analysis (3% OV-225, 190 °C) showed the presence of two peaks in a ratio of 15:85. VPC–mass spectrometric analysis showed the minor peak to have a molecular ion of 290 mass units. This is consistent with the fully saturated tricyclic structure x. The Raman spectrum of the mixture showed no acetylenic absorption, and the intensity of the olefinic absorption was approximately one-half of that found in "Hydrocarbon A". Vinyl proton absorption was absent in the ^{1}H NMR spectrum; however, angular and vinyl methyl absorption was still present. Further hydrogenation of the above mixture with platinum oxide in the presence of a trace amount of concentrated hydrochloric acid gave an oil which upon VPC analysis (3% OV-225, 180 °C) showed two peaks in a ratio of 31:69. Coinjection with the 15:85 mixture above showed only two peaks. However, VPC analysis under slightly different conditions (3% OV-17, 155 °C) showed four components as two partially resolved doublets, which upon VPC–mass spectrometric analysis showed the first three peaks to have nearly identical mass spectra with molecular ions of 290 mass units. The fourth peak showed a molecular ion of 288 mass units. The Raman spectrum showed negligible olefinic absorption and the ^{1}H NMR spectrum showed negligible olefinic absorption and the ^{1}H NMR spectrum showed negligible vinyl proton absorption. Presumably, the first three components are fully saturated isomeric tricyclic hydrocarbons x. Hydrogenation of "Hydrocarbon A" over tris(triphenylphosphine)rhodium(I) iodide afforded, after short-path distillation, a colorless oil showing mainly one peak on VPC which represented ca. 90% of the total peak area. Coinjection with the mixture obtained from the palladium-on-carbon hydrogenation showed this product to correspond to the major peak in the 15:85 mixture above. The first peak in the mixture was present in <3%. Hydrogenation of "Hydrocarbon A" seemed to afford predominantly a mixture of ix and x, the ratio of the products varying with the hydrogenation conditions. This assumption was supported by the ozonolysis studies described below which indicated the presence of tri- and tetrasubstituted olefinic bonds in at least two of the hydrogenation mixtures. Ozonolysis of the hydrogenated (palladium-on-carbon) "Hydrocarbon A" mixture followed by a reductive workup gave, after chromatography, ca. 33% of hydrocarbon material (eluted with hexane) corresponding (by VPC coinjection) to the hydrogenated "Hydrocarbon A" mixture from the platinum oxide reduction. Elution with hexane–ether afforded ca. 67% of an oil which showed carbonyl absorption at 5.87 μ in the IR spectrum. The ^{1}H NMR spectrum showed absorption at δ 2.05 ppm as a singlet which could be attributed to the presence of a methyl group adjacent to a ketone function. Ozonolysis of the hydrogenated [tris(triphenylphosphine)rhodium(I) iodide] "Hydrocarbon A" mixture followed by an oxidative workup gave acidic material which exhibited typical carboxylic acid absorption in the IR spectrum. The neutral material, after chromatography, afforded a "hydrocarbon fraction" and a "ketonic fraction". The IR spectra of these fractions were identical with the corresponding products obtained from the ozonolyses of hydrogenated (palladium-on-carbon) "Hydrocarbon A" described above.

(19) Recent work by Robert G. Finn of these laboratories has shown that the lower retention time peak, regarded as the 17α epimer, contains in addition another isomer as the principal component (ca. 90%). Higher resolution VPC (capillary column) indicated that the 17α isomer was present in minor amounts only and that the lower retention time peak corresponded predominantly to what is most probably a C/D cis (13α) epimer. See Johnson, W. S.; Hughes, L. R.; Kloek, J. A.; Niem, T.; Shenvi, A. *J. Am. Chem. Soc.* **1979,** *101,* 1279–1281, and Johnson, W. S.; Hughes, L. R.; Carlson, J. *Ibid* **1979,** *101,* 1281–1282, for a discussion of the occurrence of 13α impurities in cyclizations terminated by the methylacetylenic group. The very small amount of the 17α epimer in the original mixture is presumably due to kinetic protonation which favors the 13β,17β isomer.
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A Spectrophotometric Method for Studying the Rates of Reaction of Disulfides with Protein Thiol Groups Applied to Bovine Serum Albumin

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Abstract: Protein thiol groups that are buried often react slowly with Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid)], ESSE. The site at which these thiol groups reside may be studied kinetically by using mixtures of ESSE and another disulfide, RSSR, which does not produce a chromophore. If RSSR competes successfully for the protein thiol group, the RSH generated reacts with ESSE to produce ES$^-$. The rate of reaction of a variety of disulfides with the protein may be determined. This method was applied to bovine serum albumin, BSA, and a large variation in rate was found, depending upon the structure of the disulfide. After appropriate corrections for the inherent reactivity of the disulfide, a clear picture of the favorability of the interaction of the R group on RSSR with the thiol site arose. The data for BSA suggest that the thiol sits in a constricted hydrophobic site. A β-amino group on the disulfide increases the rate, presumably by an internal ion pair formation. The physiological role of the thiol function is apparently not to react with the cystine or oxidized glutathione.

Introduction

Because of the importance of thiol and disulfide groups in biochemistry,[1,2] substantial effort has been expended in designing specific reagents for their study and quantitation.[3,4] One of the most widely used is Ellman's reagent,[5] 5,5′-dithiobis(2-nitrobenzoic acid), which will be symbolized as ESSE hereafter. This reagent is useful because it is commercially available, water soluble, reacts with a favorable equilibrium constant with alkyl thiols,[6,7] and especially because it generates an intensely chromophoric product, ES$^-$, which can be easily monitored spectrophotometrically.

A vast array of proteins have been studied with ESSE, and it is commonly found that external, unhindered thiol functions can be titrated rapidly, often with no loss of enzymic activity.[8-10] In many other cases, the slower reaction of less readily

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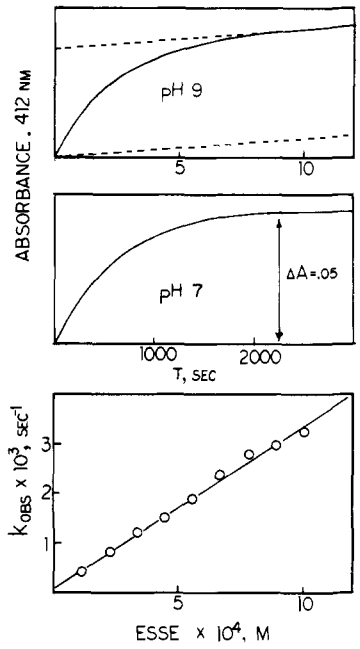


Figure 1. The upper two figures show the absorbance change that occurred when 8×10^{-6} M BSA reacted with 5.3×10^{-4} M ESSE at either pH 9 or 7. The reaction of ESSE with OH^- at the higher pH produced a linear change in absorbance that was subtracted before the rate constant for the pseudo-first-order reaction was calculated. The lowest plot shows the linear dependence of these rate constants on the concentration of ESSE with [BSA] = 10^{-6} M, μ = 0.02, and pH 7.

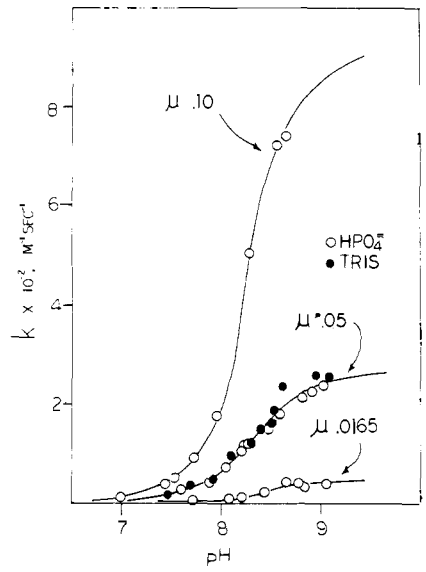
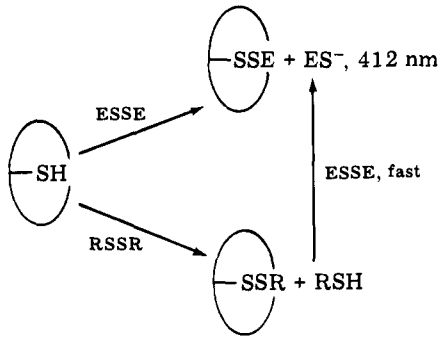


Figure 2. The pH-rate profiles for the reaction of BSA + ESSE determined with [BSA] = 9.6×10^{-6} M and [ESSE] = 5.3×10^{-4} M. The buffer concentration was 0.005 M in each case with the ionic strength raised to the indicated level with KCl. The rates are very sensitive to ionic strength but apparently independent of the buffer that is used. The rest of the studies were done in the presence of 0.005 M Tris buffer with an ionic strength of 0.02.

Scheme I



accessible thiols is coupled with the loss of activity.[12-17] Some thiol functions may be completely buried from the reagent unless the protein is denatured.[8,12,16] It is quite clear, therefore, that the rate of reaction of ESSE with a protein thiol is strongly influenced by the site in which the thiol resides.

The fact that the charge and size of the groups on ESSE are fixed limits the amount of information that may be obtained about the thiol site. It would be more useful to measure the rates of reaction of the thiol with a variety of disulfides, RSSR, so that the influence of charge, size, or stereochemistry might be delineated. Normally, however, such a reaction would not produce a chromophore and could only be followed with difficulty, for example, by monitoring the incorporation of labeled disulfide into the protein.[18]

There is presented in Scheme I a method whereby a protein thiol group that reacts slowly with ESSE may be studied kinetically with a wide variety of nonchromogenic disulfides by a simple spectrophotometric method. As shown in Scheme I, a protein thiol group can react with ESSE or RSSR in competition if both are present in solution. If the rate of reaction of RSH with ESSE is fast[6,7] compared to the preceding step in which it is generated, then the rate of appearance of ES^- at 412 nm would be the sum of the rates of reaction of the protein thiol with ESSE and RSSR. The useful properties of the favorable equilibrium constant and ability to generate a visible chromophore would be maintained with this method, but the flexibility of varying RSSR would be introduced.

The protein chosen for this study is bovine serum albumin, referred to hereafter as BSA. BSA was chosen because of availability and because it has one thiol group that reacts at a moderate rate with ESSE.[19] Despite considerable study,[1,20] it is not clear that the thiol function serves a physiological role. It has, however, been suggested that this group might provide a means of transport for equivalents of cystine.[21] This study was therefore done not only to demonstrate the utility of this approach but also to determine the nature of the site at which the thiol group of BSA resides and the type of functional groups with which the site is compatible.

Experimental Section

Most disulfides were purchased from Aldrich or Sigma and purified by distillation or recrystallization as appropriate. 4,4′-Dithiobis-(benzoic acid) was prepared as described previously.[22] Crystallized and lyophilized bovine serum albumin (A4378) was obtained from Sigma. Water doubly distilled from basic permanganate was used throughout, and this was boiled prior to use to remove oxygen. Solutions were kept under an argon or nitrogen atmosphere.

Experiments were designed so that the ionic strength was constant. A variation in pH was obtained by using various ratios of acidic and basic solutions of the Tris buffer that had each been adjusted to the appropriate ionic strength. In the calculation of ionic strength, dianionic or dicationic disulfides were treated as dianions or dications rather than as two monoanions or two monocations. Since the contribution to the total ionic strengths from the charged disulfides was less than 5%, it is unlikely that this assumption is a source of error.

Preparing solutions of ESSE from the diacid by adding KOH to a slurry of the insoluble acid was unsatisfactory because of the unavoidable reaction of hydroxide ion with the reagent.[23,24] The dipotassium salt was therefore prepared in a modification of a previously described[6] procedure. A slurry of 10 g of the diacid (Aldrich) in 75 mL of distilled water was vigorously stirred in an ice bath, while 2 equiv of 2 M KOH was slowly added using a buret. The pH of the solution was monitored continuously and began to rise above 4 as the equivalence point was reached. The last drop of base drove the pH above 7. To this solution was added 5 g of activated charcoal and then it was warmed and filtered. This procedure was repeated a second

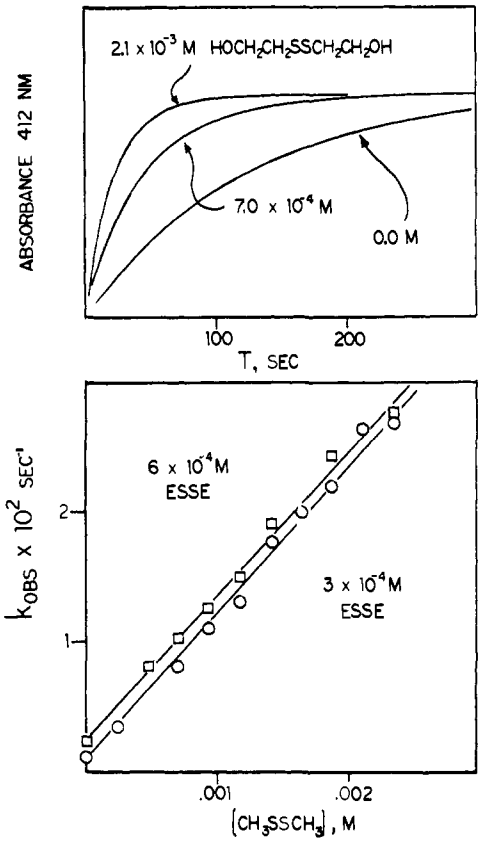


Figure 3. The upper plot demonstrates the effect of adding various concentrations of $HOCH_2CH_2SSCH_2CH_2OH$ on the rate of appearance of ES^- when [BSA] = 9.4×10^{-6} M, [ESSE] = 5.3×10^{-4} M, and the pH is 7.6. The lower plot demonstrates that the increase in rate of ES^- product ion observed when CH_3SSCH_3 is added is independent of the concentration of ESSE. For this experiment, 5.5×10^{-3} M phosphate buffer was used at pH 7.9 with an ionic strength of 0.017.

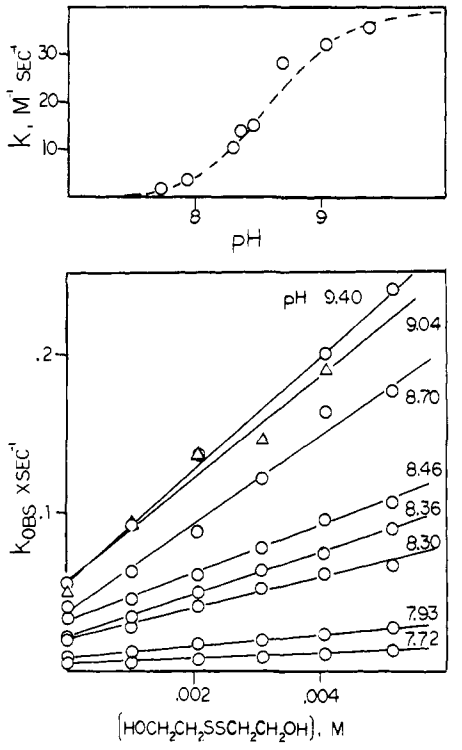


Figure 4. The lower plot shows the least-squares slopes of plots of the rate of appearance of ES^- vs. the concentration of added $HOCH_2CH_2SSCH_2CH_2OH$ as a function of pH. These data were obtained with [BSA] = 9.6×10^{-6} M, [ESSE] = 5.3×10^{-4} M, $\mu = 0.02$, and $T = 25$ °C. The upper plot shows the variation of these slopes with pH, with an arbitrary dashed line included.

time, and the resulting yellow solution was mixed with an equivalent volume of ethanol, cooled in ice, and filtered to yield 6.5 g of the dipotassium salt. This material was readily soluble in water and was used to make concentrated solutions of ESSE that did not have substantial background absorbance.

In a typical experiment, stock solutions of reagents were prepared such that the final concentrations after mixing would be BSA, 9.6×10^{-6} M; ESSE, 5.3×10^{-4} M; Tris/Tris-HCl, 5×10^{-3} M. Enough KCl was added to the acidic and basic buffer solutions so that the same ionic strength (0.02) would be obtained regardless of the pH chosen. Various ratios of water or RSSR were used to give the desired concentration of disulfide. (If RSSR was charged, an appropriate concentration of KCl was used as the diluent instead of water in order to maintain the ionic strength.) All of the solutions were incubated in a water bath at 25 °C, and the cuvettes were incubated at this temperature also. Water, buffer, disulfide, and ESSE were added to the cuvette and allowed to equilibrate at 25 °C. The reaction was then initiated by the addition of an aliquot of the BSA solution. The reaction was monitored by measuring the appearance of ES^- at 412 nm (ϵ 13 600) using a Cary 219 spectrophotometer.

The curves obtained in this manner were pseudo first order. A linear increase in absorbance due to the reaction of OH^- and ESSE had to be subtracted for data obtained at high pH.[23,24] The rate constants were calculated as described previously[25] with correlation coefficients of greater than 0.995.

Results

As shown in Figure 1, the reaction of BSA with excess ESSE at neutral pH generates an easily measurable change in absorbance at 412 nm due to ES^- production. The production of only approximately 0.6–0.7 equiv of ES^- per mol of BSA is well precedented[19,20,25,26] and due to 40% of the protein existing as a mixed disulfide with cysteine and perhaps also glutathione. There is a reaction of ESSE with OH^- that produces a linear change in absorbance that must be subtracted at higher pH values,[23,24] as shown in Figure 1. The rate of production of ES^- is dependent upon the pH and ionic strength and is linearly dependent upon the concentration of ESSE, which is in vast excess over the BSA concentration, as shown in Figures 1 and 2. As is also shown in Figure 2, the rate is independent of the buffer used to maintain the pH.

In Figure 3 is shown the effect of adding another disulfide to a solution of BSA and ESSE. The A_∞ value remains constant, but the rate of formation of ES^- increases dramatically. The increase in rate is linear with increasing concentration of the added disulfide. The slope of a plot of k_{obsd} vs. RSSR gives the second-order rate constant for reaction of RSSR with BSA under the conditions measured. This slope, as required, is independent of the concentration of ESSE, as is shown in Figure 3.

When slopes such as these are measured as a function of pH, as demonstrated in Figure 4, a pH-rate profile is obtained that is similar to that described above for ESSE. As was found for ESSE, there is a sigmoid pH-rate profile that is considerably steeper than the curve that would be expected for the ionization of a single group. The value of the rate constant for the reaction of the ionized thiol with the disulfide was estimated from the high pH data in each case and listed in Table I. Typical sets of data are shown in Figures 4, 5, and 6. Comparable sets of data with similar numbers of points and pH values studied were obtained for the other disulfides listed in Table I.

Discussion

It is evident that this procedure works well in allowing an accurate determination of the rates of reaction of disulfides with a protein thiol group. Since the method relies on the relative slowness of ESSE, it is of interest to know how much slower this reaction is than the reaction of a comparably basic

Table I

RSSR	pK_a, RSH[a]	k_2, M^{-1} s^{-1}	calcd[i] rate with unhindered RS^-, pK_a = 8.5	k_2/calcd k_2
ESSE	4.50[b]	70	200 000	0.00035
$(^+H_3NCH_2CH_2S)_2$	8.23[c]	1000	366	2.7
$(CH_3CH_2S)_2$	10.35[b]	45	0.9	50.0
$(CH_3S)_2$	9.30[d]	90	8.0	11.0
$(HOCH_2CH_2S)_2$	9.61[b]	38	4.3	8.8
p-$^-O_2CPhS)_2$	5.80[e]	75	13 800	0.005
$(^-O_2CCH_2S)_2$	10.68[e]	0 (<10)	0.1	0 (<100)
$(^-O_2CCH_2CH_2S)_2$	10.84[e]	0 (<5)	0.07	0 (<70)
$(^-O_2CCH_2CH_2CH_2S)_2$	10.50[f]	0 (<5)	0.1	0 (<50)
cystine	8.54[e]	0 (<10) (0.1)[h]	38	0 (<0.26) (0.0026)
glutathione disulfide	8.75[c]	0 (<5)	6	0 (<0.8)
cystine dimethyl ester	6.50[g]	35	13 200	0.0027

[a] Microscopic pK_a of RSH when amino group is protonated and carboxylate is deprotonated when these other groups are present. [b] Reference 6. [c] Jules Shafer, personal communication. [d] Reference 28. [e] Reference 29. [f] Estimated. [g] Reference 30. [h] Calculated from data in ref 18, which were measured by incorporation of radioactive cysteine. [i] Reference 33.

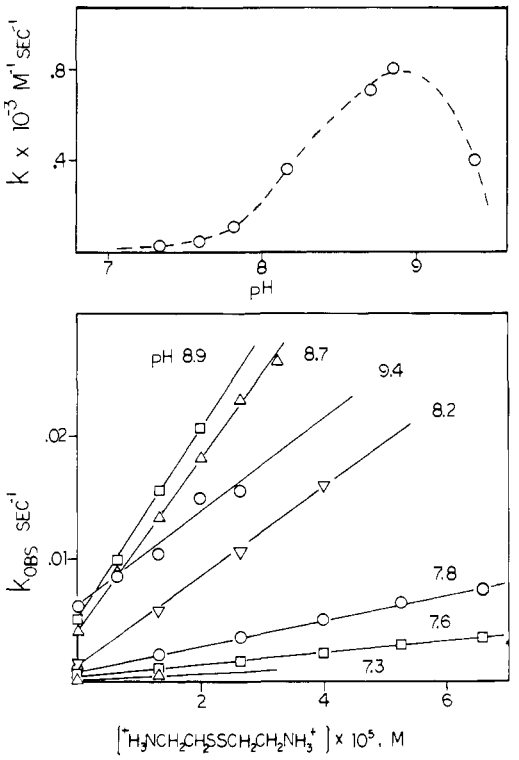


Figure 5. The lower plot shows the least-squares slopes of plots of the rate of appearance of ES^- vs. the concentration of added $H_3N^+CH_2CH_2SSCH_2CH_2N^+H_3$ as a function of pH. These data were obtained with [BSA] = 9.6×10^{-6} M, [ESSE] = 5.3×10^{-4} M, μ = 0.02, and T = 25 °C. The upper plot shows the pH dependence of these slopes with an arbitrary dashed line included.

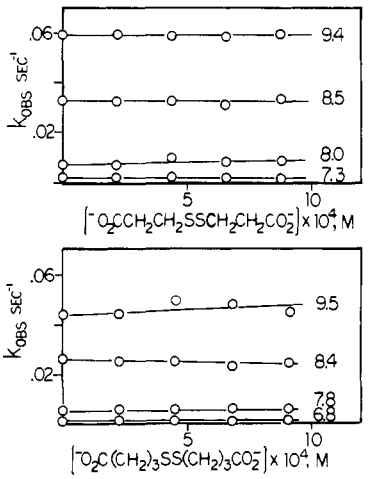


Figure 6. Two plots are shown that demonstrate the apparent lack of measurable reaction of dianionic disulfides with the thiol group of BSA. The pH values at which the measurements were made are indicated, and the rate constant for these two disulfides is listed in Table I as <10 M^{-1} s^{-1}. The concentration of BSA was 9.6×10^{-6} M, ESSE was 5.3×10^{-4} M, Tris buffer was 5×10^{-3} M, and μ = 0.02.

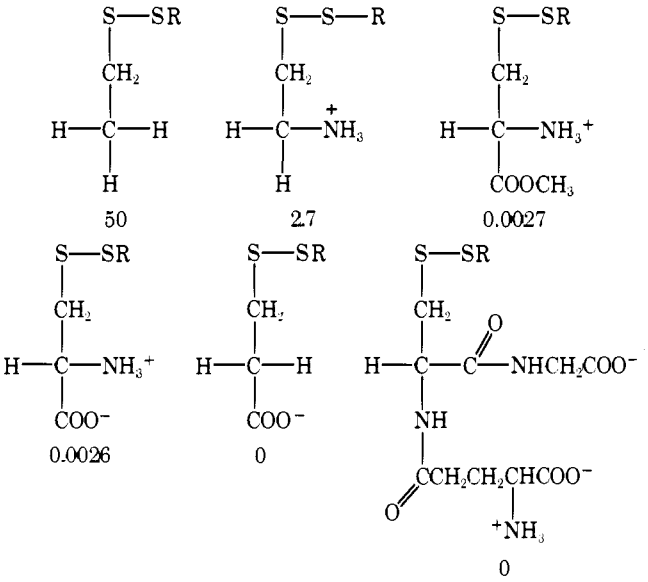
unhindered thiol anion. The structure-reactivity correlations for the reaction of simple thiol anions with ESSE predict a rate of approximately 2×10^5 M^{-1} s^{-1} for the reaction of ESSE with an unhindered thiol anion (where pK_a(RSH) = ~8.5) in aqueous solution.[6,7] The rate of 70 M^{-1} s^{-1} for the reaction of BSA with ESSE is about 3000 times slower, and it is this rate decrease that allows this method to be useful.

The highest absolute rate was found for the disulfide of cysteamine, which is postively charged. All of the neutral disulfides give moderate rates, whereas those disulfides that are aliphatic carboxylates give no apparent rate. The aromatic disulfides deviate from this trend and show a moderate rate of reaction.

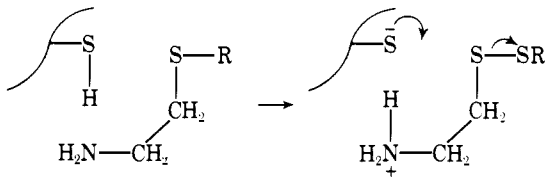
These absolute rates cannot be translated directly into a picture of the environment of the thiol group, since there are vast differences in the inherent reactivity of the disulfides used. It is possible to predict the rates of reaction of $R_1S^- + R_2SSR_3 \rightarrow R_1SSR_2 + R_3S^-$ if the pK_a values of R_1SH, R_2SH, and R_3SH are known.[6,7,31,32] With a constant R_1S^-, increasing the pK_a of R_2SH decreases the rate ($\beta_{CT} = -0.4$), and increasing the pK_a of the leaving group also decreases the rate ($\beta_{LG} = -0.5$). If the presumption is made that the reaction of the BSA thiol is subject to these same constraints, then the rate constants measured may be corrected for inherent reactivity. For example, the disulfide $(HOCH_2CH_2S)_2$ has a measured rate with BSA of 38 M^{-1} s^{-1}, whereas the computed rate[33] of this disulfide with an unhindered pK_a = 8.5 thiol anion in aqueous solution is 4.3 M^{-1} s^{-1}. The ratio of observed to calculated rates listed in Table I gives an index of the ability of $HOCH_2CH_2-$ to interact with the thiol site that is independent of the inherent reactivity of the disulfide.

These ratios are listed for all of the disulfides studied. There is an apparent preference for hydrophobic R groups, since the highest rate ratio was found for diethyl disulfide. Those disulfides with carboxylate anions gave no perceptible rate of reaction unless the carboxylate was held away from the di-

Chart I



Scheme II



sulfide site by an aromatic ring. As shown in Chart I, the rates corrected for inherent reactivity are profoundly influenced by charged or polar groups. The ability of a β-amino group to enhance the rate initially seems to be inconsistent with the affinity of the site for hydrophobic groups. This may be due to the ability of cysteamine disulfide to provide a counterion to the negative charge on the protein thiol anion within a region of low dielectric constant, as shown in Scheme II. Alternatively, this may reflect the existence of a nearby negatively charged group. These observations are consistent with spin-label[36,37] and other[38] studies, which conclude that the thiol group is in a sterically restricted environment that has hydrophobic character. The primary sequence of the protein in this region is hydrophobic also.[20] Iodoacetamide reacts readily with the thiol group of albumin, but iodoacetate does not,[39] which is consistent with this proposal also.

The sigmoid dependence of the rate of reaction with pH suggests that the rate law for the process is:

$$\frac{d[ES^-]}{dt} = k_2[PS^-][RSSR]$$

where PS^- is the ionized form of the thiol. This is the same rate law that was found for unhindered thiols in aqueous solution.[6,7] The pH-rate profiles shown in Figures 2, 4, and 5 are significantly steeper than those expected for a single ionization. The titration curve, however, for the ionization of the thiol group on BSA shows a similar steep dependence on pH and is consistent with the ionization of three proximate groups.[40] The pH-rate profiles of cysteamine and cystine dimethyl ester differ from the others because the rate drops off significantly at higher pH values. This is consistent with the loss of a proton from the amino group and the resulting lack of ability to undergo ion pair formation.

Although this study does not define any metabolic role for the BSA thiol group, the rate data in Table I are suggestive. The thiol site was clearly not designed to react rapidly with either cystine or oxidized glutathione. The rapid reaction with cystamine disulfide may be an indication of a type of disulfide that the thiol is designed to react with, but more likely this just reflects the ability of this particular disulfide to react rapidly with a thiol in a hydrophobic environment. The metabolic role of the thiol may be to induce internal covalent changes in the disulfide bonds of BSA that eventually result in catabolism.[41-43] The effectiveness of cysteamine to react with hydrophobic thiols is consistent with the suggestion that this disulfide might be the agent that transports oxidizing equivalents to a folding protein so that disulfide bonds may be formed.[44]

Acknowledgments. This work was supported by grants from the Research Corporation and the National Science Foundation (Grant CHE-7808723) for which we are grateful. J.M.W. was a National Science Foundation undergraduate research participant. The authors thank Margaret Hunter and Jules Shafer for helpful suggestions.

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