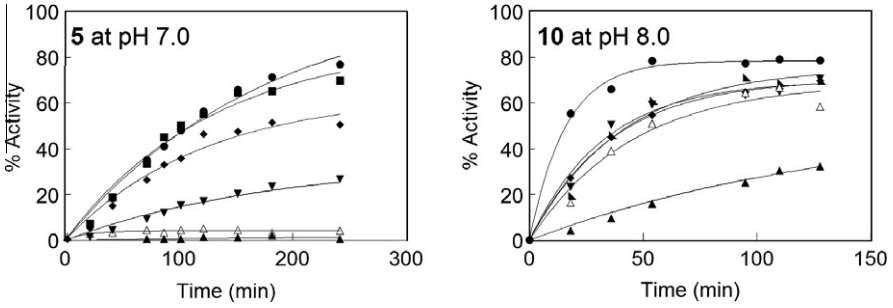
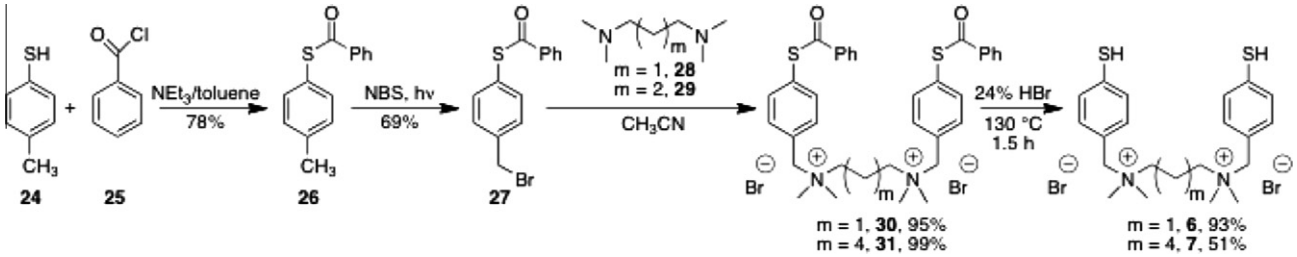


Figure 2. Folding of lysozyme (0.1 mg/mL) under (●) standard conditions (7 mM GSH/2 mM GSSG) or 0.2 mM GSSG and various concentrations of *ortho*-substituted aromatic tetraethylene glycol dithiol **5** at pH 7.0 or triethylene monothiol **10** at pH 8.0: (■) 0.25 mM, (♦) 0.50 mM (▼) 1.0 mM (◣) 2.0 mM (Δ) 3.0 mM and (▲) 10.0 mM.



Scheme 3. Synthesis of aromatic quaternary ammonium salt dithiols **6** and **7**.

monothiol **8** to determine the effect of the second thiol group on the folding rate and yield of a disulfide-containing protein.

2.2.1. Synthesis of aromatic dithiols 6 and 7

The first step in the synthesis of aromatic dithiols **6** and **7** was the reaction of *p*-toluene thiol with benzoyl chloride to yield compound **26**, Scheme 3. Compound **26** was then treated with *N*-bromosuccinimide to provide brominated compound **27**,[51,52] which was further treated with *N*,*N*,*N*′,*N*′-tetramethyl-1,3-propane diamine **28** or *N*,*N*,*N*′,*N*′-tetramethyl-1,6-hexane diamine **29** to form compounds **30** and **31**, respectively. Compounds **30** and **31** were then hydrolyzed with a 1:1 mixture of concd HBr and water at 130 °C for 90 min to provide aromatic dithiols **6** and **7**, respectively.

2.2.2. Folding of lysozyme with aromatic dithiols 6 and 7

Initially, aromatic dithiols **6** and **7**, from 1 to 10 mM, along with 2 mM GSSG were utilized for the folding of reduced lysozyme at 0.1 mg/mL concentration and at pH 7.0 and 8.0. Folding experiments were conducted simultaneously with the standard condition, 7 mM GSH/2 mM GSSG. The folding of lysozyme was followed using the recovery of enzymatic activity as described above. Folding rates increased with increasing concentration of dithiols **6** and **7**, Figure 3. In comparison with glutathione, the folding rates at pH 7.0 with the dithiols were much faster but at pH 8.0 they were similar, with **6** being slightly slower and **7** being slightly faster. At pH 7.0 and 8.0, aromatic dithiol **7** showed similar yields of native lysozyme as 7 mM GSH/2 mM GSSG at all concentrations, however, with aromatic dithiol **6** the folding yield decreased slightly with increasing concentration.

Given the successes in terms of improved folding rates and to a lesser extent folding yields at a protein concentration of 0.1 mg/mL, we also tested protein folding with dithiols, **6** and **7**, and monothiol **8** at a higher protein concentration (1 mg/mL), which is more challenging and practical. Higher protein concentrations reduce folding volumes, a significant advantage for larger scales, and can simplify subsequent protein purification steps. However, lower yields are normally obtained at higher protein concentrations and thus folding aids, for example Gdn HCl, are usually added at higher concentration.[53] Higher concentrations of Gdn HCl, generally slow down the folding rate and improve the yield.[53] Previously, it was demonstrated by our group that at high lysozyme concentration (1 mg/mL), quaternary ammonium salt **8** increased the folding rate and yield significantly, as compared to standard conditions.[19] Initial studies were performed at 10, 20, 40, 70, and 100 mM of aromatic dithiols, **6** and **7**, and aromatic monothiol **8** with 0.5, 1, and 2 mM GSSG to find the best concentrations at both pH 7.0 and 8.0. At pH 7.0 and 8.0, two combinations of aromatic dithiols, **6** and **7**, and GSSG and one combination of aromatic monothiol **8** and GSSG were selected as they provided the best yields, Table 2.

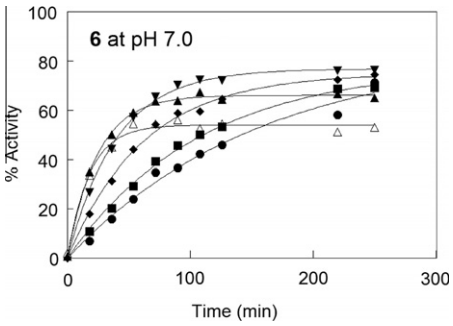


Figure 3. Folding of lysozyme (0.1 mg/mL) at pH 7.0 in the presence of 2 mM GSSG and 0.5 M Gdn HCl. Comparison of the standard conditions (●) 7 mM GSH, with various concentrations of **6**, (■) 1 mM (♦) 2 mM (▼) 3 mM (▲) 7 mM, and (Δ) 10.0 mM.

At pH 7.0, aromatic dithiol **6** produced the best yield and aromatic monothiol **8** provided the best folding rate, Table 2. Comparative folding studies were conducted at pH 7.0 with the best combinations of aromatic dithiols, aromatic monothiol, and GSH with GSSG, Figure 4. The data were fit to a single exponential function, $y = A(1 - e^{-kt})$, where A is the maximal enzymatic activity, k is the apparent folding rate constant, and t is time. The results indicated a significant increase in the folding rates and yields for aromatic dithiols **6** and **7**, and monothiol **8** relative to GSH at pH 7.0. The folding rates increased from 11 to 17 times and the yields increased by 26–42% relative to GSH. The best folding rate was observed with 40 mM **8**, whereas the best yield was observed with 20 mM **6**. When comparing aromatic dithiols versus aromatic monothiols, aromatic monothiol **8** showed a significant increase in folding rate relative to aromatic dithiols **6** and **7**. However, aromatic dithiol **6** significantly increased the yield of native lysozyme relative to aromatic monothiol **8** based on 10 measurements and a 95% confidence interval. The initial folding rates, $A * k$, for 20 mM **6** and 40 mM **8** were 24 and 28 times greater, respectfully, than that of 7 mM GSH/ 2 mM GSSG at a protein concentration of 1 mg/mL.

Table 2
Folding of lysozyme (1 mg/mL) with aromatic dithiols **6** and **7**, aromatic monothiol **8**, and GSH at pH 7.0 and 8.0[a]

Redox buffer	A (%)	k (min^{-1})	$A * k$ (%/min)
pH 7.0			
10 mM **6**/1.0 mM GSSG	83 ± 5	0.012 ± 0.001	1.0 ± 0.2
20 mM **6**/1.0 mM GSSG	86 ± 4	0.015 ± 0.003	1.2 ± 0.3
10 mM **7**/0.5 mM GSSG	70 ± 5	0.013 ± 0.002	0.9 ± 0.2
10 mM **7**/1.0 mM GSSG	74 ± 6	0.011 ± 0.001	0.8 ± 0.2
40 mM **8**/1.0 mM GSSG	82 ± 3	0.017 ± 0.002	1.4 ± 0.2
7 mM GSH/2.0 mM GSSG	44 ± 2	0.001 ± 0.0002	0.05 ± 0.01
pH 8.0			
20 mM **6**/1.0 mM GSSG	88 ± 5	0.022 ± 0.003	2.0 ± 0.3
40 mM **6**/1.0 mM GSSG	77 ± 10	0.023 ± 0.010	1.9 ± 1.0
10 mM **7**/1.0 mM GSSG	78 ± 6	0.016 ± 0.002	1.3 ± 0.2
20 mM **7**/1.0 mM GSSG	49 ± 26	0.017 ± 0.002	0.8 ± 0.4
40 mM **8**/1.0 mM GSSG	81 ± 6	0.018 ± 0.002	1.5 ± 0.1
7 mM GSH/2.0 mM GSSG	73 ± 4	0.003 ± 0.001	0.19 ± 0.02

[a] Errors represent the standard deviation.

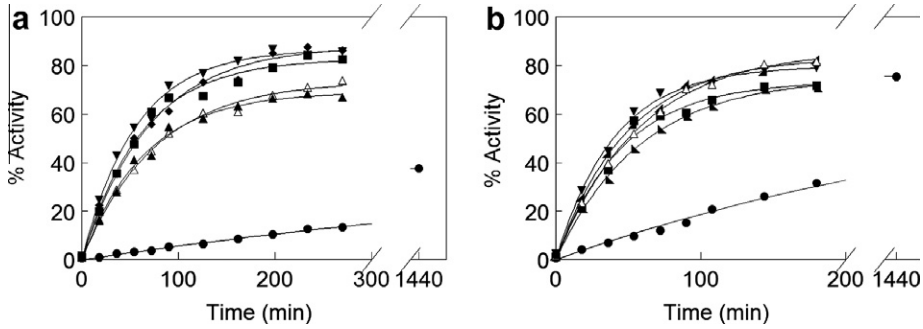


Figure 4. Folding of lysozyme (1 mg/mL) in the presence of 1.7 M Gdn HCl at (a) pH 7.0 and (b) pH 8.0, (●) 7 mM GSH/2 mM GSSG (standard conditions), (♦) 10 mM **6**/1 mM GSSG, (▼) 20 mM **6**/1 mM GSSG, (▲) 10 mM **7**/0.5 mM GSSG, (Δ) 10.0 mM **7**/1 mM GSSG, (■) 40 mM **8**/1 mM GSSG, (◢) 40 mM **6**/1 mM GSSG, and (◣) 20 mM **7**/1 mM GSSG.

At pH 8.0, aromatic dithiol **6** produced the best yield and folding rate, (Table 2 and Fig. 4a and b). The folding rates with 20 mM aromatic dithiol **6** and 40 mM aromatic monothiol **8** were 8 and 6 times higher, respectively, than that of 7 mM GSH. The yield improvements over 7 mM GSH were up to 15%. Comparison of 20 mM aromatic dithiol **6** and 40 mM aromatic monothiol **8** at pH 8.0, revealed that aromatic dithiol **6** was better in terms of rate and yield based on 6 measurements and a 95% confidence interval. With 20 mM aromatic dithiol **7**, protein precipitation was sometimes observed by the naked eye and correspondingly the yield was lower and erratic. The initial folding rate, $A * k$, with 20 mM **6** was 10 times larger than that with GSH at a protein concentration of 1 mg/mL.

When the concentrations of lysozyme and the folding aid Gdn HCl increased, folding rates decreased, as expected, and the yields with glutathione also decreased. With 10 mM aromatic dithiols **6** and **7** folding rates decreased approximately sixfold while with GSH they decreased 8 and 15-fold, at pH 7.0 and 8.0, respectively. The yields with 10 mM aromatic dithiols **6** and **7** were similar or increased slightly when the protein and salt concentrations were increased while those with glutathione decreased. When the aromatic thiol concentration was kept the same, a change in pH from 7.0 to 8.0 had little effect upon the yields, but this was not the case with glutathione. The slower folding rate at pH 7.0 with glutathione might allow other processes to become more significant.

3. Conclusion

Several new aromatic dithiols were designed and successfully prepared to increase the oxidative folding rate and yield of lysozyme. Aromatic dithiols, **6** and **7**, and an aromatic monothiol **8** significantly increased protein folding rates and yields as compared to the traditional aliphatic thiol, glutathione, at a high protein concentration (1 mg/mL) and at both pH 7.0 and 8.0. The folding rate enhancements observed with an aromatic monothiol **8** and two aromatic dithiols, **6** and **7**, were between 8–17 times, and yield improvements of 15–42% at both pH 7.0 and 8.0 were achieved. Moreover, aromatic dithiol **6** significantly increased the in vitro folding yield of lysozyme as compared to aromatic monothiol **8** at both pH 7.0 and 8.0 and high protein concentration (1 mg/mL). Therefore, protein folding with aromatic dithiols should be useful, especially for larger scales where high protein concentrations are used.

4. Experimental

Hen egg white lysozyme was purchased from Roche Applied Sciences and used without further purification. Di-, tri-, and tetraethylene glycol thiols, the reduced forms of **16**, **17**, and **18**, respectively, and **8** were synthesized as described previously.[51,54,55] ^{1}H NMR spectra were referenced to residual monoprotonated solvent at 7.26 ppm ($CDCl_3$), 3.31 ppm (CD_3OD), 4.79 ppm (D_2O), or 2.50 ppm (DMSO). ^{13}C NMR spectra were referenced to solvent at 77.00 ppm ($CDCl_3$), 49.00 ppm (CD_3OD), or 39.52 ppm (DMSO). High resolution mass spectroscopy (HRMS) was conducted by the facility at Florida State University, Tallahassee, FL. Solubility of all compounds (**1–5**, **9**, and **10**) was determined by Ellman's method.[56]

4.1. Synthesis of di-, tri-, and tetraethylene glycol disulfides (16–18) (general procedure using 17 as an example)

Triethylamine (0.5 mL, 3.58 mmol) was added to a solution of 2-[2-(2-mercaptoethoxy)ethoxy]ethanol (triethylene glycol thiol) (3.5 g, 21.08 mmol) in 25 mL of distilled water. The solution was stirred vigorously for 1 week under air. The reaction mixture was acidified with conc HCl, and the aqueous layer was then extracted with $CHCl_3$ (3 × 25 mL). The organic layers were combined and washed with brine (2 × 25 mL), dried over $MgSO_4$, filtered, and reduced under vacuum to give 2.93 g of triethylene glycol disulfide (**17**) as a yellow oil, 84% yield.

4.1.1. 2,2′-(2,2′-Disulfanediylbis(ethane-2,1-diyl)bis)oxy))-diethanol [diethylene glycol disulfide (16)][41]

45% Yield, ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.78–3.73 (m, 8H), 3.60 (t, J = 4.4 Hz, 4H), 2.94 (t, J = 6.3 Hz, 4H), 2.61 (t, J = 6.2 Hz, 2H).

4.1.2. 3,6,13,16-Tetraoxa-9,10-dithiaoctadecane-1,18-diol [triethylene glycol disulfide (17)][41]

84% Yield, ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.75–3.70 (m, 8H), 3.66–3.63 (m, 8H), 3.59 (t, J = 4.5 Hz, 4H), 3.04 (t, J = 5.5 Hz, 2H) 2.90 (t, J = 6.6 Hz, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 72.6, 70.4, 70.3, 69.6, 61.7, 38.4.

4.1.3. 3,6,9,16,19,22-Hexaoxa-12,13-dithiatetracosane-1,24-diol [tetraethylene glycol disulfide (18)][40]

91% Yield, ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.72 (t, J = 6.7 Hz, 4H), 3.68–3.63 (m, 20H), 3.60 (t, J = 4.5 Hz, 4H), 2.89 (t, J = 6.7 Hz, 4H), 2.74 (t, J = 5.7 Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 72.4, 70.4, 70.3, 70.1, 69.4, 61.4, 38.2.

4.2. Synthesis of *para*- and *ortho*-substituted aromatic ethylene glycol dithiols (1–5)

4.2.1. Synthesis of *para*- and *ortho*-substituted aromatic ethylene glycol disulfide polymers[57] (general procedure using polymer 20 as an example)

4,4′-Dithiobenzoic acid (**11**)[23,58,59] (0.213 g, 0.696 mmol) was dissolved in 4 mL of THF, and then DMF (10 μL) was added. The solution was cooled to 0 °C, and oxalyl chloride (**13**) (0.883 g, 6.96 mmol) was slowly added into the solution via syringe. The

solution was stirred for 3 h and then reduced under vacuum to give a yellow oil. The yellow oil was dissolved in 6 mL of CH_2Cl_2 and then transferred to a flask containing triethylene glycol disulfide (**17**) (0.981 g, 2.98 mmol), Et_3N (0.77 mL, 5.57 mmol), and 46 mL of CH_2Cl_2 at 0 °C. The reaction mixture was stirred at 0 °C for 30 min and gradually warmed to room temperature, and then stirring continued overnight. The reaction mixture was dissolved in 50 mL of CH_2Cl_2 and then washed with 50 mL of 0.1 N HCl. The organic layer was then washed with water (2 × 50 mL), dried ($MgSO_4$), filtered, and reduced under vacuum to provide 0.81 g of polymer **20**.

4.2.2. Reduction of *para*- and *ortho*-substituted aromatic ethylene glycol disulfide polymer with dithiothreitol (DTT) (general procedure using the reduction of compound 20 as an example)

DTT (1.032 g, 6.70 mmol) and Et_3N (0.5 mL, 3.59 mmol) were added to a concentrated solution of polymer **20** (0.81 g) in 15 mL of CH_2Cl_2. The reaction mixture was stirred (2 h for di- and triethylene glycol polymer, and 3 h for tetraethylene glycol polymer) under argon, and then the mixture was extracted with 50 mL of ethyl acetate. The organic layer was washed with water (2 × 50 mL), dried over $MgSO_4$, filtered, and concentrated under reduced pressure to give the crude product. The crude product was then purified by silica gel chromatography using CH_2Cl_2/hexane (2:1 for diethylene glycol dithiol, 4:1 for triethylene glycol dithiol, and 6:1 for tetraethylene glycol dithiol) to provide 0.124 g of *para*-substituted aromatic triethylene glycol dithiol (**2**) as a yellow oil, 30% overall yield from compound **11**.

4.2.2.1. 2-(2-Mercaptoethoxy)ethyl-4-mercaptobenzoate [*para*-substituted aromatic diethylene glycol dithiol (1)]. 48% Overall yield, ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.90 (d, J = 8.4 Hz, 2H), 7.28 (d, J = 8.4 Hz, 2H), 4.45 (t, J = 4.7 Hz, 2H), 3.79 (t, J = 4.8 Hz, 2H), 3.67–3.62 (m, 2H), 2.70 (dt, J = 8.1, 6.3 Hz, 2H), 1.59 (t, J = 8.2 Hz, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 166.1, 138.7, 130.4, 128.2, 127.1, 72.9, 69.0, 64.1, 24.4. HRMS (ESI$^+$) calcd for $C_{11}H_{14}O_3S_2$ (M+Na)$^+$ 281.0282, obsd 281.0289.

4.2.2.2. 2-(2-(2-Mercaptoethoxy)ethoxy)ethyl-4-mercaptobenzoate [*para*-substituted aromatic triethylene glycol dithiol (2)]. 30% Overall yield, ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.90 (d, J = 8.4 Hz, 2H), 7.28 (d, J = 8.4 Hz, 2H), 4.46 (t, J = 4.8 Hz, 2H), 3.83 (t, J = 4.8 Hz, 2H), 3.71–3.60 (m, 6H), 2.68 (dt, J = 8.1, 6.4 Hz, 2H), 1.58 (t, J = 8.2 Hz, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 166.1, 138.6, 130.4, 128.2, 127.1, 73.0, 70.7, 70.3, 69.3, 64.1, 24.4. HRMS (ESI$^+$) calcd for $C_{13}H_{18}O_4S_2$ (M+Na)$^+$ 325.0544, obsd 325.0538, calcd for $C_{13}H_{18}O_4S_2$ (M+H)$^+$ 303.0724, obsd 303.0721.

4.2.2.3. 2-[2-(2-(2-Mercaptoethoxy)ethoxy)ethoxy]ethyl-4-mercaptobenzoate [*para*-substituted aromatic tetraethylene glycol dithiol (3)]. 34% Overall yield, ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.90 (d, J = 8.4 Hz, 2H), 7.28 (d, J = 8.4 Hz, 2H), 4.45 (t, J = 4.8 Hz, 2H), 3.82 (t, J = 4.8 Hz, 2H), 3.71–3.58 (m, 10H), 2.68 (dt, J = 8.1, 6.5 Hz, 2H), 1.59 (t, J = 8.2 Hz, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 166.1, 138.6, 130.4, 128.2, 127.2, 73.0, 70.8, 70.8, 70.7, 70.3, 69.3, 64.2, 24.4. HRMS (ESI$^+$) calcd for $C_{15}H_{22}O_5S_2$ (M+Na)$^+$ 369.0806, obsd 369.0807.

4.2.2.4. 2-[2-(2-Mercaptoethoxy)ethoxy]ethyl-2-mercaptobenzoate [*ortho*-substituted aromatic triethylene glycol dithiol (4)]. 32% Overall yield, ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.03 (d, J = 7.6 Hz, 1H), 7.30–7.28 (m, 2H), 7.13 (ddd, J = 8.1, 5.6, 2.9 Hz, 1H), 4.67 (s, 1H), 4.46 (t, J = 4.8 Hz, 2H), 3.82 (t, J = 4.8 Hz, 2H), 3.69–3.57 (m, 6H), 2.66 (dt, J = 8.2, 6.5 Hz, 2H), 1.55 (t, J = 8.2 Hz, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 166.7, 138.3, 132.6, 131.9, 130.9, 126.0, 124.7, 73.0, 70.7, 70.3, 69.2, 64.3, 24.4. HRMS (ESI$^+$) calcd for $C_{13}H_{18}O_4S_2$ (M+Na)$^+$ 325.0544, obsd 325.0536, calcd for (M+H)$^+$ 303.0724, obsd 303.0718.

4.2.2.5. 2-[2-(2-(2-Mercaptoethoxy)ethoxy)ethoxy]ethyl-2-mercaptobenzoate [*ortho*-substituted aromatic tetraethylene glycol dithiol (5)]. 29% Overall yield, ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.03 (d, J = 7.5 Hz, 1H), 7.31–7.29 (m, 2H), 7.15 (ddd, J = 8.1, 5.6, 3.0 Hz, 1H), 4.70 (s, 1H), 4.47 (t, J = 4.8 Hz, 2H), 3.83 (t, J = 4.8 Hz, 2H), 3.70–3.57 (m, 10H), 2.67 (dt, J = 8.2, 6.5 Hz, 2H), 1.57 (t, J = 8.2 Hz, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 166.7, 138.7, 132.6, 132.0, 131.0, 126.1, 124.8, 73.0, 70.8, 70.8, 70.7, 70.4, 69.2, 64.4, 24.4. HRMS (ESI$^+$) calcd for $C_{15}H_{22}O_5S_2$ (M+Na)$^+$ 369.0806, obsd 369.0788.

4.3. Synthesis of aromatic triethylene glycol monothiol (9)

Compound **9** was prepared in 57% overall yield using the general procedures for compounds **1–5** with benzoyl chloride and triethylene glycol disulfide (**17**) as starting materials.

^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.04 (d, J = 7.0 Hz, 2H), 7.55 (t, J = 7.4 Hz, 1H), 7.42 (t, J = 7.7 Hz, 2H), 4.47 (t, J = 4.8 Hz, 2H), 3.83 (t, J = 3.7 Hz, 2H), 3.70 (t, J = 4.0 Hz, 2H), 3.65–3.58 (m, 4H), 2.66 (q, J = 6.4 Hz, 2H), 1.55 (t, J = 8.2 Hz, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 166.6, 133.1, 130.2, 129.8, 128.4, 73.0, 70.8, 70.4, 69.4, 64.2, 24.4. HRMS (ESI$^+$) calcd for $C_{13}H_{18}O_4S$ (M+Na)$^+$ 293.0823, obsd 293.0823.

4.4. Synthesis of quaternary ammonium salt dithiols (6 and 7)

4.4.1. Synthesis of protected quaternary ammonium salt dithiols 30 and 31[60] (general procedure using 30 as an example)

Benzyl bromide derivative **27**[51,52] (2.10 g, 6.84 mmol) was added to a stirred solution of *N*,*N*,*N*′,*N*′-tetramethyl-1,3-propane diamine (**28**) (0.38 g, 2.92 mmol) in 20 mL of acetonitrile. The reaction mixture was stirred overnight, diluted with diethyl ether, and filtered. The solid was then washed with diethyl ether and dried yielding 2.07 g of a white powder (**30**), 95% yield.

4.4.1.1. N^1,N^3-Bis(4-benzoylthio)benzyl)-N^1,N^1,N^3,N^3-tetramethylpropane-1,3-diaminium bromide [propane salt (30)]. 95% Yield, ^{1}H NMR (DMSO, 400 MHz) δ 8.00 (d, J = 7.4 Hz, 4H), 7.78–7.70 (m, 10H), 7.62 (t, J = 7.8 Hz, 4H), 4.74 (s, 4H), 3.37 (t, J = 8.0 Hz, 4H), 3.11 (s, 12H), 2.50–2.46 (m, 2H). ^{13}C NMR (DMSO, 100 MHz) δ 188.6, 135.7, 135.2, 134.6, 134.0, 129.6, 129.5, 129.3, 127.2, 66.5, 60.1, 49.7, 16.9. HRMS (ESI$^+$) calcd for $C_{35}H_{40}Br_2N_2O_2S_2$ (M−Br)$^+$ 663.1714, obsd 663.1685.

4.4.1.2. N^1,N^6-Bis(4-benzoylthio)benzyl)-N^1,N^1,N^6,N^6-tetramethylhexane-1,6-diaminium bromide [hexane salt (31)]. 99% Yield, ^{1}H NMR (DMSO, 400 MHz) δ 7.99 (d, J = 7.3 Hz, 4H), 7.79–7.69 (m, 10H), 7.62 (t, J = 7.9 Hz, 4H), 4.67 (s, 4H), 3.33 (br s, 4H), 3.03 (s, 12H), 2.07 (br s, 4H), 1.38 (br s, 4H). ^{13}C NMR (DMSO, 100 MHz) δ 188.5, 135.6, 135.1, 134.5, 133.8, 129.5, 129.3, 127.1, 65.5, 63.5, 49.3, 25.3, 21.7. HRMS (ESI$^+$) calcd for $C_{38}H_{46}Br_2N_2O_2S_2$ (M−Br)$^+$ 705.2184, obsd 705.2163.

4.4.2. Synthesis of quaternary ammonium salt dithiols 6 and 7 (general procedure using 6 as an example)

The quaternary ammonium salt (**30**) (1.557 g, 2.09 mmol) dissolved in 45 mL of water and 45 mL of HBr (48 wt % in water) was refluxed under argon. After 90 min, the reaction mixture was

cooled to 0 °C, filtered, and then washed with EtOAc (3 × 100 mL). The aqueous layer was reduced partially and then lyophilized several times by dissolving the residue in water to provide 1.042 g of **6** as a white powder, 93% yield.

4.4.2.1. N^1,N^3-Bis(4-mercaptobenzyl)-N^1,N^1,$N^3$$N^3$-tetramethylpropane-1,3-diaminium bromide (6). 93% Yield, ^{1}H NMR (D_2O, 400 MHz) δ 7.46 (d, J = 8.4 Hz, 4H), 7.41 (d, J = 8.4 Hz, 4H), 4.52 (s, 4H), 3.37 (t, J = 8.3 Hz, 4H), 3.09 (s, 12H), 2.41 (quintet, J = 8.3 Hz, 2H). ^{13}C NMR (D_2O, 100 MHz) δ 135.8, 133.5, 129.0, 123.5, 68.5, 60.1, 49.7, 17.0. HRMS (ESI$^+$) calcd for $C_{21}H_{32}Br_2N_2S_2$ $(M-Br)^+$ 455.1190, obsd 455.1175.

4.4.2.2. N^1,N^6-Bis(4-mercaptobenzyl)-N^1,N^1,$N^6$$N^6$-tetramethylhexane-1,6-diaminium bromide (7). 51% Yield, ^{1}H NMR (D_2O, 400 MHz) δ 7.50 (d, J = 7.2 Hz, 4H), 7.45 (d, J = 7.7 Hz, 4H), 4.48 (s, 4H), 3.30 (t, J = 8.0 Hz, 4H), 3.08 (s, 12H), 1.92 (br s, 4H), 1.47 (br s, 4H). ^{13}C NMR (D_2O, 100 MHz) δ 135.3, 133.4, 128.9, 124.1, 67.4, 63.8, 49.6, 25.2, 22.0. HRMS (ESI$^+$) calcd for $C_{24}H_{38}Br_2N_2S_2$ $(M-Br)^+$ 497.1659, obsd 497.1638.

4.5. Enzymatic assays

4.5.1. Refolding of denatured reduced lysozyme (0.1 mg/mL)

Reduced lysozyme[18,25,61] (10 mg/mL) was dissolved in 0.1 M acetic acid containing 6 M Gdn HCl at pH 2.5 (ε_{280nm} = 2.37 mL mg^{-1} cm^{-1}).[46,47] The pH of the stock solutions containing the thiol or disulfide were also adjusted to the appropriate pH (7.0 or 8.0) with 1 M KOH prior to use. Refolding of reduced lysozyme was carried out in a deoxygenated renaturation buffer [(0.1 M bis tris propane–HCl for pH 7.0 or 0.1 M Tris–HCl for pH 8.0), 1 mM EDTA, 0.5 M Gdn HCl, (various concentrations of GSH, aromatic monothiol, or aromatic dithiols), and GSSG]. In a 1.5 mL-Eppendorf tube, 10 μL of reduced lysozyme (10 mg/mL) was added to 990 μL of renaturation buffer. For reproducible mixing conditions, the renaturation buffer was placed in an Eppendorf tube, the protein was added 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, 20 μL aliquots were removed at specific times and assayed for enzymatic activity.[18,46,47,49]All the refolding experiments were performed at 25 °C.

4.5.2. Refolding of denatured reduced lysozyme (1 mg/mL)

Reduced lysozyme (11 mg/mL) was dissolved in 0.1 M acetic acid containing 6 M Gdn HCl at pH 2.5. The pH of the protein solution was then adjusted to that of the folding experiment (pH 7.0 or 8.0) by the addition of 3 M KOH. After adjusting the pH, the concentration of the protein solution was determined by UV–vis, and the final concentration was adjusted to 10 mg/mL with 6 M Gdn HCl, if necessary (ε_{280nm} = 2.37 mL mg^{-1} cm^{-1}).[25,34,47] Stock solutions of GSH, aromatic monothiol, aromatic dithiols, and GSSG were prepared in deoxygenated 0.1 M bis tris propane–HCl (for pH 7.0) or 0.1 M Tris–HCl (for pH 8.0), 1.25 M Gdn HCl, and 1 mM EDTA. From the stock solutions, varying amount of GSH and GSSG, aromatic monothiol and GSSG, or aromatic dithiols and GSSG were mixed in 0.1 M bis tris propane–HCl at pH 7.0 or 0.1 M Tris–HCl at pH 8.0, 1.25 M Gdn HCl, and 1 mM EDTA and adjusted to the appropriate pH with 1 M KOH, if necessary (renaturation buffer). In a 1.5 mL-Eppendorf tube, 900 μL of renaturation buffer was mixed with 100 μL of reduced lysozyme solution (10 mg/mL) to give a 10-fold dilution and a final concentration of 1 mg/mL protein and 1.7 M Gdn HCl. Subsequently, at specific intervals, 10 μL aliquots were withdrawn and each aliquots was diluted with 90 μL of buffer (0.1 M bis tris propane–HCl at pH 7.0 or 0.1 M Tris–HCl at pH 8.0, 1.25 M Gdn HCl, and 1 mM EDTA) followed by withdrawing 20 μL of the resulting solution and then measuring the enzymatic activity.[19,46,47,49]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.11.049.

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