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Determination of Interactive Thiol Ionizations in Bovine Serum Albumin, Glutathione, and Other Thiols by Potentiometric Difference Titration†

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ABSTRACT: A potentiometric difference titration (PDT) method is used to study the ionization behavior of the thiol group in bovine serum albumin and in the following less complex compounds: glutathione, cysteine, 2-mercaptoethanol, 3-mercaptopropionic acid, 2-mercaptoethylamine, *cis*-2-mercaptocyclobutylamine, 2-aminothiophenol, and 5-mercapto-2-nitrobenzoic acid. In the PDT method the pH dependence of the amount of protons released in the reaction $RSH + CH_3SO_2SCH_3 \rightarrow RSSCH_3 + CH_3SO_2^- + H^+$ is measured in order to obtain the pH dependence of the molar proton content of the thiol (h_u) relative to the molar proton content of its methylthio derivative (h_m). The pH dependence of $h_u - h_m$ reflects the ionization behavior of the thiol group and of other groups whose ionization is thermodynamically linked to that of the thiol group. Data presented here indicate that the ionization behavior of the single thiol group in albumin is strikingly different in the native and the urea-denatured proteins. Three ionizable groups appear to affect ionization of the thiol in the native protein whereas only one group appears to affect ionization of the thiol in the urea-denatured protein. Furthermore, the measured PDT curves are consistent with an abnormally high acidity ($pK < 5$) for the thiol in native albumin and a normal acidity for the thiol in the urea-denatured protein. Comparisons of microscopic ionization constants determined for cysteine by using the PDT method with those determined by other methods indicate that the PDT method should be useful in characterizing the ionization behavior of thiol groups in proteins and other polyprotic substances.

Assignments of catalytic functions to individual groups at the active site of enzymes are based to a large extent upon an understanding of how the pH dependence of catalytic competence is related to the ionization behavior of the catalytically important groups. Unfortunately, it is no easy matter to determine the ionization behavior of a specific group in a protein. In a previous paper (Lewis et al., 1976), we presented a potentiometric difference titration (PDT)[1] method for studying the ionization behavior of the thiol group of Cys-25 at the active site of papain. In this method the difference in proton content between unmodified papain and the methylthio derivative of Cys-25 was determined by measuring the amount

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[1] Abbreviations used: BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; MMTS, methyl methanethiosulfonate; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); PDT, potentiometric difference titration.

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of protons released in the reaction of methyl methanethiosulfonate (MMTS) with the thiol group (eq 1). The pH

$$RSH + CH_3SO_2SCH_3 \rightarrow RSSCH_3 + CH_3SO_2^- + H^+ \quad (1)$$

dependence of the difference in proton content between the unmodified protein and the methylthio derivative wherein ionization of the active-site thiol group is blocked was used to characterize the ionization behavior of the active-site thiol group as well as another group in the protein whose ionization is thermodynamically linked to that of Cys-25.

The PDT method is different from the kinetic methods usually employed to study the ionization behavior of thiol groups in proteins, wherein the ionization behavior of the thiol group is related to the pH dependence of its nucleophilic reactivity. Application of the kinetic approach is straightforward when there exists a pH-independent linear relationship between the measured nucleophilic reactivity and the fraction of thiol present as a thiolate anion. However, the existence of such a relationship for a thiol group in a protein is problematical. The reactivity of a thiolate anion in a protein might well be a sensitive function of the protonation state of other groups in the protein which affect the accessibility of the thiolate anion or the relative orientation of the thiolate anion and the electrophilic reagent in the activated complex.[2] Thus it is often impossible to deduce unambiguously the ionization behavior of a thiol group in a protein from the pH dependence of its nucleophilic reactivity.[3]

In the PDT method, however, protonation states are measured directly by titration so that the pH dependence of the difference in proton content of the unmodified protein and its methylthio derivative reflects only the ionization behavior of the thiol group and the ionization behavior of those groups whose ionization is thermodynamically linked to that of the thiol group. In this work, the PDT method is used to study the ionization behavior of the thiol group in BSA and several low molecular weight thiols. Additionally, values of microscopic ionization constants for the amino and thiol groups in cysteine as determined by the PDT method are compared to values obtained by other methods in order to determine possible errors in using the PDT method to measure interactive ionizations involving thiol groups in complex molecules.

Experimental Procedures

Materials

Carbonate-free KOH analytical concentrate for preparation of standard base for titrations was from J. T. Baker. HCl (1 N) for preparation of standard acid was from Fisher. Methyl methanethiosulfonate (MMTS) was prepared according to the method of Smith et al. (1975). Solutions of urea (ultrapure, from Schwarz/Mann) were passed through a 1 × 14 cm column of mixed-bed resin (AG501-X8, from Bio-Rad) just prior to use. The thiols used (and their sources) were the following: 2-mercaptoethanol (Matheson Coleman and Bell), 2-mercaptopropionic acid (Aldrich), cysteine (Cyclo Chemical), glutathione (Calbiochem-Behring Corp.), 2-mercaptoethylamine hydrochloride (K & K Laboratories), BSA (fraction V, Calbiochem-Behring Corp.), *cis*-2-mercaptocyclobutylamine hydrochloride (a generous gift from Dr. Sweeney, Department of the Army, Walter Reed Army Medical Center, Washington, DC), 5-mercapto-2-nitrobenzoic acid (prepared by reduction of a 1.2 mM solution of Nbs_2 from Sigma with 1 mM DTT), and 2-aminothiophenol (from Aldrich, converted to its HCl salt and recrystallized from ethanol–ether).

Methods

Preparation of Solutions. Solutions of low molecular weight thiols at 10^{-4}–10^{-3}M were prepared fresh each day, and prior to use their concentrations were determined spectrophotometrically by the method of Ellman (1959). Solutions of BSA were prepared fresh each day by dissolving the solid protein in water and adding KCl, EDTA, and urea so as to obtain the desired concentrations. BSA concentrations were calculated assuming an M_r of 67 000 and an $E^{1\%}_{1cm}$ of 6.67 at 279 nm (Janatova et al., 1968). The thiol content of the BSA (usually 50–60% of the protein concentration) was determined by diluting 0.15 mL of protein with 2.5 mL of 0.05 M phosphate buffer, pH 7.3, containing at least 200-fold excess of Nbs_2 over thiol in a manner similar to that described by Janatova et al. (1968). EDTA was added to thiol solutions in order to decrease the rate of thiol oxidation. The thiol content of all solutions was checked periodically in order to correct values of thiol concentrations for any loss of thiol due to oxidation. The decrease in thiol concentrations from one determination to the next was never more than 10%. In most determinations the concentration of MMTS was 96% that of the thiol so that it was not necessary to correct for losses of thiol that were less than 4%. Concentrations of stock solutions of MMTS in 0.15 M KCl were determined from the decrease in absorbance of a solution of 5-mercapto-2-nitrobenzoic acid at pH 7.2 upon addition of a known volume of MMTS.

Measurements of pH were made by using a Radiometer PHM 4c pH meter standardized at the appropriate temperature with a 1:1 phosphate National Bureau of Standards primary standard solution (Bates, 1964) and either phthalate or tetraborate standards depending on the pH range of interest.

Determination of Protons Released upon Reaction with MMTS Using the Glass Electrode. Solutions (2.4 mL) consisting of 0.1–1.5 mM thiol, 1–15 mM EDTA, and sufficient KCl to bring the $\Gamma/2$ to 0.15 or 1.0 were equilibrated at 25 °C and adjusted to the desired pH. HCl roughly equivalent to the amount of thiol present was added and back-titrated with KOH in order to calibrate the system. MMTS 0.96–1.04 times the molar amount of thiol was added in a KCl solution at the $\Gamma/2$ of the sample, and the acid produced upon methylthiolation was back titrated with KOH as described elsewhere (Lewis et al., 1976). The acid content of the MMTS reagent was checked and found to be negligible. KCl blanks were determined and used to correct titers. The necessity of correcting for hydrolysis of excess reagent in alkaline solutions was avoided either by using less than 4% excess MMTS or by making the MMTS the limiting reagent. After back-titration with MMTS, the system was recalibrated with HCl and KOH. The correspondence (which was always within 10%) between the KOH titers before and after reaction with

[2] Neighboring ionizations have been shown to effect the reactivity of low molecular weight thiolate anions. For example, Kallen (1971b) has shown that the rate constant for reaction of the thiolate anion of cysteine with formaldehyde decreases by a factor of 2.4 upon protonation of the carboxylate group of cysteine. Also, the kinetic data of Lindley (1960) together with the microscopic ionization constants for cysteine listed in Table II indicate that the rate constant for reaction of the thiolate anion of cysteine with chloroacetamide decreases at least 3-fold upon protonation of the amino group of cysteine. These effects are predominantly inductive in nature. In a protein, the effects of neighboring ionizations on the accessibility of the thiolate anion and the orientation of the electrophilic reagent may well result in much larger changes in the reactivity of the thiolate anion.

[3] For an example of a quantitative treatment of the problems associated with using the pH dependence of a signal such as reactivity to determine pK's of a thiol group in a polyprotic acid, see Lewis et al. (1976) and Lindley (1960).

Table I: Representative Points from a PDT of BSA[a]

initial pH	V_{s1} (μL)	V_r (μL)	V_b (μL)	V_{s2} (μL)	~ΔpH	ΔH (μmol)	$h_u - h_m$
5.01	51.5	34.0	1.5	53.0	0.020	0.320	0.78
5.48	51.0	39.0	1.5	53.5	0.025	0.370	0.90
5.95	51.5	50.5	<0.5	52.5	0.040	0.486	1.18
6.49	49.6	52.0	<0.5	49.8	0.065	0.523	1.28
7.23	51.5	61.5	<0.5	55.5	0.073	0.575	1.40
7.96	52.0	37.5	<0.5	52.5	0.045	0.359	0.88
8.76	57.0	16.0	<0.5	57.0	0.020	0.140	0.34
9.49	60.0	20.0	−1.0	60.0	0.020	0.172	0.42

[a] For each point in the titration, a 2.4-mL sample of 1 mM EDTA containing 0.442 μmol of BSA with a functional thiol group (as judged by reaction of an aliquot of the BSA stock solution with Nbs_2) was adjusted to the desired initial pH value. The titration involved addition of the following to each 2.4-mL sample: 50 μL of 10^{-2} M HCl in 0.15 M KCl, 50 μL of 8.2 mM MMTS in 0.15 M KCl, 50 μL of 0.15 M KCl, and 50 μL of 10^{-2} M HCl in 0.15 M KCl. After each addition, the sample was back-titrated to the initial pH value with KOH. The volumes (μL) of KOH (~0.01 M) required for back-titrations of the additions listed above are denoted by V_{s1}, V_r, V_b, and V_{s2}, respectively. ΔpH denotes the approximate pH change resulting upon addition of MMTS. ΔH was calculated by using eq 2 and 3, the relationship $V_s = (V_{s1} + V_{s2})/2$, and 0.5 μmol for S, the amount of standard HCl. Since MMTS was limiting, $h_u - h_m$ was determined by dividing ΔH by 0.41 μmol, the amount of MMTS added (see eq 5).

MMTS was taken as an indication of the precision of the titration. The precision of the titration was a function of the buffering capacity of the solution. As the buffering capacity increased, the pH response decreased, making it more difficult to determine end points. pH changes of several hundredths of a pH unit were usually observed for a change in proton content equivalent to the molar amount of thiol present.

The total amount of protons released in a reaction (ΔH) was calculated from eq 2 and 3

$$\Delta H = C_{OH}(V_r - V_b) \quad (2)$$

$$C_{OH} = S/(V_s - V_b) \quad (3)$$

where V_r, V_b, and V_s are the volumes of KOH required to back-titrate the reagent, a KCl blank, and the protons (S) from the HCl standard, respectively; C_{OH} is the concentration of the KOH titrant. When an addition produced an increase in pH, the negative of the volume of KOH titrant which would be required to produce the same increase in pH as the addition was set equal to the corresponding value of V in eq 2 and 3. The determination of ΔH and $h_u - h_m$ from representative points in a titration of BSA is illustrated in Table I.

The increase in the volumes, V_{s1} and V_{s2}, of KOH required to back-titrate the HCl standard observed for the pH 8.76 and 9.49 points in Table I is due to the presence of carbonate in the KOH. Carbonate mediates this variability in the KOH titer because its ability to neutralize acid decreases above pH 8. Calibration of the KOH titer for each point on the titration curve as was done here eliminates errors due to the presence of carbonate in the KOH. The data listed in Table I were obtained from sample solutions containing 1 mM EDTA, 0.18 mM BSA with a functional thiol group, and about 0.18 mM BSA containing no functional thiol. Although the extra ionizable groups from EDTA and the inert BSA do not perturb the PDT curve, they increase the buffering capacity of the sample solution and thereby lower the sensitivity of the titration. When it is necessary to minimize the concentration of protein thiol, care should be taken to minimize the concentration of extra ionizable groups. Finally, it should be noted that protein samples used for determination of points on a PDT curve can be regenerated by treatment with dithiothreitol (to remove the methylthio group) followed by gel filtration to separate the protein from low molecular weight material.

Determination of Proton Release upon Reaction with MMTS Using Indicator Dyes. The feasibility of using indicator dyes to measure proton release was demonstrated with 2-mercaptoethanol. Solutions at $\Gamma/2 = 0.15$ containing 500 μM 2-mercaptoethanol, 15 mM EDTA, and 15–30 μM indicator dye (cresol red, pH 7.7–8.6, 568 nm; phenolphthalein, pH 8.6–9.9, 550 nm; thymol blue, pH 10.5, 590 nm) were adjusted to the desired pH value by using a glass electrode. For each determination, three samples (2 mL) were transferred to three cuvettes in a GCA/McPherson EU700 series double-beam spectrophotometer thermostated at 25 °C. The first two samples were used to zero the instrument and to measure any base-line drift during the titration. The absorbance of the third sample was offset to 0.100 relative to the first two samples. A 50-μL aliquot of a solution of MMTS in 0.15 M KCl was added to the third sample so that the amount of added MMTS was 4% in excess over the thiol, and 50 μL of 0.15 M KCl was added to the first two samples. The absorbance decrease (ΔA_R) of the third sample vs. the first two samples was noted. The relationship between the absorbance decrease (ΔA_R) and the protons released (ΔH) was obtained by noting the increase in absorbance (ΔA_B) after the addition of a known amount (B) of base (10–50 μL) in 0.15 M KCl to the third sample and the addition of the same volume of 0.15 M KCl to the first two samples. The amount of base added was similar to the amount of protons released. The amount of protons released (ΔH) was calculated from the relationship

$$\Delta H = \Delta A_R B/\Delta A_B \quad (4)$$

For avoidance of errors due to carbonate in the KOH, the effective amount of base added (B) was determined at each pH value by comparing the value of ΔA_B to the absorbance change produced by adding a known amount of standard HCl (similar in amount to B and in 0.15 M KCl) to the third sample and the same volume of 0.15 M KCl to the first two samples.

Calculation of the Difference in Molar Proton Content of the Thiol and the Methylthio Derivative. The quantity of protons released in the reaction (ΔH) was related to the difference in the molar proton content between the unmodified thiol and the methylthio derivative ($h_u - h_m$) by eq 5

$$h_u - h_m = \frac{\Delta H}{R} + h_s \quad (5)$$

where R is the molar amount of thiol or MMTS present (whichever was limiting in the reaction) and h_s is the molar proton content of the methylsulfinic acid ($pK = 2.12$ at $\Gamma/2 = 0.15$) produced in the reaction. The value of h_s which is negligible above pH 4 was calculated from the relationship

$$h_s = \frac{10^{-pH}}{10^{-pH} + 10^{-2.12}} \quad (6)$$

Rates of reaction of 2-mercaptoethanol with MMTS were measured in a stopped-flow spectrometer (Aminco-Morrow) at 275 nm under pseudo-first-order conditions by using 2 mM 2-mercaptoethanol and 50 mM MMTS in acetate buffered solutions (pH 4–5.7, $\Gamma/2 = 0.15$, 25 °C). Pseudo-first-order rate constants were obtained from linear plots of $-\ln(A_f - A_t)$ vs. time where A_f is the final absorbance and A_t is the absorbance at time t. Second-order rate constants for the attack of thiolate anion on MMTS were calculated by dividing the pseudo-first-order rate constants by the product of the fraction of thiolate anion and the concentration of MMTS. The fraction of thiolate anion $[10^{-pK}/(10^{-pK} + 10^{-pH})]$ was calcu-

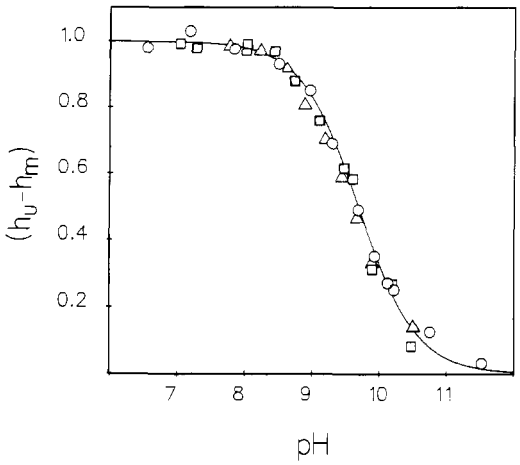


FIGURE 1: PDT curve for 2-mercaptoethanol at 0.1 mM (□), 0.5 mM (△), and 1 mM (○). The titrations were performed in the presence of 15 mM EDTA at $\Gamma/2 = 0.15$ and 25 °C. The data represented by △'s were obtained by using indicator dyes rather than a glass electrode to determine the end point of the back-titration. The solid line is the least-squares fit to eq 7 ($pK = 9.65$).

lated from the pH by using a pK of 9.65 for 2-mercaptoethanol.

Rates of solvolysis of MMTS (1 mM) in buffered solutions of sodium tetraborate ($\Gamma/2 = 0.15$, pH 9–10) were determined by following the approach of the absorbance at 235 nm to its final value in a GCA/McPherson spectrophotometer at 25 °C. Rate constants were obtained from the slopes of linear lots of $-\ln (A_f - A_t)$ vs. time.

Analysis of Titration Curves. pK values and standard deviations were obtained from fits of titration data to the indicated equations by using a nonlinear least-squares program (BMDX85) from the Health Science Computing Facility at The University of California, Los Angeles. The program obtains a least-squares fit to functions by means of Gauss–Newton iterations of the parameters.

Results

In the PDT method for determining the ionization behavior of thiol groups, a solution of thiol-containing compound is adjusted to the desired pH value, allowed to react with MMTS (eq 1), and back-titrated to the original pH value. The amount of base required for the back-titration after correction for blanks and the proton content of methylsulfinic acid is equal to the difference in proton content of the unmodified compound and its methylthio derivative at the pH value of the titration. For simple thiols, the pH dependence of the difference in molar proton content ($h_u - h_m$) is equivalent to a potentiometric titration for the unmodified thiol since the proton content of the methylthio derivative is zero. Figure 1, which depicts a PDT curve for 2-mercaptoethanol, exemplifies the use of the PDT method for determining the ionization behavior of thiols at low concentrations. The solid line in Figure 1 is the best fit of the pH dependence of $h_u - h_m$ to that expected for a single ionizable group (eq 7). A pK of 9.65 (±0.02) at $\Gamma/2$

$$h_u - h_m = \frac{10^{-pH}}{10^{-pH} + 10^{-pK}} \quad (7)$$

= 0.15 (25 °C) was obtained from the data in Figure 1. This pK value is in reasonable agreement with the value of 9.61 previously determined for this thiol by Jencks & Salvensen (1971) at $\Gamma/2 = 1.0$ (25 °C).

The points for the PDT curve in Figure 1 were determined in the presence of a 15–150-fold molar excess of EDTA over thiol to inhibit oxidation. The failure of the pK = 6.2 and 10.2 ionizable groups in EDTA (Tillotson & Staveley, 1958) to perturb the PDT curves demonstrates the usefulness of the PDT method for determining the ionization behavior of thiols in the presence of other ionizable groups. The amount of protons released upon reaction with MMTS could be estimated by using either a glass electrode or indicator dyes (Figure 1). The concentration of thiol required for a titration is dependent upon the pH range and the buffering capacity of the solution. In general, solutions consisting of 50–100 μM thiol could be titrated when a glass electrode was used to measure pH changes whereas 250–500 μM thiol was required for accurate titrations by using indicator dyes.

Rates of Reaction of MMTS with Water and Thiols. The base-catalyzed solvolysis of MMTS was measured in order to determine whether this side reaction limited the utility of the PDT method. The apparent first-order rate constant (k_{obsd}) for solvolysis of MMTS was measured in buffered solutions between pH 9 and 10 and found to be proportional to the hydroxide ion concentration. The proportionality between k_{obsd} and pH indicates that at pH 11, the highest pH which is likely to be used in the PDT method, MMTS would decompose with a first-order rate constant of 24.2 min^{-1}. Although this rate constant is large, the usefulness of MMTS is dependent upon its rate of reaction with thiolate anions relative to its solvolysis rate. Therefore, the rate of reaction of MMTS with 2-mercaptoethanol was determined. This reaction was too rapid to measure even in the stopped-flow spectrometer unless the pH value was kept well below the pK (9.65) of the thiol group so that only a very small fraction of the thiol was present in its reactive anionic form. The second-order rate constant for reaction of MMTS with the thiolate anion was determined from the observed pseudo-first-order rate constant for the reaction of 2-meraptoethanol with excess MMTS at pH 4.0, 5.0, and 5.65. These pseudo-first-order rate constants were divided by the product of the MMTS concentration and the fraction of thiolate anion present to yield a value of 1.8 (±0.1) × 10^8 M^{-1} min^{-1} for the second-order rate constant for the attack of thiolate anion on MMTS. Using this rate constant, one can calculate that at pH 11 a reaction of 50 μM MMTS with a thiol (at 50 μM) which has a reactivity similar to that of 2-mercaptoethanol (and a pK < 10) will be 95% complete in 2.1 × 10^{-5} min, a time over which only 5% of the MMTS would have undergone solvolysis had there been no thiol present. At lower pH values or higher initial concentrations of MMTS and thiol, the ability of solvolysis to compete with thiol attack is further reduced. Thus, solvolysis of MMTS should not effectively compete with the reaction of MMTS with thiol. If MMTS is present in substantial excess of thiol during the titration, a blank should be determined in which the proton release corresponding to the hydrolysis of the excess MMTS is measured. In order to avoid the necessity of such corrections, the amount of MMTS used in the titrations reported here was between 0.96 and 1.04 times the amount of thiol present. The use of a limited amount of MMTS also minimizes possible errors that might arise as a result of a slow reaction of MMTS with a group other than the thiol group.

Ionization of Thiols with More Than One Ionizable Group. The ionization behavior of 5-mercapto-2-nitrobenzoic acid, glutathione, and 3-mercaptopropionic acid as determined by the PDT method is shown in Figure 2. Data plotted in Figure 2 indicate that the titration curve for the thiol group in 5-mercapto-2-nitrobenzoic acid as determined by the PDT method (●) matches that determined spectrophotometrically (□). Although the pH dependence of the proton content of

Table II: Ionization Constants of Amino Thiols

compound	condition[a]	pG_1[b]	pG_2	$pG_{1m} = pK_1$	pK_1	pK_{12}	pK_{21}	K_2/K_1
glutathione	a	8.78 (±0.02)	9.68 (±0.02)	9.16 (±0.02)	8.93	9.31	9.53	1.7
cysteine	b	8.20 (±0.06)	10.37 (±0.02)	8.57 (±0.05)	8.44	10.00	10.14	1.4
2-mercaptoethylamine	a	8.44 (±0.02)	10.88 (±0.02)	9.73 (±0.02)	8.46	9.60	10.87	19
	c	8.63 (±0.02)	11.34 (±0.03)	10.23 (±0.02)	8.64	9.72	11.34	39
cis-2-mercaptocyclobutylamine	a	7.81 (±0.04)	10.18 (±0.03)	8.59 (±0.05)	7.89	9.40	10.11	5.0
	d	8.17 (±0.03)	10.78 (±0.03)	9.14 (±0.04)	8.22	9.81	10.73	8.3
BSA in urea	a	8.79 (±0.03)	10.27 (±0.06)	9.42 (±0.06)	8.88	9.63	10.17	3.5

[a] The conditions for the PDT titrations were the following: (a) 25 °C, $\Gamma/2 = 0.15$; (b) 25 °C, $\Gamma/2 = 1.0$; (c) 12 °C, $\Gamma/2 = 0.15$; (d) 10 °C, $\Gamma/2 = 0.15$. [b] Standard deviations are given in parentheses.

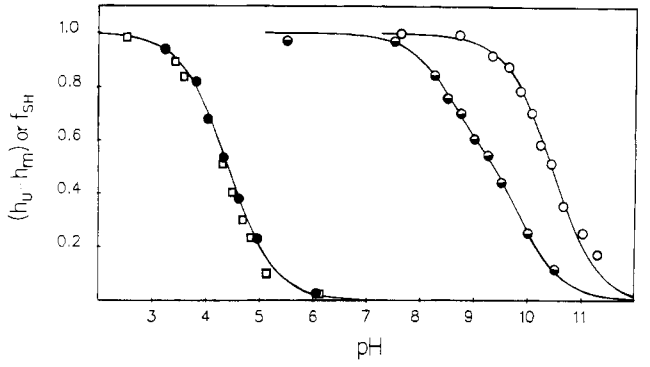


FIGURE 2: PDT curves for 5-mercapto-2-nitrobenzoic acid (●), glutathione (◒), and 3-mercaptopropionic acid (○). The titrations were performed with 1 mM thiol in the presence of 15 mM EDTA at $\Gamma/2 = 0.15$ and 25 °C. The squares depict the pH dependence of the fraction of thiol present in the protonated form (f_{SH}) for 5-mercapto-2-nitrobenzoic acid as determined by spectrophotometric titration at 410 nm. The solid lines for 5-mercapto-2-nitrobenzoic acid (pK = 4.41) and 3-mercaptopropionic acid (pK = 10.44) are least-squares fits to eq 7. The solid line for glutathione is the least-squares fit to eq 8 ($pG_1 = 8.78$, $pG_2 = 9.68$, $pG_{1m} = 9.16$).

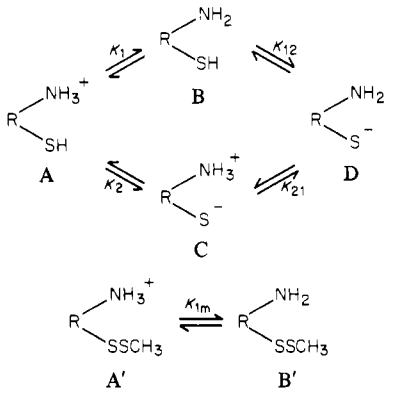
5-mercapto-2-nitrobenzoic acid ($pK = 4.41 \pm 0.02$) and that of 3-mercaptopropionic acid ($pK = 10.44 \pm 0.02$) is that expected for a single ionizable group, the ionization behavior of the thiol group in glutathione appears more complex. The complexity of the titration curve is most simply explained by a dependence of the ionization constant of the thiol group in glutathione on the protonation state of the α-amino group. This point is illustrated by Scheme I which depicts the ionization scheme for a simple amino thiol wherein the pK of each group is dependent on the protonation state of the other.

The simplest way to analyze the pH dependence of the proton content of a polyprotic substance such as glutathione is to use the approach of Simms (1926). In this treatment the titration curve of a polyprotic substance with n ionizable groups is analyzed as if it were a mixture of n hypothetical groups which independently ionize with dissociation constants G_1, G_2, . . . G_n. Thus we may write

$$h_u - h_m = \sum_{i=1}^{i=n+1} \frac{1}{1 + 10^{pH-pG_i}} - \sum_{i=1}^{i=n} \frac{1}{1 + 10^{pH-pG_{im}}} \quad (8)$$

where G_i and G_{im} are the dissociation constants of the hypothetical groups required to represent the pH dependence of the proton content of the unmodified thiol and the methylthio derivative, respectively. These dissociation constants are referred to as titration constants to emphasize the fact that they may not be numerically equivalent to true acid dissociation constants of groups in the protein. The first summation represents the contribution to the proton content of groups in the unmodified thiol, and the second summation represents the contribution of groups in the methylthio derivative. Naturally, the pH dependence of $h_u - h_m$ will only reflect the ionization

Scheme I



behavior of groups that are altered by methylthiolation. Thus, the number of groups altered by methylthiolation and the values of titration constants can be obtained from the fit of the observed pH dependence of $h_u - h_m$ to eq 8. Exact values for the macroscopic ionization constants for removal of the first through the *nth* proton may be calculated from the values of the titration constants.[4] For example, when $n = 1$, Scheme I obtains, and

$$K_I = \frac{([B] + [C])[H]}{[A]} = G_1 + G_2 \quad (9)$$

$$K_{II}^{-1} = \frac{([B] + [C])}{[D][H]} = G_1^{-1} + G_2^{-1} \quad (10)$$

$$K_{Im} = \frac{[B'][H]}{[A']} = G_{1m} \quad (11)$$

The data in Figure 2 for GSH could be fit to eq 8 with a value of $n = 1$ (solid line) to yield values of pG_1, pG_2, and pG_{1m} which appear in Table II. The values of 8.73 for pK_I and 9.73 for pK_{II} obtained from the values of G_1 and G_2 must be macroscopic ionization constants for removal of the first and second proton from the thiol and ammonium pair in GSH since these groups are the only ones that ionize in the pH range studied. These values of K_I and K_{II} are in good agreement with pK values of 8.75 and 9.65 for successive removal of two protons

[4] See Simms (1926) for derivations of relationships between macroscopic ionization constants and titration constants. One could of course circumvent the need for separately evaluating titration constants by using equations which contain the true macroscopic ionization constants instead of titration constants. Such equations, however, are much more complex and are more difficult than eq 8 to use in the analysis of PDT curves. Furthermore, if a polytropic substance ionizes so that the first proton is removed almost entirely from group 1, the second proton is removed almost entirely from group 2, etc., then the titration constants become numerically equivalent to microscopic ionization constants for individual groups. The titration constants also become equivalent to microscopic ionization constants when the pK of each group is independent of the protonation state of the other groups in the molecule.

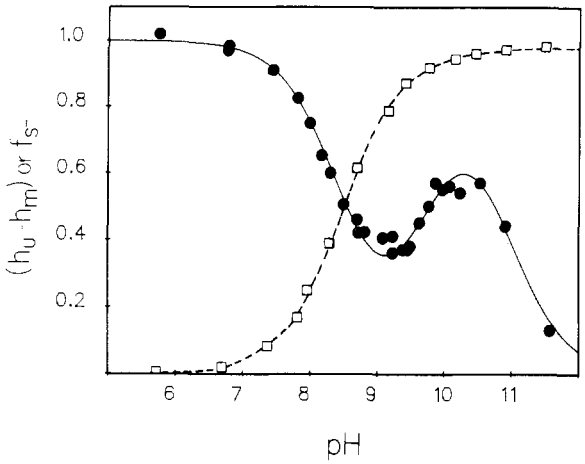


FIGURE 3: PDT curve for 1 mM 2-mercaptoethylamine in 15 mM EDTA at $\Gamma/2 = 0.15$, 25 °C. The solid line is the least-squares fit to eq 8 ($pG_1 = 8.44$, $pG_2 = 10.88$, $pG_{1m} = 9.73$). The dashed line is a least-squares fit to the relationship $f_{s^-} = (10^{-pK})/(10^{-pK} + 10^{-pH})$ which relates the degree of ionization (f_{s^-}) of a simple thiol to the pH (pK = 8.44). The squares were calculated by using the relationship $f_{s^-} = (10^{pK_I - pK_2} + 10^{pH - pK_{II}})/(1 + 10^{pH - pK_{II}} + 10^{pK_I - pH})$ for the fraction of thiolate anion present (f_{s^-}) as derived from Scheme I ($pK_I = 8.44$, $pK_{II} = 10.88$, and $pK_2 = 8.46$).

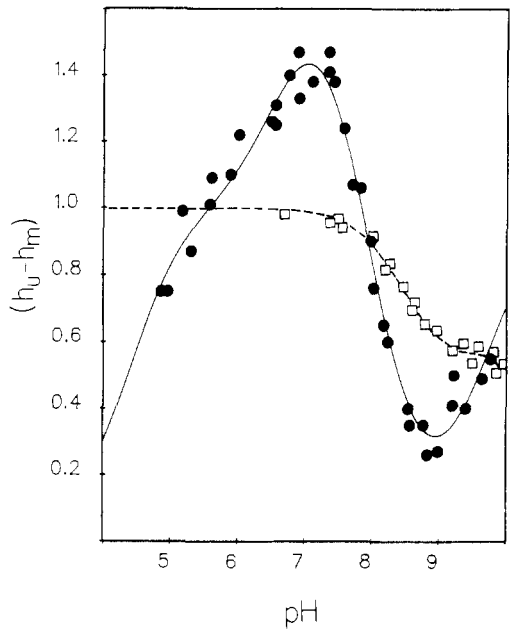


FIGURE 4: PDT curve for BSA in 1 mM EDTA at $\Gamma/2 = 0.15$, 25 °C, in the absence (●) and the presence (□) of 8 M urea. The concentration of BSA having a functional thiol group was 0.15–0.20 mM. The dashed line is the least-squares fit to eq 8 ($pG_1 = 8.79$, $pG_2 = 10.27$, and $pG_m = 9.42$). No measurements were made in the pH range 10–12 where values of $h_u - h_m$ would be expected to approach zero. The solid line is the least-squares fit to eq 8 ($pG_2 = 7.88$, $pG_3 = 7.89$, $pG_{1m} = 4.37$, $pG_{2m} = 6.70$, $pG_{3m} = 9.65$, and, in the pH range studied, $G_1/10^{-pH} \gg 1$ and $G_4/10^{-pH} \ll 1$). It should be noted that in the PDT method degrees of protonation are measured directly so that it is often possible to estimate pG values such as pG_{1m} from measurements which extend only over a small part of the pH range for the ionization. (Such a determination is analogous to a determination of the pK of an acid from the pH of a partially neutralized solution of the acid.) The term in eq 8 containing pG_{1m} accounts for about 25% of the observed value of $h_u - h_m$ at pH 5.

from GSH in the pH range 6–10.5 which have been determined previously by direct potentiometric titration (Li et al., 1954).

The macroscopic ionization constants K_I and K_{II} are related to the microscopic ionization constants defined in Scheme I by eq 12–14 (Edsall & Wyman, 1958). If one assumes that

$$K_I = K_1 + K_2 \tag{12}$$

$$K_{II}^{-1} = K_{12}^{-1} + K_{21}^{-1} \tag{13}$$

$$K_1 K_{12} = K_2 K_{21} \tag{14}$$

replacement of the thiol hydrogen with a methylthio group does not change the pK of the ammonium group (i.e., $K_{Im} = K_I$), the three remaining ionization constants in Scheme I can be determined by using eq 12–14. The four microscopic ionization constants for glutathione are listed in Table II along with the corresponding constants for cysteine, 2-mercaptoethylamine, and *cis*-2-mercaptocyclobutylamine which have been determined by using the PDT method. Examination of the data in Table II reveals that deprotonation of the ammonium group in these thiols increases the pK of the thiol group and vice versa. As might be expected from the proximity of the ammonium and thiol groups, this effect is more pronounced in the vicinal amino thiols than it is in glutathione (compare values of pK_2 and pK_{12}).

Figure 3 illustrates the pronounced effects on the PDT curves caused by the thiol-linked ammonium ionizations in a vicinal amino thiol. The deviation in the pH dependence of $h_u - h_m$ from that expected for a simple thiol can be visualized in terms of a difference in the protonation state of the ammonium group in the unmodified compound and the corresponding methylthio derivative. In the pH range where the deviation of the PDT curve from that expected for a simple thiol is maximal, the ammonium group is adjacent to a thiolate anion in the unmodified compound whereas it is adjacent to an uncharged methylthio group in the derivative. The presence of an adjacent negative charge results in the ammonium group having a higher pK and a higher degree of protonation in the unmodified compound than in the methylthio derivative.

Figure 3 also depicts the pH dependence of the fraction of thiol existing as thiolate anion (f_{s^-}). The points (□) were calculated by using the microscopic pK values listed in Table II to relate f_{s^-} to the pH. As shown by the dashed line in Figure 3, the pH dependence of f_{s^-} is close to that expected for a single ionizable group with a pK of 8.44. This result is obtained because 95% of the thiol group ionizes with a pK of 8.46 prior to ionization of the ammonium group (pK = 9.73). Thus, interactions between the ammonium and thiol groups are not reflected in the pH dependence of f_{s^-} for this amino thiol, as well as others wherein the thiol group is much more acidic than the ammonium group. The ratios of K_2/K_1 obtained from PDT curves for 2-mercaptoethylamine, *cis*-2-mercaptocyclobutylamine, and cysteine (Table II) indicate that the thiol group is 1.4–39 times more acidic than the ammonium group and that the predominant monoprotonated tautomer is the ion pair in these three vicinal amino thiols. The PDT curve for 2-aminothiophenol, on the other hand, indicates that the thiol group in this compound is much less acidic than the adjacent ammonium group. 2-Aminothiophenol exhibits a PDT curve (not shown) which is identical with the titration curve for a single ionizable group with a pK of 6.20 ± 0.03 ($\Gamma/2 = 0.15$, 25 °C). Such a PDT curve suggests that the pK of the ammonium group in the conjugate acid of 2-aminothiophenol is much lower than 6 so that in the pH range studied any cationic ammonium group that is present would be adjacent to an uncharged group in both the unmodified compound and in the methylthio derivative. A pK for the ammonium group substantially below 6 also is consistent with pK values of 4.6 and 4.4 reported for anilinium and toluidinium ions (Zawidzki et al., 1959) and the pK value of 3 determined by direct titration (Danehy & Noel, 1960) for the loss of the

first proton from the conjugate acid of 2-aminothiophenol.

Interactive Ionizations in BSA. PDT curves for the single thiol group in native BSA and urea-denatured BSA are depicted in Figure 4. The PDT curve for the urea-denatured protein could be fit by eq 8, with $n = 1$. This result is consistent with the view that only one ionizable group interacts with the thiol group in the urea-denatured protein. The PDT curve for the native protein is much more complex than that of the denatured protein. The complex nature of the PDT curve cannot be attributed to reaction of MMTS with groups in BSA other than the thiol group since assays of the thiol content of the reacted BSA indicate that in the pH range of the PDT curve more than 90% of the added MMTS reacted with the thiol group. The fit of the PDT curve for the native protein to eq 8 requires three negative terms (Figure 4), indicating that the ionization behavior of three groups is altered in the methylthio derivative. It follows that eq 8 must contain four positive terms in order to account for the ionization behavior of these three groups and that of the thiol group in the unmodified protein. Three positive terms are obtained from the fit of the PDT curve for BSA in the pH range studied. Two of the positive terms are pH dependent, and one term has a value of unity. The value of the missing fourth term must, of course, be zero. The pH-independent terms with values of zero and unity indicate the existence of one group which is unprotonated and one group which is protonated in the pH range of the PDT curve. Since the PDT curve extends from pH 5 to 10, the pK of the unprotonated group must be less than 5 and that of the protonated group must be greater than 10 in unmodified BSA.

It is apparent from the values of the titration constants that striking changes in the ionization behavior of three groups must occur upon modification of the thiol group in BSA. One could argue that the observed alterations in ionization behavior arise because methylthiolation mediates a change in the conformation of BSA which results in altered environments and ionization constants for three groups. On the other hand, the observed altered ionization behavior which accompanies methylthiolation can be attributed entirely to altered electrostatic interactions of the thiolate anion as was done with the simple amino thiols. If this electrostatic model can also be applied to BSA, the thiol group should be the most acidic group of the four ionizable groups in the unmodified protein. Groups more acidic than the thiol group would ionize in the presence of a neutral thiol group and would not be expected to exhibit an altered ionization behavior upon methylthiolation. It follows from this argument that the thiol group in BSA has an abnormally low pK of less than 5 and exists primarily as a thiolate anion at physiological pH values. An abnormally low pK of the thiol group in native BSA might well arise from electrostatic interactions between the thiol group and one or more of the three groups whose ionization behavior is altered by methylthiolation.

Although it is possible to calculate macroscopic ionization constants from the observed titration constants, more information is required before we can establish how the values of the observed parameters are related to the microscopic ionization constants of individual groups in BSA. If one assumes for purposes of illustration that the ionization behavior of the three groups is affected only by the protonation state of the thiol but that they otherwise ionize independently of one another, then the titration constants would be equivalent to microscopic ionization constants for individual groups. This assumption together with the electrostatic model (which requires a decrease in each microscopic pK value upon methylthiolation) leads to the conclusion that the pK values of two groups shift from 7.9 to 4.4 and 6.7 and that the pK of the third group decreases from a value greater than 10 to 9.75 upon methylthiolation of BSA. Additional studies are needed to characterize the unusual interactions and the physiological role (if any) of the single thiol group in BSA.

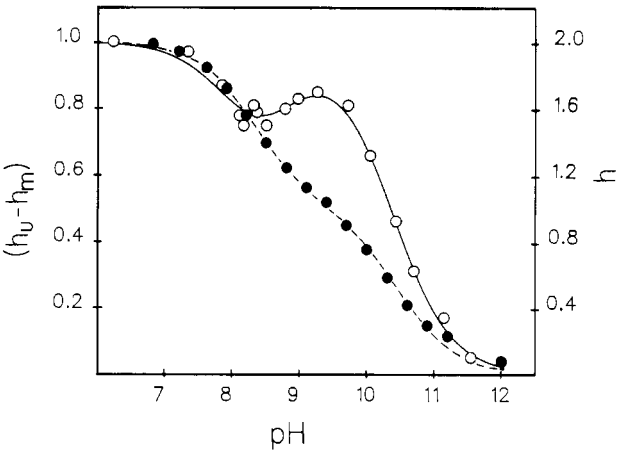


FIGURE 5: PDT and direct potentiometric titration curves for cysteine in 15 mM EDTA at $\Gamma/2 = 1.0$, 25 °C. Open circles (left axis) are PDT data obtained with 1 mM cysteine. The solid line is the least-squares fit to eq 8 ($pG_1 = 8.20$, $pG_2 = 10.37$, and $pG_{Im} = 8.57$). The close circles (right axis) are direct potentiometric titration data obtained with 0.02 M cysteine. Values of h were calculated from the amount of cysteine initially present and the amount of titrant (1 M KOH) required to reach each pH value in the presence of absence of cysteine. The dashed line is the least-squares fit to the equation ($pG_1 = 8.31$ and $pG_2 = 10.32$) $h = [1/(1 + 10^{pH-pG_1})] + [1/(1 + 10^{pH-pG_2})]$.

Table III: Macroscopic pK Values for Cysteine and Two Cysteine Derivatives (25 °C, $\Gamma/2 = 1.0$)

compound	method	pK_I or pK_{Im}	pK_{II}
cysteine	PDT	8.20	10.37
	PT[a]	8.34	10.47
	PT[b]	8.38	10.37
	ST[c]	8.21	10.35
	ST[b]	8.38	10.38
S-(methylthio)cysteine	PDT	8.57	
	PT[d]	8.65	
S-methylcysteine	PT[e]	8.83	
	PT[b]	8.88	

[a] By potentiometric titration as described in the legend to Figure 5. [b] From potentiometric (PT) and spectrophotometric (ST) titrations reported by Kallen (1971a). [c] From spectrophotometric titrations carried out in this work by using the method of Benesch & Benesch (1955). [d] By potentiometric titration of 0.02 M *S*-(methylthio)cysteine which was prepared by addition of an equivalent amount of MMTS to a solution of cysteine. [e] By potentiometric titration of 0.02 M *S*-methylcysteine.

Discussion

Cysteine, an amino thiol whose ionization behavior is well characterized,[5] was used in studies designed to test further the agreement between the PDT method and other methods for determining the ionization behavior of thiols. A PDT curve for cysteine appears in Figure 5, and values of pK_I, pK_{II}, and pK_{Im} obtained from the best fit of data in Figure 5 to eq 8–11 are listed in Table III along with values of pK_I and pK_{II} ob-

[5] A comprehensive review of investigations of the ammonium-linked thiol ionization in cysteine appears in Kallen's (1971a) in-depth study of the ionization of this amino acid.

Table IV: Comparison of Microscopic pK Values for Cysteine (25 °C, $\Gamma/2 = 1.0$)

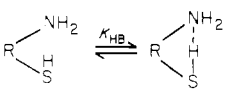
method	pK_1	pK_2	K_2/K_1	pK_{12}	pK_{21}
PDT[a]	8.57	8.44	1.4	10.00	10.14
ST[b]	8.72	8.37	2.2	9.84	10.19
ST[c]	8.90	8.54	2.3	9.85	10.22
PT[d]	8.65	8.63	1.0	9.83	10.18
PT[e]	8.83	8.51	2.1	9.98	10.30
PT[f]	8.88	8.55	2.1	9.87	10.20

[a] The PDT curve in Figure 6 was fit to eq 8, and K_1 was set equal to K_{Im} for *S*-(methylthio)cysteine. [b] The spectrophotometric titration curve was fit as described by Benesch & Benesch (1955), wherein the absorptivity of the thiolate anion at its λ_{max} was assumed to be equal in species B and D of Scheme I. [c] Reported by Kallen (1971a). [d] Separate potentiometric titrations were carried out to determine the value of K_I and K_{II} for cysteine and K_{Im} for *S*-(methylthio)cysteine. K_1 was set equal to K_{Im}, and the relationships in the text were used to calculate the other microscopic constants. [e] Same as d only K_1 was set equal to K_{Im} for *S*-methylcysteine. [f] Determined by Kallen (1971) as described in footnote *e*.

tained from the direct potentiometric titration curve of cysteine depicted in Figure 5. A value for pK_{Im} determined by direct potentiometric titration of *S*-(methylthio)cysteine is also listed in Table III. As expected, the pK values determined by the PDT method are in reasonable agreement with the corresponding values determined by direct titration in this work as well as with the titrimetrically determined values of pK_I and pK_{II} (Table III) previously reported by Kallen (1971a).

Although no assumptions are involved in obtaining pK_I, pK_{II}, and pK_{Im} by PDT, estimation of the microscopic ionization constants of cysteine involved application of the assumption that the pK of the ammonium group in *S*-(methylthio)cysteine is equal to the pK of the ammonium group in cysteine when the thiol group is protonated. This assumption is analogous to that used in published studies wherein Wegscheider's principle of equivalence of a methyl group and a proton is applied. In this method, the pK of the ammonium group in *S*-methylcysteine is used as an estimate of pK_1 (Edsall & Wyman, 1958; Grafius & Neilands, 1955; Kallen, 1971a). The agreement between microscopic pK values determined by the two methods will be dependent of course upon the correspondence of the pK values for *S*-(methylthio)cysteine and *S*-methylcysteine. As shown in Table III, the pK's of the ammonium groups in these two compounds differ by 0.2–0.3 pK unit. This difference results in small differences in the microscopic pK values of cysteine (see Table IV).

A question which has been addressed by previous workers (see Edsall, 1965) is the appropriateness of using Wegscheider's principle. For example, one might argue that hydrogen bonding in the amino thiol as depicted in eq 15 is



(15)

important. Such hydrogen bonding might occur without formation of any ion pair. The PDT curve and the different ionization behavior of the ammonium group in cysteine and *S*-methylcysteine would then be explained in terms of eq 16.

$$K_1 = K_{Im}(1 + K_{HB}) \quad (16)$$

The existence of a thiolate ammonium ion pair, however, has been established by examining the ionization behavior of cysteine spectroscopically. In one approach for measuring thiolate anion levels developed by Benesch & Benesch (1955), the pH dependence of the UV absorbance due to the thiolate anion is determined. A problem associated with this method is that ionization of the ammonium group perturbs the UV spectrum of the thiolate anion. Benesch and Benesch circumvented this problem in their analysis by assuming that the ammonium ionization shifted the absorption maximum of the thiolate anion without changing the molar absorptivity of the thiolate anion at the wavelength of maximal absorbance.[6] Values for the ionization constants for cysteine determined by using the method of Benesch and Benesch at $\Gamma/2 = 1.0$ (25 °C) appear in Table III and IV. Examination of the data in Table IV reveals that this spectrophotometric titration procedure and the use of pK_{Im} (for *S*-methylcysteine) as an estimate for pK_1 result in similar values of 2.1–2.3 for the equilibrium constant for ion-pair formation (K_2/K_1).

These values of K_2/K_1 for cysteine correspond closely to the value of 2.2 determined under similar conditions by Elson & Edsall (1962) who used the intensity of S–H stretching band in the Raman spectrum to monitor ionization of the thiol group. The observation that the UV spectrophotometric titration (which monitors the thiolate anion) and the Raman spectrophotometric titration (which monitors the thiol group) yield similar values for K_2/K_1 close to those estimated by using the Wegscheider principle supports the view that, at least in the case of cysteine, hydrogen bonding in the uncharged amino thiol is not important. [For a more detailed discussion of this point see Edsall (1965).] The PDT curve for 2-aminothiophenol provides additional evidence that low molecular weight amino thiols do not undergo significant intramolecular hydrogen bonding (in water) of the type shown in eq 15. The PDT curve for 2-aminothiophenol is that expected for a single ionizable group whereas deviations in the PDT curve should have been observed if significant intramolecular hydrogen bonding had occurred.[7] In the light of the low electronegativity of sulfur and the relative instability of hydrogen bonds involving thiol groups (Marthur et al., 1963; Edsall, 1965), it would be surprising if hydrogen bonding of the type depicted in eq 15 were found to be important in proteins.

The results presented in this work indicate that the PDT method should be useful for studying the ionization behavior of thiol groups in proteins. At the very least, the PDT method reveals the presence in a thiol-containing protein of groups whose ionization behavior is altered upon blocking ionization of the thiol group. X-ray crystallographic analysis of the ummodified protein and its methylthio derivative should allow one to determine whether a conformational change or altered electrostatic interactions between the thiolate anion and its neighboring groups are responsible for the ionizations reflected in the PDT curves. Interpretation of the PDT curves in terms of an electrostatic model yields information about possible interactive ionizations of the thiol group. The 0.2–0.3 unit difference between the pK's of *S*-(methylthio)cysteine and *S*-methylcysteine suggests that application of the PDT method will result in small underestimates of the extent of the ionic

[6] Experimental evidence for the validity of this assumption has been presented by Kallen (1971a) who showed that at the absorption maximum the value of the molar absorptivity of a thiolate anion is essentially independent of the charge of neighboring groups.

[7] A significant hydrogen bonding interaction between the thiol group and the uncharged amino group in 2-aminothiophenol would be expected to stabilize the uncharged amine form relative to the ammonium ion form. Upon methylthiolation, the putative hydrogen bonding interaction would be lost, and the pK of the ammonium group would be expected to increase. This increase in pK should be reflected in the PDT curve in the pH region where the amino group titrates. The failure of the PDT curve to reflect any change in the state of protonation of the amino group upon modification of the thiol group argues against the existence of significant hydrogen bonding between the uncharged amino and thiol groups.

interactions which effect the thiol group.[8] In light of the standard deviations of 0.05–0.1 associated with pK values obtained from PDT curves of proteins (Lewis et al., 1976), this 0.2–0.3 pK unit discrepancy is tolerable. It would be desirable of course to be able to use UV absorbance spectroscopy and Raman spectroscopy in addition to the PDT method (as was done with cysteine) in order to establish the ionization behavior of thiol groups in proteins since the application of each of these methods is based upon a different assumption. Unfortunately, the high background absorbance of proteins near the absorption maximun for a thiolate anion and the insensitivity of Raman spectroscopy severely limit the usefulness of these spectroscopic techniques in studies of the ionizations of thiol groups in proteins.

It should also be noted that the PDT method can be used to measure the extent of strong ionic interactions that cannot be detected spectrophotometrically. For example, the values of K_2/K_1 for 2-mercaptoethylamine and *cis*-2-mercaptocyclobutylamine listed in Table II cannot be obtained from spectrophotometric titrations since the pH dependence of f_{s^-} as measured in a spectrophotometric titration would reflect only one ionization (K_2) for these compounds, wherein essentially all of the thiol group loses a proton while it is adjacent to a cationic ammonium group (see Figure 3).

In the past, ionizations of thiol groups in proteins have been studied almost exclusively by using kinetic methods, wherein the protonation state of the thiol group is estimated from its nucleophilic reactivity. As already pointed out, such estimates of the protonation state of the thiol group are uncertain since the nucleophilic reactivity of the thiolate anion may be dependent upon the protonation state of other ionizable groups in the protein. Recently published pH dependencies of the nucleophilic reactivity of the thiol group in BSA toward low molecular weight disulfides (Wilson et al., 1980) indicate that more than one ionizable group in BSA affects the ionization behavior of the thiol group or the nucleophilic reactivity of the thiolate anion (or both). These pH dependencies are consistent with the results presented here, indicating that the thiol group in BSA interacts with three other ionizable groups. Although the rate of reaction of the thiol group in BSA decreases as the pH is decreased from 9 to 7 (Wilson et al., 1980), the ionization behavior of the thiol group in BSA (as determined by the PDT method) indicates that the decreased rate of reaction at pH 7 relative to that at pH 9 may *not* reflect protonation of the thiolate anion, as one might expect from the behavior of simple alkyl thiols. Instead, the decreased rate of reaction may well reflect a decreased reactivity of the thiolate anion caused by protonation of other groups in the protein. In light of these findings, it is important to reinvestigate the ionization behavior of other protein thiol groups whose ionization behavior has been deduced entirely from kinetic studies. It also would be interesting to use the PDT method in conjunction with detailed kinetic studies in order to characterize more completely the ionic interactions affecting the nucleophilic reactivity of thiolate anions in proteins.

[8] When the pK of *S*-(methylthio)cysteine is used instead of that of *S*-methylcysteine as an estimate of pK_1, the value of the equilibrium constant obtained for ion pair formation (K_2/K_1) from the amino thiol is between 1.5- and 2.1-fold lower. The difference between the pK's of the two compounds may be due in part to the greater electron-withdrawing inductive effect of the methylthio group relative to that of the methyl group (Kallen, 1971b).

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