Ionization−Reactivity Relationships for Cysteine Thiols in Polypeptides†

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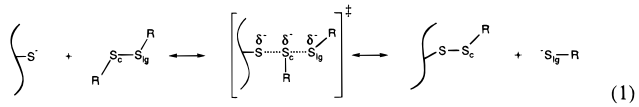
ABSTRACT: Thiol−disulfide exchange reactions are required for many aspects of cellular metabolism including the folding of disulfide-bonded proteins, electron transfer, and numerous regulatory mechanisms. To identify factors influencing the rates of these reactions in polypeptides, the reactivities of Cys thiols in 16 model peptides were measured. For each of the peptides, which contained single Cys residues with thiol pK_as ranging from 7.4 to 9.1, the rates of exchange with four disulfide-bonded compounds were measured. In reactions with two of the disulfide reagents, cystine and 2-hydroxyethyl disulfide, the peptide thiols displayed Brønsted correlations between reaction rate and pK_a similar to those observed previously with model compounds ($\beta_{nuc} = 0.5$ and 0.3, respectively). For two reagents with net charges, oxidized glutathione and cystamine, however, the apparent Brønsted coefficients were 0 and 0.8, respectively. These observations are in striking contrast with those obtained with model compounds, for which the Brønsted coefficients for the nucleophilic thiolates are largely independent of the disulfide-containing compound. The differences in the apparent Brønsted coefficients can be largely accounted for by electrostatic interactions between charged groups on the peptides and disulfide reagents and demonstrate that such interactions can play a dominant role in determining the rates of thiol−disulfide exchange in biological molecules. The results presented here provide an improved basis for predicting the rates of these reactions and suggest ways in which differences in the rates of competing reactions can be either minimized, to simplify the analysis of disulfide-coupled folding reactions, or enhanced, to favor formation of particular disulfides.

The thiol group of the amino acid cysteine is readily deprotonated under physiological conditions, and the resulting thiolate is one of the most reactive functional groups found in proteins (*1*). A particularly prevalent reaction of ionized Cys residues is thiol−disulfide exchange (*2*, *3*), in which the thiolate acts as a nucleophile, displacing one sulfur atom in an existing disulfide bond and forming a new bond with the other sulfur atom:



(1)

The preexisting disulfide can be part of a small molecule or another protein, or it may involve Cys residues in the same polypeptide chain as the reacting thiolate. This reaction plays a central role in the formation of disulfide bonds in newly synthesized proteins and is involved in many aspects of cellular metabolism, including electron-transfer reactions and the regulation of enzymatic activity (*2*). It has also recently been implicated in signal transduction (*4*) and the regulation of transcription factors (*5*). As indicated in eq 1, the reaction takes place via a concerted process in which the rate-determining step is the formation of a transition state with the negative charge of the thiolate partially transferred to the other two participating sulfur atoms, which are generally identified as the central atom (S_c) and the leaving group atom (S_{lg}) (*3*).

Because the reaction involves negatively charged species in both the ground and transition states, its rate is particularly sensitive to electrostatic factors, including the presence of nearby charges and through-bond inductive effects that alter the electron distribution around the sulfur atoms. For most Cys residues in proteins, the thiol pK_a lies in the range of 8 to 9 (*2*). Cysteine thiols are thus found predominantly in the un-ionized state under physiological conditions, and an increase in the pK_a will decrease the fraction of ionized molecules and, other factors being equal, decrease the exchange rate. Negative charges near the thiol, for instance, tend to destabilize the thiolate and decrease the observed reaction rate, while positive charges are expected to increase the rate.

Once formed, however, the reactivity of the thiolate is also influenced by electrostatic effects. Model compound studies have shown that the reactivity is correlated with the thiol pK_a, as expressed by a Brønsted relationship of the form:

$$\log(k_{s^-}) = C + \beta_{nuc} \times pK_a \qquad (2)$$

where k_{s^-} is the rate for the thiolate, C is a constant applicable to the reactions of a particular disulfide with a series of thiolates under specified conditions, and β_{nuc} is the Brønsted coefficient. For the families of small molecules examined

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so far, in which the thiol pK_as are influenced primarily by polar groups or charges only a few covalent bonds away, the value of β_{nuc} is typically in the range of 0.4−0.6 (*3*, *6*, *7*). This positive correlation between reactivity and basicity indicates that those factors that favor the transfer of electron density from the thiolate to a proton, to form the conjugate acid, also tend to favor the transfer of negative charge to other atoms in the transition state for exchange.

The reaction rate is also correlated with the pK_as of the thiols generated upon reducing the disulfide (S_c and S_{lg}). Higher pK_as for these thiols generally lead to *decreased* reaction rates, consistent with negative charge being transferred to the central and leaving group atoms in the transition state (*7*−*10*). These correlations can be incorporated into a more general relationship:

$$\log(k_{S^-}) = D + \beta_{nuc} \times pK_a + \beta_c \times pK_{a,c} + \beta_{lg} \times pK_{a,lg} \quad (3)$$

where β_c and β_{lg} are the Brønsted coefficients applicable to the central atom in the transition state and the leaving group sulfur atom, respectively, p$K_{a,c}$ and p$K_{a,lg}$ represent the pK_as of the corresponding thiols derived from the disulfide, and D is a constant (*3*). Implicit in eq 3 is the assumption that the groups attached to the three sulfur atoms influence the reaction rate independently. The limited data presently available indicate that β_c and β_{lg} both lie in the range of −0.5 to −0.3. The similarity in the absolute values of all three coefficients suggests that the charge is distributed nearly evenly among the three atoms. There is, however, considerable uncertainty in the exact values of the coefficients, and it has been noted that they can be used to draw only qualitative conclusions about the transition-state structure (*3*, *7*).

In addition to providing mechanistic insights, the Brønsted coefficients have been used to predict and interpret the reaction rates for peptide thiols with different pK_as (*11*−*13*). Relatively little is known, however, about the applicability of the Brønsted relationships determined with small molecules to Cys residues in folded or unfolded polypeptide chains. In the context of a protein, a thiol pK_a may be influenced by a variety of factors, including charges on spatially adjacent side chains, the orientation and strength of nearby dipoles, and solvent accessibility. It is unclear whether changes in pK_a caused by these different factors will all be correlated with similar changes in reactivity. In addition, the disulfide-containing molecules with which peptide thiols react are often themselves highly charged, and electrostatic interactions may alter the probability of an initial collision between the reacting molecules (*14*−*16*).

As a particular example, recent studies have shown that two of the six Cys residues of fully reduced and unfolded bovine pancreatic trypsin inhibitor (BPTI)[1] react with oxidized glutathione (GSSG) at unusually high rates, contributing to the rapid formation of one of the 15 possible one-disulfide intermediates in the refolding of this protein (*17*). The high reactivities of these thiols (those of Cys 14 and 38) have been attributed to the presence of nearby positive charges (*17*, *18*), but this hypothesis has not been tested directly, and it is not known whether the charges alter the ionization of the thiols, the nucleophilicity of the thiolates, or interactions with the disulfide reagent.

To characterize the factors influencing the reactivities of Cys thiols in proteins, we have measured the rates of reaction of four different disulfide reagents with the Cys thiols in each of 16 peptides with different electrostatic properties. These measurements reveal significant deviations from the relatively simple behavior found for model compounds. In particular, the relationship between nucleophile pK_a and reactivity was found to depend greatly on the net charge of the disulfide reagent used, indicating that electrostatic interactions between the reagent and peptide can play a major role in determining the rate of thiol−disulfide exchange.

EXPERIMENTAL PROCEDURES

Peptides and Reagents. Peptides were synthesized using solid-phase F-moc chemistry and were purified by reversed-phase HPLC. Concentrations of the BPTI-related peptides were determined from their absorbance at 274.5 nm, assuming an extinction coefficient of 1400 M^{-1} cm^{-1} for the single Tyr residue in each (*19*). Peptides DsbH, DsbU, TrxU, and GrxU contained no Tyr residues, and their concentrations were determined by titrating the cysteine thiols with Ellman's reagent (*20*).

Iodoacetamide, oxidized glutathione, L-cystine, 2-hydroxyethyl disulfide (HED), and cystamine were obtained from commercial sources and used without further purification.

Determination of Peptide Thiol pK_as. To determine the pK_as of the thiols in the BPTI-related peptides, the rates of alkylation with iodoacetamide were measured as a function of pH. Reaction mixtures contained 0.1 M buffer, 0.2 M KCl, 1 mM EDTA, 30 μM peptide, and 0.5 to 1.5 mM iodoacetamide. The following buffers were used to establish the solution pH over the indicated ranges: 3-(*N*-morpholino)-propanesulfonic acid (MOPS)-NaOH (pH 6.0−7.6), Tris-HCl (pH 7.9−9.4), and glycine-NaOH (pH 9.9−10.5).

The reactions were carried out at 25 °C in septum vials flushed with N_2. After appropriate reaction times, 150 μL aliquots were withdrawn and mixed with 30 μL of 95% formic acid. The mixtures were fractionated by reversed-phase HPLC using a Vydac C_{18} column (4.6 mm × 25 cm) eluted with a linear gradient of acetonitrile in 0.1% TFA. The gradient conditions were adjusted for each peptide to optimize the separation of the alkylated and unmodified forms. The initial concentrations of acetonitrile ranged from 4.5% to 22.5% and increased at a rate of 1−2% per minute over a 10 min period. The flow rate was 1 mL per minute. The elution of the peptides was monitored by measuring UV absorbance at 220 nm.

Pseudo first-order rate constants were determined by fitting the data to a single exponential and were then divided by the iodoacetamide concentration to yield second-order rate constants. The thiol pK_as were estimated by fitting plots of the second-order rate constants versus pH to the Henderson−Hasselbalch equation. Data were fit by the method of least-squares, using the KaleidaGraph program from Synergy Software.

Kinetics of Thiol−Disulfide Exchange. Rate constants for the reactions between peptide thiols and disulfide reagents

[1] Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; GSSG, oxidized glutathione; HED, 2-hydroxyethyl disulfide; HPLC, high-performance liquid chromatography; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; TFA, trifluoroacetic acid.

Table 1: Peptide Sequences, Thiol pK_as, and Rates of Thiol−Disulfide Exchange at pH 7.3

peptide	sequence	pK_a[a]	observed rate of reaction (s^{-1} M^{-1}) with: cystine	HED	GSSG	cystamine
Pti5	Ac-RPDF*C*LEPPY-NH_2	9.08	2.4	0.30	0.17	11
Pti5a	Ac-RPNF*C*LQPPY-NH_2	8.84	3.5	0.57	0.57	7.0
Pti5b	Ac-RPNL*C*LQPPY-NH_2	8.79	3.7	0.63	0.51	7.4
Pti14	Ac-PYTGP*C*KAR-NH_2	8.34	7.1	1.0	1.6	8.9
Pti14a	Ac-PYTGP*C*MAR-NH_2	8.40	6.1	1.0	1.1	10
Pti30	Ac-YNAKAGL*C*QTF-NH_2	8.50	4.4	0.64	0.61	8.0
Pti38	Ac-YGG*C*RAKRNN-NH_2	8.26 (8.00)	9.0	1.1	2.6	7.8
Pti38a	Ac-YGG*C*AAKRNN-NH_2	8.31	7.4	1.1	1.6	8.4
Pti38b	Ac-YGG*C*AASQNN-NH_2	8.43 (8.45)	5.3	0.65	0.74	9.1
Pti51	Ac-YKSAED*C*MRT-NH_2	8.69 (8.77)	3.5	0.39	0.37	10
Pti51a	Ac-YKSAQN*C*MRT-NH_2	8.38 (8.23)	5.4	0.76	1.0	7.2
Pti55	Ac-YKSAEDAMRT*C*GGA-NH_2	8.46	5.8	0.62	0.85	11
DsbH	Ac-GG*C*PHAAAA(RAAAA)$_2$RAA-NH_2	7.35	7.9	1.7	4.5	12
DsbU	Ac-GG*C*PHAAPA(RAAPA)$_2$RAA-NH_2	8.16	6.2	1.7	2.2	12
TrxU	Ac-GG*C*GPAAPA(RAAPA)$_2$RAA-NH_2	8.77	4.0	0.38	0.49	6.6
GrxU	Ac-GG*C*PFAAPA(RAAPA)$_2$RAA-NH_2	8.11	7.6	2.2	2.7	16

[a] Thiol pK_as of the BPTI-derived peptides were determined at 25 °C in 0.1 M buffer solutions containing 0.2 or 0 M KCl (shown in parentheses). The pK_as of peptides DsbH, DsbU, Trx U, and GrxU were determined at 25 °C in the presence 0.3 M KCl and 1 mM each of citrate, phosphate, and borate. The estimated uncertainties in the pK_as are ±0.04. Each of the peptides was acetylated (AC) at the amino terminus and amidated (NH_2) at the carboxyl terminus.

were measured using essentially the same procedure as described above for the alkylation reactions. These reactions were carried out at pH 7.3 and 25 °C in solutions containing 15−30 μM peptide, 0.1 M MOPS-NaOH, 0.2 M KCl, 1 mM EDTA, and the appropriate disulfide reagent. The reagent concentrations used were 2−5 mM GSSG, 1−2 mM cystamine, 0.24 mM cystine, or 1−4 mM HED.

The reactions involving GSSG, cystamine, or cystine were quenched by mixing 150 μL aliquots of the reaction solutions with 30 μL of 95% formic acid. The mixed disulfides formed with HED, however, were not easily separated from the unmodified peptides. These reactions were quenched by adding cystamine to a final concentration of 0.1 M, thereby converting the unreacted peptide to the cystamine mixed-disulfide, which was readily separated from the HED mixed-disulfide. The reaction mixtures were fractionated by reversed-phase HPLC, and the areas of the peaks were used to determine the progress of the reactions.

The reactions with cystamine, GSSG, and HED were carried out under pseudo first-order conditions with the disulfide reagent present in an excess of at least 30-fold, and rate constants were determined by fitting the resulting data to a single exponential. Because of the limited solubility of cystine, however, the reactions with this reagent were carried out at lower reagent excess (11-fold), and the resulting data were fit to the integrated form of the second-order rate expression. All of the reported rates represent means of two or more independent measurements. For most of the peptide−reagent combinations, the rates were measured at two different reagent concentrations, and the rates were found to be proportional to the disulfide concentration, as expected for the bi-molecular reactions. Based on possible errors in solution concentrations, errors in the integration of HPLC peaks and other experimental variations, the uncertainties in the rate constants are estimated to be about 20%. The reported uncertainties in the Brønsted coefficients are the standard errors from the least-squares fits.

RESULTS AND DISCUSSION

A total of sixteen chemically synthesized peptides, each containing a single Cys residue, were used in this study. The sequences of these peptides, listed in Table 1, were designed to represent segments of four well-characterized disulfide-containing proteins. Six peptides (Pti5, Pti14, Pti30, Pti38, Pti50, and Pti55) correspond to the sequences surrounding the Cys residues of wild-type BPTI and are similar to those described previously by Dadlez and Kim (*17*). Six additional BPTI-related peptides, with amino acid replacements in the vicinity of the Cys residues, were also characterized. The other four peptides studied here were designed as models of the active-site regions of proteins in the thioredoxin family. One of these peptides, DsbH, was designed both to mimic the local sequence surrounding Cys 30 of the *E. coli* protein DsbA and to place this residue near the N-terminus of an α-helix, as in the native protein. The three other peptides, DsbU, TrxU, and GrxU, were designed to represent the active site sequences of DsbA, thioredoxin, and glutaredoxin, respectively, in unfolded conformations. Far-UV circular dichroism spectra confirmed that DsbH is predominantly helical and that DsbU and TrxU have little or no regular secondary structure.

Ionization Equilibria of Cysteine Thiols. For the BPTI-derived peptides, the thiol pK_as were determined by measuring the rates of reaction with iodoacetamide as a function of pH (Figure 1). For each peptide, the observed rate constants (k_{obs}) were fit to the following form of the Henderson−Hasselbalch equation:

$$k_{obs} = k_s/(1 + 10^{(pK_a - pH)}) \quad (4)$$

where k_{s^-} is the pH-independent rate constant for the reaction of the thiolate form (Figure 1b). The pK_as of the thiols in the peptides DsbH, DsbU, TrxU, and GrxU were determined using the absorbance of the thiolate ion at 240 nm to monitor ionization as a function of pH, as described previously (*21*).

The measured pK_a values for the 16 peptide thiols range from 7.35 (for DsbH) to 9.08 (for Pti5) (Table 1). Previous studies of peptides similar to DsbH indicate that the unusually low thiol pK_a for this peptide is due to conformational factors, especially interactions of the thiolate with the peptide dipoles at the N-terminus of the α-helix (*21*). At the other extreme, the high thiol pK_a of Pti5 appears to be due in part to the

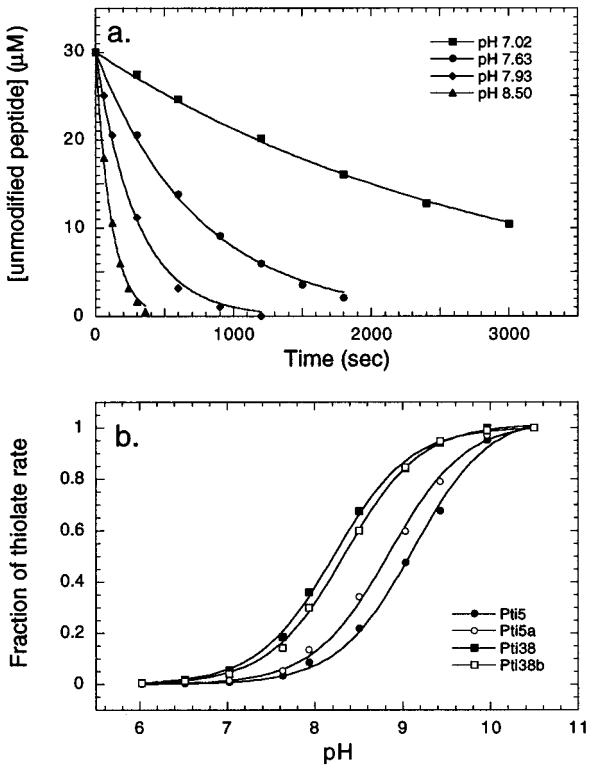

FIGURE 1: Determination of thiol pK_as. (*a*) Kinetics of reaction between iodoacetamide and peptide Pti5 at four different pH values. At the indicated times, the reactions were quenched by acidification, and the concentration of unreacted peptide determined by reversed-phase HPLC. (*b*) pH-dependence of the alkylation rates for four peptides. The observed rate constants are expressed as a fraction of the pH-independent rate for the thiolate, and the curves represent least-squares fits to the Henderson−Hasselbalch equation.

presence of two nearby negatively charged residues. Even with these residues replaced with neutral ones, however, (in peptide Pti5a) the sequence surrounding Cys 5 of BPTI results in a pK_a greater than that seen for any of the other peptides examined here. Further replacement of Phe 4 (Pti5b) caused only a very small decrease in the thiol pK_a (0.05 units), indicating that the unusually high pK_a of Cys 5 is not due to interactions with the adjacent aromatic ring. While NMR studies of a peptide derived from the first 15 residues of the BPTI sequence indicate the presence of transient nonrandom conformations in the vicinity of Cys 5 (*22*), the structural basis of the high pK_a of this thiol is presently unclear.

For the 12 peptides other than DsbH and those related to Pti5, the thiol pK_as ranged from 8.1 to 8.7, comparable to the values observed for other peptides and proteins (*2*). For the BPTI-related peptides, the lower pK_as were generally associated with the presence of positively charged residues within 1 to 4 residues of the cysteine (in peptides Pti14, Pti38, Pti38a, and Pti51a), while higher pK_as were associated with the presence of negative charges (in Pti51). Replacing two negatively charged residues, in either peptide Pti5 or Pti51, was found to decrease the pK_a by 0.24 or 0.33 pH units, while replacing positive residues, in peptide Pti14 or Pti38, increased the thiol pK_a, by as much as 0.17 units.

For four of the peptides, Pti38, Pti38b, Pti51, and Pti51a, the thiol pK_as were also determined at a reduced ionic strength, where Coulombic interactions are expected to be enhanced. In the absence of the 0.2 M KCl used in the other

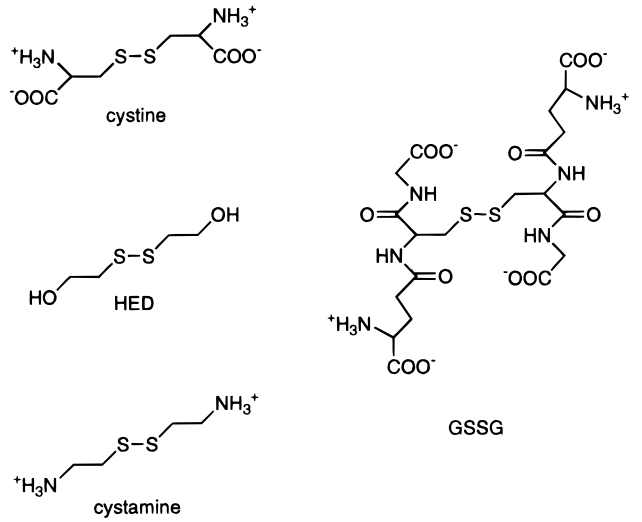

FIGURE 2: Structures of the disulfide reagents used in this study: cystine, 2-hydroxyethyl disulfide (HED), cystamine, and oxidized glutathione (GSSG).

experiments, the thiol pK_as of Pti38 and Pti51a were decreased by 0.26 and 0.15 pH units, respectively, consistent with electrostatic stabilization of the thiolates by the nearby positive charges. Pti51 contains two positively and two negatively charged side chains, and decreasing the ionic strength caused only a very small increase in the thiol pK_a (0.08 units). For peptide Pti38b, which contains neither acidic nor basic residues, the thiol pK_a also did not change measurably upon decreasing the ionic strength.

For the peptides derived from thioredoxin-related proteins, the variations in pK_a are not correlated with the presence of charged side chains. Studies of a closely related set of peptides indicate instead that these pK_as are influenced primarily by conformational factors (*21*). It is particularly striking that the variations in pK_a seen in these peptides are considerably larger than those attributed to nearby side chain charges in the BPTI-related peptides. While the helical conformation of DsbH appears to lower the pK_a by 0.8 pH units, relative to that of DsbU, altering as many as three nearby charged side chains changes the pK_a by 0.54 units or less, even at the lower ionic strength.

Kinetics of Thiol−Disulfide Exchange. As summarized in Table 1, rates were measured for reactions between each of the 16 peptides and each of four different symmetrical disulfide reagents. The reagents used were oxidized glutathione (GSSG), cystine, cystamine, and 2-hydroxyethyl disulfide (HED, the disulfide form of 2-mercaptoethanol). As shown in Figure 2, the structures of these reagents vary substantially, particularly with respect to the distribution of charged functional groups.

In Figure 3a, the rates observed at pH 7.3 for the exchange reactions with the two neutral reagents, cystine and HED, are plotted as a function of the peptide thiol pK_as, and the rates of the reactions with the positively charged cystamine and negatively charged GSSG are plotted in Figure 3b. For the two neutral reagents and GSSG, the observed rates were found to decrease as a function of increasing nucleophile pK_a, as expected if the thiolate is the reactive species. For the positively charged cystamine, however, the rates were nearly independent of the pK_a. Furthermore, the dependence on pK_a was significantly greater for GSSG than for the neutral reagents.

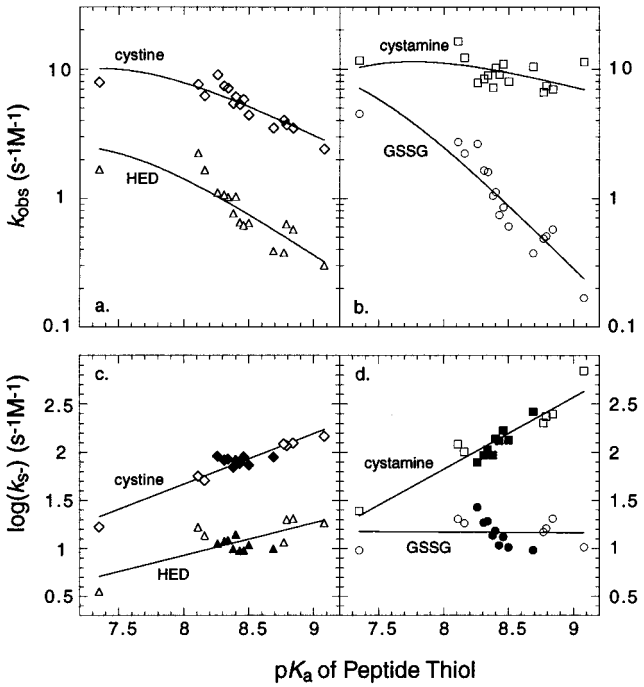

FIGURE 3: Rate constants for thiol−disulfide exchange reactions as a function of the peptide thiol pK_a. (*a*,*b*) Observed second-order rate constants for the reactions with the neutral reagents, cystine and HED (*a*), or the charged reagents, cystamine and GSSG (*b*) determined at pH 7.3, 25 °C. The curves shown in *a* and *b* were calculated from the Henderson−Hasselbalch equation and the Brønsted relationships determined in *c* and *d*. (*c*, *d*) Brønsted-type plots of the logarithms of the thiolate reaction rates versus pK_a for the reactions with the neutral (*c*) and charged disulfide reagents (*d*). The rate constants for the thiolates were calculated from the observed rate constants and the pK_as listed in Table 1. The lines represent least-squares fits to the data. Filled symbols are used to emphasize the behavior of the peptides with thiol pK_as in the range of 8.2 to 8.7, as discussed in the text.

In addition to the dependence on the pK_a of the nucleophile, the observed rates were also correlated with the pK_a of the thiol generated by reduction of the disulfide reagent. For each peptide, the rate with cystamine [$pK_a = 8.2$ (*14*)] was greater than that with GSSG [$pK_a = 8.9$ (*23*)], and the rates with cystine [$pK_a = 8.3$ (*24*)] were all greater than those with HED [$pK_a = 9.6$ (*16*)]. These pairwise comparisons are qualitatively consistent with the results of other studies (*7*−*10*) and suggest that the mechanism of the reaction is not fundamentally different from that characterized with smaller model compounds. As in the previously characterized reactions, there appears to be a significant transfer of negative charge from the nucleophile to the other sulfur atoms in the transition state. However, the magnitudes of the rate differences seen with the various disulfide reagents depend greatly on the pK_a of the nucleophile thiol, particularly when cystamine and GSSG are compared.

These observations indicate that there is a complex relationship between the rates of thiol−disulfide exchange and the properties of the reacting molecules. In contrast to the relatively simple Brønsted relationships observed with small molecules, expressed in eq 3, the influence of the various molecules participating in the reactions are not independent, as discussed further below.

Apparent Brønsted Relationships for Peptide Thiol−*Disulfide Exchange.* To visualize more clearly the differences in the reactivities of the thiolates, the observed rates were replotted (in Figure 3c,d) as the logarithms of the rates for the thiolate form, calculated using the pK_a values listed in Table 1. When fit to a linear relationship, the slopes of these plots yield apparent Brønsted coefficients for the nucleophile, β_{nuc}.

For the reactions with the two neutral reagents, cystine and HED, the apparent Brønsted coefficients were 0.52 (±0.05) and 0.34 (±0.08) (Figure 3c), respectively, similar to those observed with model compounds (*3*, *6*, *7*). For the positively charged reagent, cystamine, the apparent Brønsted coefficient, 0.75 (±0.07), was substantially larger than that observed with the neutral reagents, while for the negatively charged GSSG, there was little or no overall correlation between thiol pK_a and the thiolate rate. Because one of the thiol pK_as, that for DsbH, was significantly lower than all of the others, the behavior of this peptide might unduly influence the estimated Brønsted coefficients. When the data for this peptide were omitted, the apparent Brønsted coefficients for cystine, HED, cystamine, and GSSG were 0.41, 0.17, 0.8, and −0.22, respectively. Thus, the data for DsbH appear to be consistent with the general trends seen with the other peptides, though the considerable scatter in the data does lead to significant uncertainties in the Brønsted coefficients.

In studies of reaction mechanisms, Brønsted correlations between nucleophile pK_a and reactivity have traditionally been used to estimate the transfer of electrical charge in the transition state (*25*). The very different slopes observed for the plots in Figure 3c,d might, then, be taken to indicate differences in the transition states for reactions with the different disulfide reagents. Several authors have warned, however, that apparent Brønsted correlations may reflect factors other than the electronic structure of the transition state, particularly when the reaction rates are influenced by nearby charges (*14*−*16*).

To a first approximation, the differences in the apparent Brønsted coefficients seen for the charged reagents can be accounted for by electrostatic interactions between the peptides and disulfide reagents, without invoking a change in the transition state structure. For the most part, the peptides with low thiol pK_as are those with nearby positive charges (or the peptide dipoles at the N-terminus of an α-helix, in the case of peptide DsbH), while the thiols with higher pK_as are generally surrounded by neutral or negatively charged side chains. The positively charged reagent, cystamine, is more likely to interact electrostatically with the peptides with net negative charges (and high thiol pK_as) than with the positively charged peptides (i.e., those with low pK_as). As a consequence, the correlation between reaction rate and nucleophile pK_a is likely to be more positive than expected for reactions with neutral reagents. The reactions with negatively charged GSSG are expected to display the opposite tendencies, thus leading to a less positive apparent Brønsted coefficient. These interactions may change the probability of forming an initial encounter complex between the reacting molecules, without necessarily affecting the subsequent transfer of charge among the sulfur atoms as the transition state is formed. Similar, though less dramatic, effects of nearby charges have been observed previously in studies of thiol−disulfide exchange in small molecules (*14*).

Although these arguments can account for the general trends illustrated in Figure 3c,d, there are substantial devia-

tions from the linear relationships implied by the Brønsted coefficients. In particular, the electrostatic effects discussed above appear to be more pronounced for the peptides with thiol pK_as in the range of 8.2 to 8.7 (highlighted with filled symbols in Figure 3c,d). When only these peptides are considered, the apparent Brønsted coefficient for the reaction with GSSG is actually negative (−1), while that for the reaction with cystamine is more positive (1.2) than seen with the full set of peptides. As discussed earlier, the thiol pK_as for this subset of peptides appear to be influenced primarily by nearby charged side chains, while some of the more extreme variations of pK_a seem to be associated with conformational effects. It thus appears that the charged side chains are particularly effective in modulating the interactions with charged reagents, even though they have only modest effects on the thiol pK_as.

The subset of peptides with thiol pK_as in the range of 8.2 to 8.7 also displayed distinctive behavior in their reactions with the neutral reagents, cystine and HED. For these reactions, the Brønsted coefficients are close to 0. Snyder and co-workers (*15*) have noted previously that charged side chains are expected to influence Cys thiol pK_as primarily via solvent-mediated Coulombic interactions, rather than inductive effects mediated by covalent bonds. It might be expected that a charged group on a side chain would interact equally with the thiolate and the transition state for exchange, since the sulfur atoms in both species have a total charge of −1, and the effective distance between this charge and other charged side chains may be similar in the ground and transition states. Thus, the rate of reaction of the thiolate with a neutral disulfide might show little or no correlation with changes in pK_a caused by through-space electrostatic interactions. This appears to be the case for the subset of peptides with thiol pK_as in the range of 8.2 to 8.7, though this limited data set is probably too small for definitive conclusions to be drawn.

Effects of Ionic Strength on Reaction Rates. To explore further the role of electrostatic interactions in determining the rates of thiol−disulfide exchange, the rates of reaction for selected peptides with GSSG or cystamine were measured at different salt concentrations: 0.1 M buffer and KCl concentrations ranging from 0 to 0.5 M (Figure 4). The thiol pK_as for the peptides used for these experiments (Pti38, Pti38b, Pti51, and Pti51a) were also measured at 0 M KCl (above).

For each of the two peptides with net positive charges, Pti38 and Pti51a, the observed rate of reaction with cystamine was nearly independent of ionic strength (filled symbols in Figure 4b). Since the thiol *p*K_as of these peptides increase by about 0.2 pH units when the KCl concentration is increased from 0 to 0.2 M, leading to a smaller fraction of ionized molecules, higher ionic strength must also lead to an increase in the thiolate reaction rate, by about 35%. This pattern is consistent with a repulsive interaction between the positive charges of the side chains and cystamine. In contrast to the reactions with cystamine, the reactions of the positively charged peptides with negatively charged GSSG decreased in rate as the KCl concentration was increased (filled symbols in Figure 4a). This change reflects both the increased pK_a at the higher ionic strengths and a small decrease in the thiolate rate. These effects, though small, are consistent with the suggestion made above that the reaction rates are influenced by electrostatic interactions between the charged peptides and reagents.

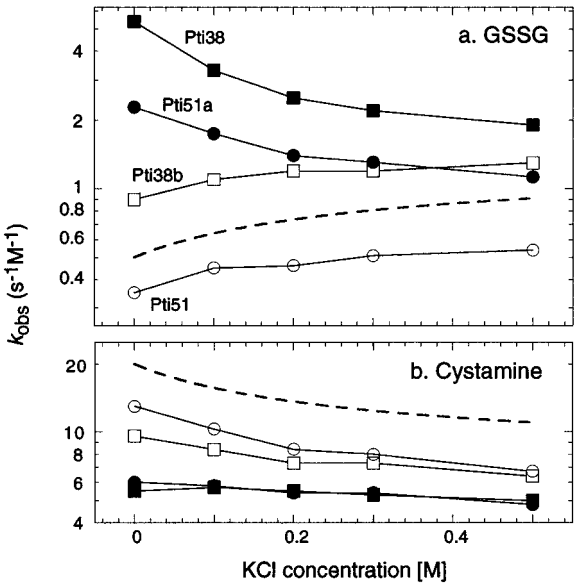

FIGURE 4: Effect of ionic strength on the reactions between peptide thiols and the charged disulfide reagents: GSSG (*a*) and cystamine (*b*). Observed rate constants were determined at pH 7.3 and 25 °C in the presence of 0.1 M MOPS-NaOH, 1 mM EDTA and the indicated KCl concentrations. The dashed lines represent calculated ionic-strength effects on the equilibrium constant for the association between molecules with net charges of −1 and −2 (*a*) or −1 and +2 (*b*). The curves were calculated assuming that the free energy changes for the reactions include a contribution for bringing the charges to a distance of 7.5 Å from one another. The positions of the curves along the vertical axis were set arbitrarily to allow comparison with the observed rates with peptides Pti38b and Pti51 (open symbols), each of which has a net charge of −1. The ionic strength dependence was calculated assuming an effective dielectric constant calculated from the Debye−Hückel theory, according to eqs 5−7 in the text.

The other two peptides examined, Pti38b and Pti51, are electrically neutral except for the charge of the thiolate. In reactions of these peptides with GSSG, the rates increased by about 30% as the KCl concentration was increased to 0.5 M (open symbols in Figure 4a). Conversely, in reactions with cystamine, both rates decreased at higher salt concentrations (open symbols in Figure 4b). Since the thiol pK_as for these peptides are only slightly affected by ionic strength, the changes in observed reaction rates probably arise from interactions between the thiolate on the otherwise neutral peptide and the charges present on the reagents.

The ionic conditions used for the other measurements presented here, 0.1 M buffer and 0.2 M KCl, were originally adopted for studies of disulfide-coupled protein folding in order to minimize electrostatic effects on the rate of thiol−disulfide exchange (*26*, *27*). As shown in Figure 4, further increases in ionic strength caused only small changes in the reaction rates, consistent with previous observations (*15*, *27*). Nonetheless, the results presented here indicate that nearby charged side chains can cause quite substantial differences in the reactivities of Cys thiols, particularly toward charged disulfide reagents, and these effects are not easily masked by salts at the concentrations typically used for biochemical experiments.

Comparison with Electrostatic Theory. It is difficult to predict accurately from first principles the effects of elec-

trostatic interactions on the reaction rates, since the total electrostatic energy will depend on the positions of the various charges and their interactions with other formal charges and dipoles in the reacting molecules and solvent. The flexibility of the peptides and disulfide reagents introduces additional uncertainties in any calculation of the electrostatic energies. Simple calculations suggest, however, that the observed effects are at least qualitatively consistent with electrostatic interactions between the charged reagents.

In the absence of other ionic species, the free energy change (ΔG_{elect}) for bringing two charged species to a distance r from one another is given by

$$\Delta G_{elect} = \frac{Z_A Z_B \epsilon^2}{Dr} \quad (5)$$

where Z_A and Z_B are the net charges on the two species, D is the dielectric constant (78 for water), and ϵ^2 is the square of the unit of electrostatic charge of an electron, and is equivalent to 5.514×10^{-19} Å $\times$ cal or, on a molar basis, 3.32×10^5 Å cal mol^{-1}. In the presence of significant salt concentrations, however, the free energies of the two reacting species and the complex they form will be modulated by interactions with the other solution ions. A simple approach to treating the effects of added salt is to replace the dielectric constant, D, in eq 5 with an "effective dielectric constant", D_{eff}, which can be estimated from the Debye−Hückel theory:

$$D_{eff} = D \exp(r\kappa) \quad (6)$$

where κ is the reciprocal of the Debye length (*28*). The value of κ, expressed in Å^{-1}, is related to the ionic strength, μ, according to:

$$\kappa = \sqrt{\left(\frac{4\pi\epsilon^2}{DkT}\right) N_a \mu 2 \times 10^{-27}} \quad (7)$$

where k is the Boltzmann constant and N_a is Avogadro's number (*29*).

Using this approach, the electrostatic free energy for bringing together charges of +2 and −2 in the presence of 0.3 M monovalent salt is predicted to lie between 0.3 and 1.4 kcal/mol for interaction distances in the range of 5 to 10 Å. These free energy differences are at least qualitatively consistent with the differences in rate constants observed for the reactions of the peptides and disulfide reagents with different net charges.

Of perhaps greater significance, the Debye−Hückel treatment predicts quite well the observed effects of ionic strength on the reaction rates, as illustrated by the dashed lines in Figure 4. The line in Figure 4a represents the relative equilibrium constant calculated for bringing charges of −1 and −2 to a distance of 7.5 Å, as a function of salt concentration. The position of the curve along the vertical axis was set arbitrarily so that the value calculated for 0.1 M monovalent salt (e.g., 0.1 M buffer and no added KCl) corresponds to a second-order rate constant of 0.5 s^{-1} M^{-1}. The shape of this curve matches closely those for the observed reaction rates of GSSG (with a net charge of −2) with peptides Pti38b and Pti51, each of which has a single negative charge. Similarly, the dashed curve in Figure 4b represents the calculated relative equilibrium constant for bringing together charges of −1 and +2, and this curve has a shape very similar to that observed for the reactions of Pti38b and Pti51 with cystamine.

This simplified treatment clearly represents only a very approximate description of the multiple electrostatic interactions formed as the peptides and disulfide reagents react with one another. However, the qualitative consistency with the experimental data suggests that such interactions can account for much of the observed variation in the Brønsted relationships for the reagents with different net charges.

Possible Applications. The results presented here suggest ways in which thiol−disulfide exchange rates might be manipulated advantageously for different purposes. In studies of disulfide-coupled refolding of reduced proteins, the conformational properties of the polypeptide chain at different stages of folding are generally of primary interest, and it is desirable to minimize differences in reaction rate arising from local sequence effects. As shown in Figure 3b, the observed reaction rates with cystamine at pH 7.3 are nearly independent of thiol pK_a. At this relatively low pH, the higher reactivity of the thiolates with higher pK_as is almost exactly balanced by the smaller fraction of ionized molecules. With GSSG used as the disulfide reagent, on the other hand, differences in pK_a lead to large differences in observed rates at pH 7.3, but the differences are expected to be minimized at high pH values where most of the thiols are ionized. For thiol−disulfide pairs where $\beta_{nuc} \approx 0.5$, the observed reaction rate at a given pH is expected to be maximum for thiols with pK_as equal to the pH (*3*). Thus, variations in the observed rates for different thiols can be minimized by choosing a solution pH near the middle of the pK_a range.

It may also be possible to choose reaction conditions or design amino acid sequences to enhance differences in the rates of forming particular disulfides in polypeptides with more than two Cys residues. This might, for instance, provide a means of enhancing the yields of the folding reactions required to produce disulfide-bonded proteins prepared by chemical synthesis or in recombinant microorganisms. As shown in Figure 3b, particularly large differentials in the rates of forming mixed disulfides at pH 7.3 are observed when GSSG is used as the disulfide reagent, with thiols with low pK_as reacting most rapidly. At higher pH, however, it is the reactions with cystamine that are expected to display the widest range of reaction rates. These effects can be potentiated at lower ionic strengths (Figure 4), and they are expected to be even more pronounced in the presence of nonpolar solvent additives (*16*).

All of these suggestions, however, are based on the assumption of simple pK_a-reactivity correlations. As illustrated in Figure 3c,d, deviations from these correlations can be significant, and the variations in the observed rates for different thiol−disulfide pairs may be larger or smaller than those predicted. Also, the relative rates at which different protein disulfides are formed can depend on the rate of a second, intramolecular, thiol−disulfide exchange reaction following formation of the mixed disulfide. The rate of this reaction will also be influenced by the electrical environment of the various sulfur atoms, as well as by the tendency of the polypeptide chain to bring the reacting atoms together in the appropriate orientation. Nonetheless, the large

differences and clear trends in the observed rates of mixed-disulfide formation indicate that there is considerable potential to manipulate the rates of competing reactions.

Summary and Conclusions. Collectively, the measurements with the different peptides and disulfide reagents indicate that the rates of thiol−disulfide exchange are influenced by multiple factors. While the Brønsted relationship between pK_a and reactivity determined previously with small molecules is observed with peptides under some conditions, especially with neutral reagents, we have observed substantial deviations from the behavior predicted by these relationships alone. The presence of net charges on both the peptide and disulfide reagent appears to cause particularly large effects; in one case involving a limited set of peptides, even the sign of the correlation is changed.

These studies also extend previous work on the folding mechanism of BPTI by demonstrating that the enhanced reactivities of Cys 14 and 38 toward GSSG are due to the nearby positive charges, which both promote ionization and favor interaction with the negatively charged reagent. In reactions with cystamine, however, the effects of the nearby charges on ionization and reactivity cancel almost exactly, and there is very little net effect on the observed reaction rates (Table 1). These patterns have also been observed in the reactivities of the thiols in intact reduced BPTI (G. Bulaj and D. P. Goldenberg, unpublished results). However, the choice of reagent has very little effect on the steady-state distributions of the intermediates, since these distributions are influenced primarily by the relative thermodynamic stabilities of the various species.

Electrostatic effects are almost certain to modulate the rates of individual steps in other disulfide-coupled protein folding pathways, as well as the rates of the many other processes involving thiol−disulfide exchange. The data presented here should aid in predicting and interpreting the rates of these important reactions.

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