Effects of Aromatic Thiols on Thiol−Disulfide Interchange Reactions That Occur during Protein Folding

Todd V. DeCollo and Watson J. Lees*

Department of Chemistry, Center for Science and Technology, Syracuse University, Syracuse, New York 13244

wjlees@syr.edu

Received February 28, 2001 (Revised Manuscript Received April 25, 2001)

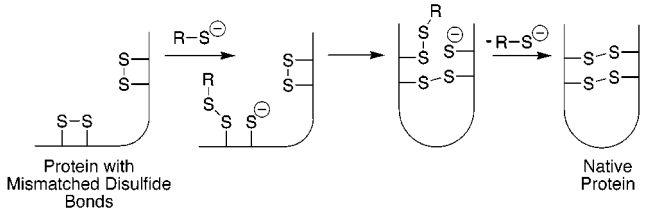
The folding of disulfide containing proteins from denatured protein to native protein involves numerous thiol−disulfide interchange reactions. Many of these reactions include a redox buffer, which is a mixture of a thiol (RSH) and the corresponding disulfide (RSSR). The relationship between the structure of RSH and its efficacy in folding proteins in vitro has been investigated only to a limited extent. Reported herein are the effects of aliphatic and especially aromatic thiols on reactions that occur during protein folding. Aromatic thiols may be particularly efficacious as their thiol pK_a values and reactivities match those of the in vivo catalyst, protein disulfide isomerase (PDI). This investigation correlates the thiol pK_a values of aromatic thiols with their reactivities toward small molecule disulfides and the protein insulin. The thiol pK_a values of nine para-substituted aromatic thiols were measured; a Hammett plot constructed using σ_p^- values yielded $\rho = -1.6 \pm 0.1$. The reactivities of aromatic and aliphatic thiols with 2-pyridyldithioethanol (2-PDE), a small molecule disulfide, were determined. A plot of reactivity versus pK_a of the aromatic thiols had a slope (β) of 0.9. The ability of these thiols to reduce (unfold) the protein insulin correlates strongly with their ability to reduce 2-PDE. Since the reduction of protein disulfides occurs during protein folding to remove mismatched disulfides, aromatic thiols with high pK_a values are expected to increase the rate not only of protein unfolding but protein folding as well.

Introduction

The production of proteins in bacteria has been essential for the elucidation of protein structure and function. Unfortunately, in many cases when proteins are overexpressed in bacteria, they are isolated as aggregates of inactive protein known as inclusion bodies. To obtain active protein from the inclusion bodies, the inactive protein is denatured and then folded in vitro.[1−4] For proteins containing disulfide bonds, the folding rate usually is limited by a thiol−disulfide interchange reaction. This reaction occurs within the protein itself or between the protein and the redox buffer, which is a mixture of a thiol (RSH) and the corresponding disulfide (RSSR, Scheme 1).[5] Despite intense examination of protein folding, only a few studies have investigated the relationship between the structure of the redox buffer and the efficiency of protein folding. The standard redox buffers have been mercaptoethanol or cysteine derivatives such as glutathione.

We sought to design small molecule thiols as redox buffers for protein folding. The design was based on the thiol pK_a value and inherent nucleophilicity of protein disulfide isomerase (PDI), nature's solution to the protein-folding problem. PDI has two similar active sites which both contain two thiols in a CXXC motif.[6] One thiol in each active site is essential for catalysis, has a pK_a value of 6.7, and is exceptionally reactive with disulfides.[7−11] Glutathione, mercaptoethanol, and almost all other aliphatic thiols have thiol pK_a values above 7. In comparison, aromatic thiols have thiol pK_a values between 4 and 7 and are more reactive toward aromatic disulfides than aliphatic thiols of similar pK_a values.[12−15] Conse-

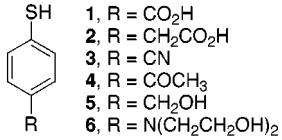
Scheme 1. Folding of a Protein with Mismatched Disulfide Bonds



(1) Woycechowsky, K. J.; Raines, R. T. *Curr. Opin. Chem. Biol.* **2000**, *4*, 533−39.
(2) De Bernardez Clark, E. *Curr. Opin. Biotechnol.* **1998**, *9*, 157−63.
(3) Chaudhuri, J. B. *Ann. N. Y. Acad. Sci.* **1994**, *721*, 374−85.
(4) Rudolph, R.; Lilie, H. *FASEB J.* **1996**, *10*, 49−56.
(5) Wedemeyer, W. J.; Welker, E.; Narayan, M.; Scheraga, H. A. *Biochemistry* **2000**, *39*, 4207−16.
(6) Edman, J. C.; Ellis, L.; Blacher, R. W.; Roth, R. A.; Rutter, W. J. *Nature (London)* **1985**, *317*, 267−70.
(7) Darby, N.; Creighton, T. E. *Biochemistry* **1995**, *34*, 16770−80.
(8) Hawkins, H. C.; Freedman, R. B. *Biochem. J.* **1991**, *275*, 335−9.
(9) Walker, K. W.; Lyles, M. M.; Gilbert, H. F. *Biochemistry* **1996**, *35*, 1972−80.
(10) LaMantia, M.; Lennarz, W. J. *Cell (Cambridge, MA)* **1993**, *74*, 899−908.
(11) Laboissiere, M. C. A.; Sturley, S. L.; Raines, R. T. *J. Biol. Chem.* **1995**, *270*, 28006−9.
(12) Wilson, J. M.; Bayer, R. J.; Hupe, D. J. *J. Am. Chem. Soc.* **1977**, *99*, 7922−6.
(13) Szajewski, R. P.; Whitesides, G. M. *J. Am. Chem. Soc.* **1980**, *102*, 2011−26.
(14) Whitesides, G. M.; Lilburn, J. E.; Szajewski, R. P. *J. Org. Chem.* **1977**, *42*, 332−8.
(15) De Maria, P.; Fini, A.; Hall, F. M. *J. Chem. Soc., Perkin Trans. 2* **1977**, 149−51.

10.1021/jo015600a CCC: $20.00
Published on Web 05/22/2001

Scheme 2



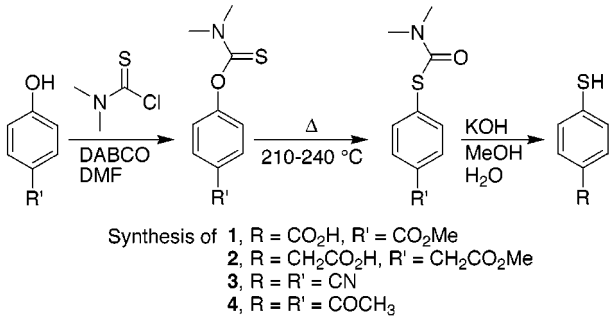
quently, the thiol pK_a and reactivity of aromatic thiols more closely match those of PDI. The correlation between aromatic thiol pK_a value and reactivity in reactions that take place during protein folding is the focus of our research. Previously, the only reaction of aromatic thiols in aqueous solution investigated was that with Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid)), an aromatic disulfide.[12,14] Results from these studies indicated enhanced reactivity of aromatic thiols relative to aliphatic thiols which was ascribed to either a "hydrophobic interaction between aryl groups on the attacking and central thiols" or to "hard−soft acid−base behavior".[12] If hydrophobic interaction between aryl groups is required for enhanced reactivity then aromatic thiols will not exhibit similar enhanced reactivity during protein folding reactions. In addition, aryl disulfides are conceivably poor models for a protein disulfide. Preliminary results from another laboratory have reported no clear correlation between reaction rates of thiols in general with Ellman's reagent and effectiveness as reducing agents for proteins.[14] Thus, it is unclear if results obtained with Ellman's reagent will be similar to results obtained with protein disulfides or even other small molecule disulfides that do not contain two aromatic groups. Therefore, initial investigations featured the reactions of small molecule aromatic and aliphatic thiols with small molecule disulfides and protein disulfides to determine if aromatic thiols have enhanced reactivity with a broad range of disulfides. In addition, to allow the rationale design of aromatic thiols we investigated the relationship between thiol pK_a and reactivity with small molecule disulfides and protein disulfides. The two-step reduction of protein disulfides to dithiols is an important component of the folding process as it removes intermediates with mismatched disulfide bonds.[5]

We report the synthesis of numerous water-soluble aromatic thiols and their impact upon reactions that take place during protein folding. The pK_a values of several aromatic thiols were determined for the first time, and a Hammett plot was constructed to create a predictive model for aromatic thiol pK_a values. The rate constants of aromatic thiols with small molecule nonbis(aromatic)-disulfides were determined and correlated with thiol pK_a values. In addition, the relative rates of reducing insulin with aromatic or aliphatic thiols or PDI were determined. The effect of thiol pK_a on reactivity and the relative reaction rates of PDI and small molecule thiols are discussed.

Results and Discussion

Synthesis. A series of para-substituted aromatic thiols was synthesized with electron-donating or electron-withdrawing substituents (**1**−**6**, Scheme 2). Thiols **1**−**4** were synthesized via a Newman-Kwart reaction (Scheme 3).[16,17] Compound **5** was prepared by reduction of **1** with

Scheme 3



Synthesis of **1**, R = CO_2H, R' = CO_2Me
2, R = CH_2CO_2H, R' = CH_2CO_2Me
3, R = R' = CN
4, R = R' = $COCH_3$

Scheme 4

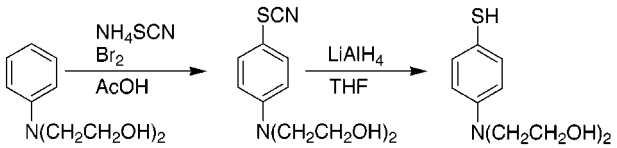


Table 1. pK_a Values of Para-Substituted Aromatic Thiols

		method of determining thiol pK_a values		
	substitutent	UV−vis	titration	lit.[24,25]
1	CO_2H	6.0		5.80
2	CH_2CO_2H	6.6		
3	CN	4.9		
4	$COCH_3$	5.0		5.33
5	CH_2OH	6.4		
6	$N(CH_2CH_2OH)_2$		7.0	
7	OH		6.9	
8	NO_2	4.5		4.71, 4.50
9	H	6.6		6.61, 6.43

lithium aluminum hydride. Compound **6** was obtained via a thiocyanate intermediate, which was subsequently reduced to the desired aromatic thiol (Scheme 4).[18,19]

pK_a Determinations. The pK_a values of the aromatic thiols were determined either by measuring the change in the UV−vis spectrum as a function of pH[20,21] or by acid−base titration.[22,23] Although the UV−vis method was preferred, it was not applicable to all compounds, as it required the spectra of the thiol and thiolate to be readily distinguishable. The error in the pK_a values at 95% confidence was less than ±0.2 for all compounds measured by the UV−vis method (Table 1). For compounds **6** and **7**, thiol and thiolate spectra were not sufficiently distinguishable requiring recourse to acid−base titration (Table 1).

A method to predict the pK_a values of aromatic thiols was sought, as this would allow the rational design of thiols. A Hammett plot comparing σ_p^- of the substituent with the observed pK_a values provided a slope of −1.62, $\rho = -1.6 \pm 0.1$ (Figure 1).[26] A previous Hammett plot yielded $\rho = -1.8 \pm 0.1$, but it was based upon nonstand-

(16) Newman, M. S.; Karnes, H. A. *J. Org. Chem.* **1966**, *31*, 3980−4.
(17) Kaji, A.; Araki, Y.; Miyazaki, K. *Bull. Chem. Soc. Jpn.* **1971**, *44*, 1393−9.
(18) Brewster, R. Q.; Schroeder, W. *Organic Syntheses*; Wiley: New York, 1943; Collect. Vol. II, pp 574−6.
(19) O'Connor, G. L.; Nace, H. R. *J. Org. Chem.* **1953**, *18*, 2118−22.
(20) Lees, W. J.; Singh, R.; Whitesides, G. M. *J. Org. Chem.* **1991**, *56*, 7328−31.
(21) Benesch, R. E.; Benesch, R. *J. Am. Chem. Soc.* **1955**, *77*, 5877−81.
(22) Houk, J.; Singh, R.; Whitesides, G. M. *Methods Enzymol.* **1987**, *143*, 129−40.
(23) Whitesides, G. M.; Houk, J.; Patterson, M. A. K. *J. Org. Chem.* **1983**, *48*, 112−15.

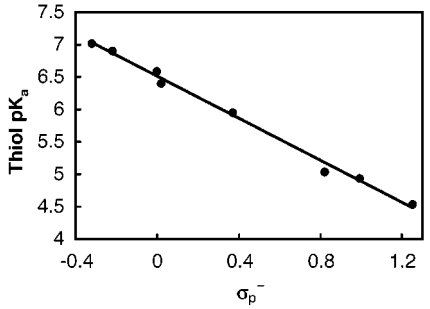
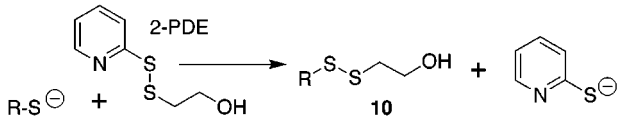


Figure 1. Hammett plot of para-substituted benzenethiol pK_a values versus σ_p^-. The σ_p^- value for a dimethylamino substituent was used for the diethanolamino substituent. No suitable σ_p^- value could be found for the $-CH_2COO^-$ substituent.

Scheme 5. Reduction of 2-PDE by Thiolates (RS−) at pH 7 (Figure 2)



ard σ_p values instead of σ_p^-, thus limiting its predictive ability for our purposes.[24] Now if the σ_p^- value of a substituent is known, the pK_a value of the corresponding para-substituted aromatic thiol is predictable.[24,25]

Kinetics of the Reduction of Small Molecule Disulfides by Thiols. The only thiol−disulfide interchange reaction of aromatic thiols investigated previously was that with Ellman's reagent.[12,14] For comparison, we initially sought to perform similar experiments with our aromatic thiols, some of which were expected to be considerably more reactive than those previously studied at pH 7. Preliminary results indicated that the assay was not practical without the use of a stop-flow apparatus due to exceptionally fast reactions. Interpretation of the data is also complicated because Ellman's reagent and the mixed disulfide between Ellman's reagent and an aromatic thiol have similar reactivities.[12,14] However, the results did confirm the enhanced reactivity of aromatic thiols relative to aliphatic thiols of similar pK_a value, at least with Ellman's reagent. Therefore, to obtain quantitative results we could either advance to a different disulfide instead of Ellman's reagent or lower the pH significantly from physiological pH, which would slow the reaction. Since we were ultimately interested in reactions with proteins at close to physiological pH we chose to advance to a less reactive disulfide, 2-pyridyldithioethanol (2-PDE) (Scheme 5).

The disulfide 2-PDE provides an almost ideal substrate for determining reaction rate constants of aromatic thiols with disulfides. We have found the reaction rate constants for **1** (Scheme 2), a slowly reacting aromatic thiol, and trifluoroethanethiol with 2-PDE at pH 7 are approximately 4% and 2% of their reaction rate constants with Ellman's reagent, respectively.[12,14] The mixed disulfide between aromatic thiol and mercaptoethanol (**10**, Scheme 5) is expected to react with aromatic thiol at a much slower rate than the reaction of 2-PDE with aromatic thiol, thereby simplifying data interpretation relative to Ellman's reagent. Unlike Ellman's reagent, which has a double negative charge at pH 7, 2-PDE is neutral at pH 7, thus minimizing charge effects between thiol and disulfide. In addition, 2-PDE will not allow π-stacking between the nucleophilic aromatic thiolate and the group attached to the most electrophilic sulfur of the disulfide. The most electrophilic sulfur is attached to an aliphatic group for 2-PDE and an aromatic group for Ellman's reagent. The extinction coefficient at λ_{max} of the thiolate of 2-PDE (ϵ_{343} = 7060 mol $^{-1}$ L^{-1} cm^{-1}) is half that of Ellman's reagent (ϵ_{412} = 13 700 mol^{-1} L^{-1} cm^{-1}), but this is more than compensated for by the enhanced accuracy enabled by the slower reaction rate of 2-PDE.[27]

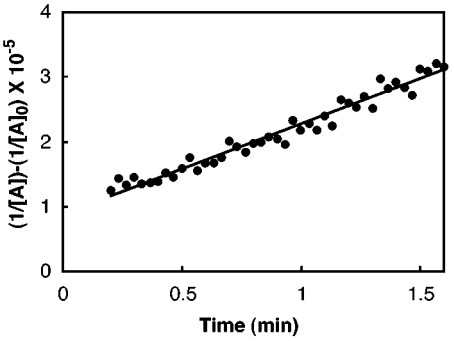


Figure 2. Determination of the second-order rate constant, k^{obsd}, for the reduction of 2-PDE by compound **6**. The slope corresponds to k^{obsd}. The values of $[A]_0$, the initial concentration of 2-PDE, and [A] are calculated from the change in absorbance at 343 nm. The 2-pyridylthiolate released in the reaction absorbs at 343 nm (ϵ = 7060 mol^{-1} L^{-1} cm^{-1}). The standard deviation of k^{obsd} is ±10%.

Table 2. Rate Constants for the Reduction of 2-PDE by Thiols at pH 7 (Scheme 5)

thiol	pK_a of thiol	k_1^{obsd} (M^{-1} min^{-1})	k_1 (M^{-1} min^{-1})
6	7.0	14×10^4	29×10^4
7	6.9	13×10^4	23×10^4
2	6.6	7.7×10^4	11×10^4
5	6.4	5.6×10^4	7.0×10^4
1	6.0	3.2×10^4	3.5×10^4
2-HOC_6H_4SH	5.7	2.9×10^4	3.0×10^4
F_3CCH_2SH	7.3	1.3×10^4	3.8×10^4
glutathione	8.7	0.24×10^4	12×10^4

The 2-PDE assay provides k_1^{obsd} for the reaction between 2-PDE and a thiol at pH 7 (Table 2, Figure 2). If the pH of the reaction is changed then k_1^{obsd} may also change. A rate constant independent of pH, k_1, can be calculated; it corresponds to the reaction of pure thiolate with disulfide and thus reflects the intrinsic reactivity of the thiolate, independent of pH effects. Under the reaction conditions, the thiol form of the compound is a very poor nucleophile and contributes negligibly to the reaction. To calculate k_1, the proportion of the compound in the thiolate form is determined from the pH of the solution and the pK_a of the thiol; $(1/(1 + 10^{pK_a - pH})$. The measured rate constant, k_1^{obsd}, is divided by the proportion of the compound in the thiolate form to obtain the reaction rate constant of the thiolate $k_1 = k_1^{obsd}(1 + 10^{pK_a - pH})$. The reaction rate constants of aromatic thiols with 2-PDE increases with increasing thiol pK_a values, as is the case with Ellman's reagent. A plot of log k_1 versus *para*-substituted aromatic thiol pK_a is linear with

(24) De Maria, P.; Fini, A.; Hall, F. M. *J. Chem. Soc., Perkin Trans. 2* **1973**, 1969−71.

(25) Jencks, W. P.; Salvesen, K. *J. Am. Chem. Soc.* **1971**, *93*, 4433−6.

(26) Exner, O. *Correlation Analysis of Chemical Data*; Plenum Press: New York, 1988.

(27) Grimshaw, C. E.; Whistler, R. L.; Cleland, W. W. *J. Am. Chem. Soc.* **1979**, *101*, 1521−32.

a positive slope, $\beta = 0.9 \pm 0.1$ ($r^2 = 0.99$). The slope is greater than the β values of 0.2−0.7 previously reported for thiol−disulfide interchange reactions.[12,14] The greater slope may be the result of aromatic thiolates reacting with a nonbis(aromatic) disulfide, a substrate type that has not been investigated previously.

The pK_a value of the thiol with the greatest k_1^{obsd} at a given pH in a thiol−disulfide interchange reaction can be calculated from β and the pH of the solution.[14] Two competing factors affect the reaction rates of thiols. The first one is the portion of thiol in the reactive thiolate form, which is determined by the thiol pK_a value and the pH of the solution. The greater the proportion of compound in the thiolate form the lower the pK_a of the thiol. The second one is the nucleophilicity of the thiolate, which for a structurally similar set of compounds increases with the pK_a of the conjugate acid (thiol) and can be determined from the thiol pK_a value and β, the proportionality constant. The more reactive the thiolate the greater the pK_a of the conjugate acid (thiol). The optimum pK_a value of the thiol can be determined from the following equation: $(1 - \beta)/\beta = 10^{pH-pK_a}$.[14] With a β value of 0.9 (2-PDE), the optimum thiol pK_a value is approximately 1 unit greater than the pH of solution. With a β value of 0.5 (reported for Ellman's reagent),[12] it is equivalent to the pH of solution. Thus, depending on whether the aromatic thiol is reacting with 2-PDE or Ellman's reagent its optimum pK_a value is quite different.

Kinetics of Reduction of Protein Disulfides by Thiols. The first assay attempted was the reduction of the protein papain-S−S-Me to papain-SH, the enzymatically active form.[28−30] Preliminary results using a coupled assay demonstrated that the reaction rates of aromatic thiols with papain-S−S-Me were considerably faster than aliphatic thiols with papain-S−S-Me at pH 7. Unfortunately, the reaction with aromatic thiols was so rapid at pH 7 that no quantitative data could be obtained. Therefore, from the papain assay we can conclude that aromatic thiols react faster with protein disulfides than aliphatic thiols at pH 7.

Increases in the rate of reduction of the protein insulin in the presence of dithiothreitol (DTT) by aromatic thiols provided quantitative data.[31] Native insulin has three disulfide bonds, two of which connect the α chain to the β chain. Cleavage of these disulfide bonds leads to the formation of two separate peptides (Scheme 6). When insulin is reduced at pH 6.5, the liberated β-chain slowly reaches a critical concentration at which it precipitates out of solution, forming a cloudy white suspension which scatters 650 nm light. The rate of reduction of insulin was followed by measuring the rate of increase in light scattering with time (Figure 3).

The rate of reduction of native insulin by a mixture of the thiol of interest (1 equiv of thiol groups relative to thiols in reduced insulin) and DTT (2 equiv) was compared to the rate of reduction of native insulin by DTT (2 equiv) alone. A control reaction containing native insulin, DTT (2 equiv), and a compound structurally similar to the thiol of interest but with the thiol group replaced by either a hydroxyl group or hydrogen was also prepared. All three reactions were run simultaneously.

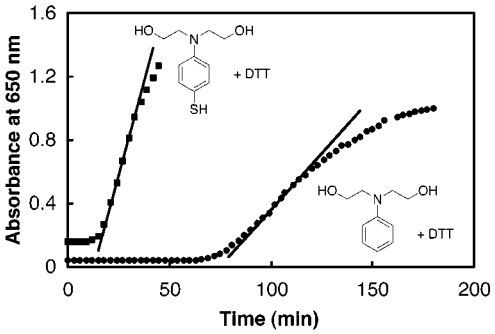
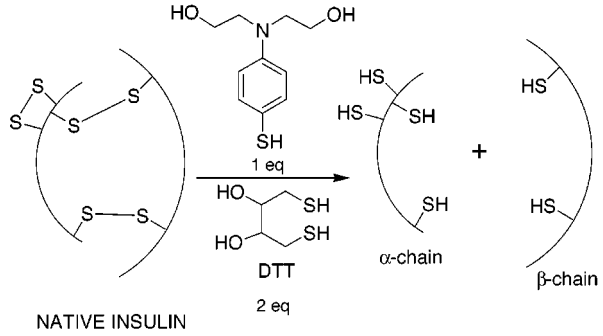


Figure 3. Following the reduction of insulin by compound **6** and DTT (squares), and an analogue of compound **6** and DTT (circles) by observing the light scattered at 650 nm. The rate of reduction is determined from the slope of the linear portion.[31] The curve observed for the reduction of insulin by an analogue of compound **6** and DTT was almost identical to that of DTT only (not shown).

Scheme 6. Reduction of Native Insulin by Aromatic Thiol + DTT at pH 6.5



The relative rate was measured to account for minor variations between batches of native insulin. The control reaction demonstrated that other functional groups in the molecule did not change the rate of reduction of insulin by DTT. DTT, a strongly reducing dithiol, was added to all reactions to ensure the equilibrium was shifted toward reduced insulin. DTT will reduced the aromatic disulfides formed along with reduced insulin back to the aromatic thiol, thus shifting the equilibrium toward reduced insulin. In all three reactions, the light scattered at 650 nm initially changed very little, but as the precipitate formed, the scattered light increased linearly with time (Figure 3). Holmgren has shown the rate of reduction of insulin is proportional to the slope of the linear portion and to the lag time before precipitation occurs.[31] We found the relative ratios of the slopes to be more reproducible than the relative ratios of the lag times. These results were qualitatively confirmed by tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS−PAGE).[32]

The addition of aromatic thiols to a mixture of insulin and DTT increased the rate of reduction of insulin but the addition of aliphatic thiols did not, within experimental error. The rate of reduction of insulin increased with increasing pK_a of the aromatic thiol (Table 3). Given the β value, 0.9, for 2-PDE the optimum pK_a value of the aromatic thiol is predicted to be approximately 1 unit greater than the pH of the solution, the data are consistent with this prediction.[14] The trend in relative reaction rates also correlates with the reactivity of the

(28) Singh, R.; Whitesides, G. M. *J. Org. Chem.* **1991**, *56*, 2332−7.
(29) Singh, R.; Blattler, W. A.; Collinson, A. R. *Anal. Biochem.* **1993**, *213*, 49−56.
(30) Mole, J. E.; Horton, H. R. *Biochemistry* **1973**, *12*, 816−22.
(31) Holmgren, A. *J. Biol. Chem.* **1979**, *254*, 9627−32.
(32) Schaegger, H.; Von Jagow, G. *Anal. Biochem.* **1987**, *166*, 368−79.

Table 3. Relative Rates of Insulin Reduction by Thiol + DTT (0.8 mM) at pH 6.5

thiol (mass, concentration)	pK_a of thiol	rate relative to DTT only
1 (123 μg, 0.7 mM)	6.0	1.6
5 (102 μg, 0.7 mM)	6.4	1.9
2 (134 μg, 0.7 mM)	6.6	2.0
6 (190 μg, 0.7 mM)	7.0	3.3
6 (380 μg, 1.5 mM)	7.0	8.1
CF_3CH_2SH (93 μg, 0.7 mM)	7.3	1.1
cysteine (98 μg, 0.7 mM)	8.3	0.9
glutathione (245 μg, 0.7 mM)	8.7	1.0
PDI (20 μg, 0.0003 mM)	6.7	3.2
PDI (10 μg, 0.0001 mM)	6.7	1.8

Table 4. Relative Reaction Rates of Thiols

thiol	pK_a	2-PDE at pH 6.5 $k_1/(1 + 10^{pKa-6.5})$	relative rates PDE	relative rates insulin[a]
6	7.0	7.0×10^4	3.3[b]	3.3
2	6.6	4.9×10^4	2.3	2.0
5	6.4	3.9×10^4	1.9	1.9
1	6.0	2.6×10^4	1.3	1.6
CF_3CH_2SH	7.3	0.52×10^4	0.25	1.1
cysteine	8.3	0.075×10^4	0.04	0.9

[a] The reaction rates of a mixture of thiol and DTT with insulin are relative to DTT only; the minimum value is approximately 1. [b] Set to 3.3 to allow a direct comparison with the reaction rates of insulin.

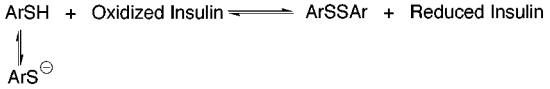
Table 5. Reduction of Insulin by Aromatic Thiols in the Absence or Presence of DTT

thiol	pK_a of thiol	rate relative to DTT only
5 (0.7 mM)	6.4	0.6
5 (0.7 mM) + DTT (0.8 mM)	6.4	1.8
6 (0.7 mM)	7.0	3.4
6 (0.7 mM) + DTT (0.8 mM)	7.0	3.4

aromatic thiols with 2-PDE, k_1, after an adjustment to account for the pH of the reaction, pH = 6.5 (Table 4).

To achieve the same rate of reduction of insulin in the presence of DTT, 10 times and 5 times the weight of aromatic thiol **6** is needed to match PDI (57 kDa) and Thioredoxin (12 kDa),[31] respectively. On a mole basis, these numbers increase to 1000 and 200, per active site, respectively. However, the expense of using thioredoxin or PDI is significant and sometimes prohibitive. Also, thioredoxin and PDI can be difficult to separate from the protein of interest, while small molecules such as **6** are not. Therefore, on a weight basis, aromatic thiol **6** compares favorably with PDI and thioredoxin, nature's protein refolding/unfolding catalysts.To allow a more direct comparison with the 2-PDE results, the aromatic thiols were tested for their ability to reduce insulin in the absence of DTT. Again, three reactions were run simultaneously. The first reaction measured the rate of reduction of insulin by the aromatic thiol (1 equiv) alone, the second measured the rate of reduction of insulin by DTT (2 equiv) alone, and the third reaction measured the rate of reduction of insulin by a mixture of aromatic thiol and DTT (Table 5). Although thiols **1**, **5**, and **6** were tested, only **5** and **6** yielded measurable rates. The reaction rate for compound **5** with insulin was 20% that of compound **6**; the same comparison for 2-PDE was 60%. The discrepancy is likely the result of a shift in the equilibrium. As the pK_a of the aromatic thiol (ArSH) decreases, the concentration of aromatic thiolate (ArS^-) increases at the expense of aromatic thiol, and the equilibrium is shifted away from reduced insulin and toward the aromatic thiolate (Scheme 7).

Scheme 7



In conclusion, we have demonstrated a strong correlation between the σ_p^- value of a substituent, the pK_a value of the corresponding para-substituted aromatic thiol, the reactivity of the corresponding para-substituted aromatic thiol with small molecule disulfides, and the ability of the corresponding para-substituted aromatic thiol to increase the rate of protein unfolding. The proportionality constant linking aromatic thiol pK_a to reactivity (log k_1) was considerably greater for 2-PDE ($\beta = 0.9$) and protein disulfides than that previously reported for Ellman's reagent ($\beta = 0.5$). Indicating, in contrast to the results with Ellman's reagent, that the most reactive aromatic thiol for protein unfolding will have a pK_a value greater than the pH of the solution.[14] The enhanced reactivity, at approximately neutral pH, of aromatic thiols with disulfides relative to aliphatic thiols was shown to occur not only for Ellman's reagent but also for nonbis-(aromatic) small molecule disulfides and, most importantly, protein disulfides, thus clearly demonstrating that the enhanced reactivity of aromatic thiols is not due to hydrophobic interactions between aryl groups on the attacking and central thiol. Furthermore, equivalent rates of protein unfolding were observed when PDI or 10 times the weight of aromatic thiol **6** as PDI was added. PDI, the in vivo protein folding catalyst, is rarely used for protein unfolding or folding, as it is expensive and can be difficult to separate from other proteins. Since the reduction of protein disulfides occurs during the folding of many proteins,[5] aromatic thiols with high pK_a values such as **6** are expected to increase the rate of protein folding as well.

Experimental Section

General Remarks. NMR spectra were recorded at 300 MHz (^{1}H) and at 75 MHz (^{13}C) on a Bruker spectrophotometer. Chemical shifts were indirectly referenced to TMS via solvent signals ($CDCl_3$, 7.26 ppm for ^{1}H and 77.00 ppm for ^{13}C; CD_3OD, 49.00 ppm for ^{13}C). Thin-layer chromatography (TLC) was conducted on Aldrich general-purpose silica gel on polyester plates with UV indicator. Silica gel chromatography was performed with E. M. Science silica gel (230−400 mesh). Dry THF was obtained by distillation from sodium metal in the presence of benzophenone. Routine drying of organic solutions was carried out with anhydrous magnesium sulfate. All reactions performed under inert atmospheric conditions were carried out under Ar. All reagents purchased, including compounds **7**−**9**, were used without purification unless otherwise noted. UV−vis spectra were recorded on a Cary 1 UV−vis spectrophotometer. All proteins were purchased from Sigma. E & R Microanalytical Laboratory Inc. performed elemental analysis.

4-[*N*,*N*-Bis(2-hydroxyethyl)amino]benzenethiocyanate.[18] A 250-mL flask equipped with a stirring bar was charged with *N*-phenyldiethanolamine (10.0 g, 0.055 mol), ammonium thiocyanate (8.4 g, 0.110 mol), and 35 mL of glacial acetic acid. After the mixture was cooled to 10 °C, a solution of bromine (8.8 g, 0.055 mol) in glacial acetic acid (20 mL) was added dropwise over 30 min while keeping the temperature below 20 °C. The mixture was then stirred for an additional 10 min at room temperature and poured onto 600 mL of H_2O. After filtration, the filtrate was extracted with ethyl acetate (3 ×

100 mL), washed with saturated $NaHCO_3$ (100 mL), dried over $MgSO_4$, and concentrated in vacuo to yield 11.20 g (86%) of 4-[*N*,*N*-bis(2-hydroxyethyl)amino]benzenethiocyanate: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.39 (d, J = 9.0 Hz, 2 H, Ar H), 6.66 (d, J = 9.0 Hz, 2 H, Ar H), 3.78 (t, J = 4.9 Hz, 4 H, NCH_2CH_2OH), 3.55 (t, J = 4.9 Hz, 4 H, NCH_2CH_2OH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 149.6, 134.6, 113.6, 112.6, 107.2, 60.1, 54.9.

2,2′-[(4-Mercaptophenyl)imino]bisethanol (6).[19] A solution of 4-[*N*,*N*-bis(2-hydroxyethyl)amino]benzenethiocyanate (5.0 g, 26.4 mmol) in 30 mL of THF was added dropwise over 1 h to a 250-mL flask containing lithium aluminum hydride (2.5 g, 65.9 mmol), 20 mL of THF, and a stirring bar. After 48 h, the reaction was quenched by the dropwise addition of 1 mL of H_2O. The mixture was then acidified to pH 1 with 1 N HCl and washed with diethyl ether (50 mL). The aqueous layer was adjusted to pH 6 and extracted with ethyl acetate (3 × 50 mL). The combined organic layers were dried over MgSO4 and concentrated in vacuo. Recrystallization from benzene/heptane (1:1) yielded 1.20 g (27%) of 4-[*N*,*N*-bis(2-hydroxyethyl)amino]benzenethiol as a light yellow powder: mp 134 °C (lit. 136 °C);[33] ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.21 (d, J = 8.8 Hz, 2 H, Ar H), 6.57 (d, J = 8.9 Hz, 2 H, Ar H), 3.78 (t, J = 4.9 Hz, 4 H, N−CH_2−CH_2−OH), 3.51 (t, J = 4.9 Hz, 4 H, N−CH_2−CH_2−OH), 3.30 (s, 1 H, SH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 146.9, 133.1, 114.7, 113.4, 60.6, 55.1. Anal. Calcd for $C_{10}H_{15}NO_2S$: C, 56.31; H, 7.09; N, 6.57; S, 15.03. Found: C, 56.59; H, 6.87; N, 6.51; S, 14.74.

Determination of pK_a Values (UV−Vis Method).[20,21] Initially, 24 buffers of varying pH were prepared (50 mM): glycine, pH 2.5, 3.0, 3.3; 2,2-dimethylsuccinate, pH 3.7, 4.0, 4.3, 4.7, 5.0, 5.3, 5.7, 6.0, 6.3, 6.7; Tris, pH 7.0, 7.3, 7.7, 8.0, 8.3, 8.7; glycine, pH 9.0, 9.5, 10.0, 10.5; H_3PO_4, pH 11.0. A 100 mM ethanolic solution of each aromatic thiol (100 μL) was diluted 90-fold with selected buffers. The buffers were chosen so that the pH of the buffer would be within 1.5 units of the pK_a of the thiol tested. The absorbances of these diluted solutions were measured at the λ_{max} of the corresponding thiolate. This absorbance data was then plotted against pH. The plot was compared with plots derived from theory. The pK_a value was determined from the best-fit curve. Additional titratable functional groups had no observable effects upon the results with the exception of the *p*-hydroxy group.

Titration Method.[22,23] Deoxygenated distilled water (10 mL) was allowed to equilibrate under Ar in a 50 mL three-neck round-bottom flask equipped with a stirring bar. A pH meter probe was inserted in the neck of the flask. The appropriate amount of the thiol was added to the flask to make a 0.010 M thiol solution. The thiol solution was titrated against 0.151 M carbonate-free KOH by adding 350 μL of KOH, waiting 1 min., recording the pH, then repeating the procedure. After 20 points (7 mL total KOH added) were measured, a titration curve was created. The plot was compared with plots derived from theory. The pK_a value was determined from the best-fit curve. All titrations were performed in duplicate.

Kinetics of the Reduction of 2-Pyridyldithioethanol by Thiols.[27] A thiol solution (approximately 18 μM) was prepared in deoxygenated buffer (pH 7.0, 0.100 M sodium phosphate buffer, 2.0 mM EDTA). The concentrations of the thiol solution was determined using Ellman's reagent (ϵ_{412} = 13 700 mol^{-1} L^{-1} cm^{-1}). A solution of 2-pyridyldithioethanol (2-PDE) (approximately 18 μM) was prepared in a fashion similar to the thiol solution. The concentration of the 2-PDE was determined by mixing 0.500 mL of 2-PDE solution with concentrated 4-mercaptobenzenemethanol (0.100 mL of a 1.25 mM solution) and 0.400 mL of pH 7.0 buffer and measuring the absorbance at 343 nm (ϵ_{343} = 7060 mol^{-1} L^{-1} cm^{-1} for the thiolate of 2-mercaptopyridine). This absorbance was compared to the absorbance of a standard containing concentrated 4-mercaptobenzenemethanol (0.100 mL of a 1.25 mM solution) and 0.900 mL of pH 7.0 buffer. In a 1 mL cuvette, appropriate amounts of the thiol and 2-PDE solutions were mixed together to form an equimolar solution and the rate of the 2-thiopyridine anion formation was measured over 20 min by observing the increase in absorbance at 343 nm. The initial concentration of PDE, $[A]_0$, will approximately equal the concentration of 2-mercaptopyridine at the end of the reaction, $[A]_0 = (Abs_{final} - Abs_{initial})/7060$, where Abs_{final} is the absorbance after the reaction has gone to completion and $Abs_{initial}$ is the absorbance prior to the beginning of the reaction. Using linear regression, the slope of a plot of $((Abs_{obsd} - Abs_{initial})/7060)/(((Abs_{final} - Abs_{initial})/7060)((Abs_{final} - Abs_{obsd})/7060))$ versus time was determined. The slope corresponds to k^{obsd}.

Kinetics of Reduction of Insulin by Aromatic Thiols.[31] All measurements were performed at room temperature and in duplicate. Insulin (1.0 mL of a 10 mg/mL stock solution)[31] was diluted with 8.0 mL of aqueous buffer (pH 6.5, 0.10 M in potassium phosphate, 2 mM EDTA). This cloudy solution was made clear by adjusting to pH 3.0 with the addition of 1.0 M HCl and rapidly titrating the solution back to pH 8.0 with 1.0 M NaOH. The final volume of the dilute insulin (1.0 mg/mL, 0.167 mM) solution was adjusted to 10.0 mL with water. A representative kinetic assay is described; others followed a similar procedure. Three cuvettes were prepared containing 1.00 mL of dilute insulin each. Dithiothreitol (40 μL of a 25.0 mM solution in buffer (pH 6.5, 0.10 M in potassium phosphate, 2 mM EDTA)) was then added to each cuvette just prior to the addition of the last component. At 1 min intervals **6** (160 μL of a 5.0 mM solution in buffer) was added to cuvette 1, a structurally similar nonthiol containing compound, *N*-phenyldiethanolamine (160 μL of a 5.0 mM solution in buffer) was added to cuvette 2, and 160 μL of buffer was added to cuvette 3. The absorbance of each cuvette at 650 nm was then measured every 3 min for 3 h. The slopes were determined from a plot of absorbance versus time. The ratio of the slopes of the data obtained from cuvettes 1 and 3 provided the relative rate of reduction of **7** compared to dithiothreitol (DTT).

Acknowledgment. Financial support from the National Science Foundation (CHE9975076), the Petroleum Research Fund (34902-G4), the Camille and Henry Dreyfus Foundation (New Faculty Award), and Syracuse University is gratefully acknowledged. We thank Dr. Rajeeva Singh of Immunogen and Dr. Roger Hahn of Syracuse University for helpful comments. We also thank Dr. Rajeeva Singh for providing 2-PDE.

Supporting Information Available: Experimental procedures and spectral data for the synthesis of compounds **1**−**5**. Experimental procedure and the results for the SDS−PAGE experiment. This material is available free of charge via the Internet at http://pubs.acs.org.

JO015600A

(33) Valu, K. K.; Gourdie, T. A.; Boritzki, T. J.; Gravatt, G. L.; Baguley, B. C.; Wilson, W. R.; Wakelin, L. P. G.; Woodgate, P. D.; Denny, W. A. *J. Med. Chem.* **1990**, *33*, 3014−19.