eluted with the same buffer at 1 ml/min for 40 min before switching to 0.5 *M* sodium acetate at the same pH. The eluate, mixed with ninhydrin (0.5 ml/min), is passed through a Teflon reaction coil heated in a boiling water bath (transit time 15 min) before measuring absorbance at 570 nm with a Dupont 410 photometer.

The concentrations of cysteic acid and glutathionesulfonic acid, the two principal sulfonic acids present in the extract, are calculated from standard curves obtained with the authentic compounds.[14] In this system, cysteic acid and glutathionesulfonic acid elute at 26 and 63 min, respectively, and both are well resolved from contaminating ninhydrin-positive peptides liberated by performic acid oxidation. This method has been used to measure the concentration of protein-mixed disulfides in normal and oxidatively stressed lens tissue from rats and monkeys.[14] The procedure is quite reproducible and gives values for protein-bound GSH and cysteine that are considerably less than that obtained by other methods.

Reductive Method

Principle. Thiols linked to proteins by disulfide bonds can be released from proteins by reduction [Eq. (4)]. The acid-soluble thiols are readily separated by precipitating the protein with trichloroacetic or perchloric acid.

$$\text{Protein—SSR} \rightarrow \text{Protein—SH} + \text{RSH} \qquad (4)$$

While most procedures[2-4] employ either sodium borohydride or dithiothreitol (DTT) to reduce disulfides, neither reductant alone or in combination has proven satisfactory. The large amount of DTT required for complete reduction frequently interferes with subsequent assays for acid-soluble thiols, and reduction of disulfides in crude tissue preparations by borohydride is rarely quantitative.[15] We have found that reduction by a system consisting of NADPH and glutathione reductase in the presence of a small amount of DTT gives the most consistent results.

Reagents

A solution containing 25 m*M* sodium pyrophosphate at pH 8.4, 5 m*M* EDTA, 1 m*M* DTT, 0.2 m*M* $NADP^+$, 2.5 m*M* glucose 6-phosphate, 2 μg/ml glucose-6-phosphate dehydrogenase (*Leuconostoc mesenteroides;* Sigma Type XXIII), and 4 μg/ml glutathione reductase (yeast; Sigma Type III) is prepared daily from stock solutions and stored on ice until needed.

Procedure. The dried protein powder is resuspended in 1.5 ml of the NADPH–glutathione reductase solution and incubated at 37° for 10 min with occasional mixing. After incubation any protein in solution is precipitated by adding 0.15 ml 3 *M* perchloric acid. Specific acid-soluble thiols liberated by reduction are then measured by procedures described in detail in other chapters of this volume. GSH in the extract liberated from protein of perfused rat liver, measured by the sensitive enzymatic recycling technique,[16] gives values that vary from 12 to 17 nmol/g (wet weight) liver.

[14] M. F. Lou, R. McKeller, and O. Chyan, *Exp. Eye Res.* **42,** 607 (1986).

[15] The reasons for lack of quantitative reduction are not immediately apparent; however, it is not related to lack of dispersion of denatured proteins. The yield of GSH from liver proteins by borohydride reduction was not improved by dispersing the pellet in urea or detergents, and was consistently less than that obtained with the DTT–glutathione reductase-dependent system.

[16] F. Tietze, *Anal. Biochem.* **27,** 502 (1969).

[21] Measurement of Thiol–Disulfide Interchange Reactions and Thiol pK_a Values

By JANETTE HOUK, RAJEEVA SINGH, and GEORGE M. WHITESIDES

Thiol–disulfide interchange (SH/S_2 interchange) reactions involving proteins are important in a number of biochemical processes including formation and cleavage of structural cystines,[1] control of enzyme activities by reversible redox reactions of enzyme thiols and disulfides,[2,3] and redox processes requiring thiols.[4] The reaction is mechanistically simple: it involves initial ionization of thiol to thiolate anion, followed by nucleophilic attack of thiolate anion on the sulfur–sulfur bond of the disulfide [Eq. (1)]:

$$\text{RSH} \rightleftharpoons \text{RS}^- + \text{H}^+ \qquad (1a)$$

$$\text{RS}^- + \text{ESSE} \rightleftharpoons \text{RSSE} + \text{ES}^- \qquad (1b)$$

$$\text{ES}^- + \text{H}^+ \rightleftharpoons \text{ESH} \qquad (1c)$$

Equation (1) makes it evident that three types of parameters must be determined to characterize fully a SH/S_2 interchange reaction: (1) the rates at which the displacement steps occur; (2) the values of pK_a of the

[1] T. Y. Liu, *in* "The Proteins" (H. Neurath and R. L. Hill, eds.), 3rd ed., Vol. 3, p. 239. Academic Press, New York, 1977.

[2] D. M. Ziegler, *Annu. Rev. Biochem.* **54,** 305 (1985).

[3] H. F. Gilbert, this series, Vol. 107, p. 330.

[4] P. C. Jocelyn, "Biochemistry of SH Group." Academic Press, New York, 1972.

participating thiols; and (3) the positions of the equilibria between the thiol (thiolate) and disulfide species. Here we discuss the characteristics of each of these parameters and describe methods of determining them. We place particular emphasis on methods that are useful for biologically relevant thiols and cysteine groups in proteins.

Rates of Thiol–Disulfide Interchange Reactions

Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid)][5,6] (see this volume [11]) has been widely used in studies[7–9] of rates of SH/S_2 interchange reactions because of several reasons. (1) It is water soluble; (2) forward rates are seldom complicated by back reactions; (3) the reduction of Ellman's reagent is easily followed spectrophotometrically; and (4) Ellman's reagent has been widely used for the determination of sulfhydryl groups, and information concerning rates of its reduction by thiols is useful in these applications.

SH/S_2 interchange between most aliphatic thiols and Ellman's reagent proceeds under thermodynamic control at pH 7 to complete reduction of the Ellman's reagent. Thus, Ellman's reagent *cannot* normally be used to explore the influence of the structure of the reducing thiol on SH/S_2 interchange equilibria. Reduction of typical cystine moieties by aliphatic thiols is thermodynamically much less favored than is reduction of Ellman's reagent. Thus, Ellman's reagent, though convenient to study, is a poor model for protein cystine groups.

Glutathione disulfide [GSSG, $(\gamma$-glutamylcysteinylglycine$)_2$] is a better cystine-containing peptide for use as a model in the study of rates of SH/S_2 interchange.[10] The rate of release of glutathione (GSH) on reduction of GSSG by thiols can be determined enzymatically. GSH can be converted to *S*-lactoylglutathione (GS-lac) by reaction with methylglyoxal in the presence of glyoxalase I (lactoylglutathione lyase), and the concentration of GS-lac monitored spectrophotometrically at 240 nm.[10,11] Equations (2)–(4) list reactions occurring in the assay for reduction of GSSG by a monothiol (RSH). The conditions of reaction can be adjusted such that

[5] G. L. Ellman, *Arch. Biochem. Biophys.* **82,** 70 (1959).
[6] P. W. Riddles, R. L. Blakeley, and B. Zerner, this series, Vol. 91, p. 49.
[7] G. M. Whitesides, J. E. Lilburn, and R. P. Szajewski, *J. Org. Chem.* **42,** 332 (1977).
[8] G. M. Whitesides, J. Houk, and M. A. K. Patterson, *J. Org. Chem.* **48,** 112 (1983).
[9] J. M. Wilson, D. Wu, R. M. DeGrood, and D. J. Hupe, *J. Am. Chem. Soc.* **102,** 359 (1980).
[10] R. P. Szajewski and G. M. Whitesides, *J. Am. Chem. Soc.* **102,** 2011 (1980).
[11] D. L. Vander Jagt, E. Daub, J. A. Krohn, and L.-P. B. Han, *Biochemistry* **14,** 3669 (1975).

the rate of conversion of GSH to GS-lac is fast relative to the rate of formation of GSH.

$$RS^- + GSSG \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RSSG + GS^- \quad (2)$$

$$RS^- + RSSG \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} RSSR + GS^- \quad (3)$$

$$GSH + CH_3COCHO \rightleftharpoons GS{-}CH(OH)COCH_3 \xrightarrow[\text{glyoxalase I}]{k_3} GS{-}COCH(OH)CH_3 \text{(GS-lac)} \quad (4)$$

Using suitable concentrations of methylglyoxal and glyoxalase I, and assuming steady-state concentration for GSH, the initial rate of formation of GS-lac is given by Eqs. (5) and (6).

$$\frac{d(\text{GS-lac})}{dt} = k_1(RS^-)(GSSG) = k_1^{obsd}[(RS^-) + (RSH)](GSSG) \quad (5)$$

where parentheses denote molar concentrations and

$$k_1 = k_1^{obsd}(1 + 10^{pK_a^{RSH} - pH}) \quad (6)$$

Mass balance and integration of Eq. (5) gives Eq. (7),

$$k_1^{obsd}t = \frac{1}{(S)_0 - (GSSG)_0} \ln\left[\frac{(GSSG)_0}{(S)_0} \times \frac{(S)_0 - (\text{GS-lac})/n}{(GSSG)_0 - (\text{GS-lac})/n}\right] \quad (7)$$

In Eq. (7), for monothiols, $(S)_0 = [(RS^-) + (RSH)]$, $n = 1$; for dithiols, $(S)_0 = [(SRS^-) + (HSRS^-) + (HSRSH)]$, $n = 2$. The following assay conditions were found suitable: 5 m*M* thiol, 0.77 m*M* methylglyoxal, 0.35 m*M* GSSG, 2.4 units/ml glyoxalase I.[10] This procedure is not completely general—aromatic thiols absorb at 240 nm, the wavelength used to monitor GS-lac; aminothiols (cysteine, *N*,*N*-diethylcysteamine) react rapidly with methylglyoxal and form species that absorb at 240 nm; 2,3-dimercaptopropanol inhibits glyoxalase I.[10]

Typical rate constants (k_1, M^{-1} min^{-1}) for reduction of GSSG by thiols are as follows: 2-mercaptoethanol ($k_1^{obsd} = 8.7$, $k_1 = 3.4 \times 10^3$); dithiothreitol (DTT) ($k_1^{obsd} = 14.1$, $k_1 = 2.2 \times 10^4$) (in 66 m*M* phosphate at pH 7.0, 30°, under argon).[10] Typical rate constants (k, M^{-1} min^{-1}) for reduction of Ellman's reagent by thiols under similar conditions are the following: 2-mercaptoethanol ($k_1^{obsd} = 3.7 \times 10^4$, $k_1 = 1.2 \times 10^7$, $k_2^{obsd} = 1.7 \times 10^3$, $k_2 = 5.2 \times 10^5$); dithiothreitol ($k_1^{obsd} = 1.5 \times 10^5$, $k_1 = 1.7 \times 10^7$, k_2^{obsd} and k_2 could not be determined).[7] Since the fraction of thiol present in the reactive thiolate form in solution depends upon the thiol pK_a and solution pH, k^{obsd} provides a more direct measure of reactivity of the thiol toward disulfide than does k.

Hupe *et al.* have described a spectrophotometric method for reaction of disulfides with buried thiol groups in bovine serum albumin.[9]

Brønsted Plots for Rates of Reduction of Disulfides with Thiols

Rates of SH/S_2 interchange follow a Brønsted correlation. A Brønsted plot (log k_1 versus pK_a of thiols) for reduction of Ellman's reagent with thiols gave $\beta_{nuc} = 0.36$.[7] This plot included data for both aryl and alkyl thiols. A similar study by Hupe *et al.* showed separate correlation lines for alkyl and aryl thiols, with $\beta_{nuc}^{alkyl} = 0.49$ and $\beta_{nuc}^{aryl} = 0.48$.[12] The Brønsted coefficients for reduction of GSSG by thiols were $\beta_{nuc} = 0.50$,[10] ($\beta_c + \beta_{lg}$) $= -1.0$[10,13] [nuc = nucleophilic; c = central; lg = leaving group (see below)]. Other Brønsted correlations have been determined with 4,4′-dipyridyl disulfide ($\beta_{nuc} = 0.34$)[14] and with 2,2′-dipyridyl disulfide ($\beta_{nuc} = 0.23$).[15] Mechanistically, reductions of Ellman's reagent and glutathione disulfide by thiols appear to be closely related reactions. Both show similar values of Brønsted coefficients. Neither shows any curvature in the Brønsted plot (a kinetic feature suggesting a change in the mechanism).

Assuming that the rate of SH/S_2 interchange [Eq. (8)] is described by an equation of the form of Eq. (9), experimental evaluation of coefficients gives Eq. (10). Equation (10) was found to be a useful kinetic model for SH/S_2 interchange.[10]

$$R^{nuc}S^- + R^cSSR^{lg} \rightarrow R^{nuc}SSR^c + SR^{lg} \quad (8)$$

$$\log k = C + \beta_{nuc}pK_a^{nuc} + \beta_c pK_a^c + \beta_{lg}pK_a^{lg} \quad (9)$$

$$\log k = 7.0 + (0.50)pK_a^{nuc} - (0.27)pK_a^c - (0.73)pK_a^{lg} \quad (10)$$

Determination of Thiol pK_a Values

Small Molecules

Values of pK_a for structurally simple, low molecular mass thiols are easily determined by conventional acid/base titration. This method can be used satisfactorily to determine values of pK_a for many thiols and dithiols.[7,8] The procedure used for low molecular mass thiols follows.

Solution pH values were measured with a Radiometer PH M82 standard pH meter equipped with a REA 160 titrigraph module, a REA 260 derivation unit, and a 25-ml thermostated titration vessel. All manipulations of thiol-containing solutions were carried out under argon. The pH meter was standardized against pH 7.00 and pH 10.00 standard buffer solutions at 25°. The water used to prepare all solutions was deionized and doubly distilled, once from glass. Degassed distilled water (10 ml) was allowed to equilibrate under argon in the thermostated titration vessel.

Mercaptoethanol (70 μl), 1,3-dithiopropan-2-ol (50 μl), or dithiothreitol (77.2 mg) was added to the titration vessel yielding a 0.1 *N* solution of the thiol. Thiols were titrated against 0.151 *M* carbonate-free potassium hydroxide.[16] All titrations were in triplicate. Complete titration curves (pH versus volume of titrant added) were obtained by using the stepped curve mode on the autotitrimeter. The KOH solution (about 7 ml) required to neutralize the thiol was added in 20 equal aliquots, and the pH of the solution was measured 1 min after each addition. The titrant was added to the thiol solution slowly enough to maintain the desired temperature throughout the titration.

For monothiols, e.g., mercaptoethanol, the pH of the solution at half-equivalence point of the titration was taken to be the pK_a (for mercaptoethanol, $pK_a = 9.5$).[7] For dithiols, e.g., 1,3-dithiopropan-2-ol or dithiothreitol, assuming *C* to be the initial concentration of the dithiol and *B* the amount of base added, the following expressions can be derived for K_1 and K_2 (the first and second acid dissociation constants of the dithiol),

$$K_1 = Z/(K_2Y - X)$$
$$K_2 = (Z + K_1X)/K_1Y$$

where $X = (B + [H^+] - C)[H^+]$; $Y = 2C - B - [H^+]$; and $Z = [H^+]^2(B + [H^+])$.

Two points on the titration curve equidistant from the midpoint of the titration curve were chosen and K_1 and K_2 were calculated as follows,

$$K_1 = (Y_1Z_2 - Y_2Z_1)/(X_1Y_2 - X_2Y_1)$$
$$K_2 = (X_1Z_2 - X_2Z_1)/(Y_1Z_2 - Y_2Z_1)$$

Using a computer program, several values of K_1 and K_2 were calculated for several pairs of points on the titration curve.[7,8,16,17] Values of pK_{a1} and pK_{a2} were calculated from the average values of K_1 and K_2. Values of pK_a obtained by this procedure (1,3-dithiopropan-2-ol, 9.2, 10.7; DTT, 9.3, 10.3) agreed with literature values.[7,18]

[12] J. M. Wilson, R. J. Bayer, and D. J. Hupe, *J. Am. Chem. Soc.* **99**, 7922 (1977).
[13] T. E. Creighton, *J. Mol. Biol.* **96**, 767 (1975).
[14] C. E. Grimshaw, R. L. Whistler, and W. W. Cleland, *J. Am. Chem. Soc.* **101**, 1521 (1979).
[15] M. Shipton and K. Brocklehurst, *Biochem. J.* **171**, 385 (1978).
[16] A. Albert and E. P. Serjeant, "Ionization Constants of Acids and Bases," p. 52. Methuen, London, 1962; H. T. S. Britton, "Hydrogen Ions," 4th ed., pp. 217ff. Chapman & Hall, London, 1954.
[17] Z. Shaked, R. P. Szajewski, and G. M. Whitesides, *Biochemistry* **19**, 4156 (1980).
[18] J. J. Christensen, L. D. Hansen, and R. M. Izatt, "Handbook of Proton Ionization Heats." Wiley, New York, 1976; J. P. Danehy and C. J. Noel, *J. Am. Chem. Soc.* **82**, 2511 (1960); E. L. Loechler and T. C. Hollocher, *ibid.* **102**, 7312 (1980); see also H. Fukada and K. Takahashi, *J. Biochem. (Tokyo)* **87**, 1105 (1980).

Values of pK_a for thiols containing groups with proton affinities similar to that of SH can be determined spectrophotometrically by measuring the UV absorption of thiolate anion as a function of pH.[19] This method was first described by Benesch and Benesch[19] for the determination of values of pK_a of sulfur and nitrogen functions in aminothiols. Ionization constants of cysteine thiols in small peptides can be determined using this method, providing the peptide does not contain tyrosines (a group that resembles cysteine in that it shows a pH-dependent absorption at 245 nm).[20]

Proteins

The determination of values of pK_a for thiol groups in proteins is less straightforward. Several investigators have developed indirect methods for studying the ionization behavior of protein SH groups. Shafer has estimated the manner in which ionization of SH groups in low molecular mass thiols and bovine serum albumin affects the behavior of other ionizable groups in the molecule.[21] Parente *et al.* have described a kinetic method for estimating values of thiol pK_a.[22] They studied the reaction kinetics of Ellman's reagent and 2,2′-dithiopyridine with low molecular mass thiols, model peptides, and monomeric bovine seminal ribonuclease (Cys-31 and Cys-32) as a function of pH. Plots of the apparent second-order rate constant for the SH group versus pH resemble titration curves. The pH at the inflection point in these curves corresponds to the thiol pK_a value. They obtain good results with model thiols and peptides. The titration curve of the monomeric enzyme exhibits, however, a discontinuity that is attributed to a pH-dependent change in protein tertiary structure.

A kinetic method based on Eq. (9) permits estimation of the pK_a values of thiol moieties in proteins. The method has been tested with papain (Cys-25), adenylate kinase, DNase, and lysozyme.[17] The rates of reduction of the disulfide groups of these several proteins and protein derivatives by low molecular mass thiols appear to follow normal Brønsted relationship(s). This observation provides a method for determining the value of the pK_a of a protein cysteine thiol group indirectly by measuring the rates of SH/S_2 interchange involving this thiol. For example, DNase and lysozyme contain two and four cystines, respectively. Reduction of the disulfide groups leads to complete loss of activity, and rates of reduction can be followed by observing the loss in activity. Papain has an active site cysteine moiety. The *S*-thioalkyl derivative of papain (papain-SSR) has no enzymatic activity; reduction of papain-SSR leads to restoration of activity as papain-SH is regenerated. Adenylate kinase (AdK) contains two cysteine SH groups. The bisthiomethyl derivative of the kinase [$AdK(SSCH_3)_2$] has 70% of the activity of the native enzyme. Reduction of the active site cysteine ($SSCH_3$) can be followed by restoration of enzymatic activity.

[19] G. H. Snyder, M. J. Cennerazzo, A. J. Karalis, and D. Field, *Biochemistry* **20**, 6509 (1981); G. H. Snyder, *J. Biol. Chem.* **259**, 7468 (1984); P. H. Connett and K. E. Wetterhahn, *J. Am. Chem. Soc.* **107**, 4282 (1985); R. E. Benesch and R. Benesch, *ibid.* **77**, 5877 (1955).

[20] G. H. Snyder, M. K. Reddy, M. J. Cennerazzo, and D. Field, *Biochim. Biophys. Acta* **749**, 219 (1983).

[21] S. D. Lewis, D. C. Misra, and J. A. Shafer, *Biochemistry* **19**, 6129 (1980).

[22] A. Parente, B. Merrifield, G. Geraci, and G. D'Alessio, *Biochemistry* **24**, 1098 (1985).

Activation of Papain and Adenylate Kinase. Commercial papain and adenylate kinase have little or no activity as a result of oxidation of essential thiol groups (in major part to disulfides). Both enzymes were activated before modification with $CH_3SSO_2CH_3$, by incubation with reducing solution as follows: for 3 mg/ml papain (<0.1 unit/mg), a solution containing 50 m*M* L-cysteine, 60 m*M* 2-mercaptoethanol, and 10 m*M* EDTA was used in a 1-hr incubation at 30°; for 1 mg/ml adenylate kinase (340 units/mg), 80 m*M* DTT was used in a 1-hr incubation at 30°. The enzyme was separated from excess reducing agent by placing in a stirred ultrafiltration cell (Amicon Diaflo, PM10 membrane) and passing 2 liters (papain) or 0.6 liter (AdK) of degassed 50 m*M* phosphate at pH 7.0 (0.1 *M* in KCl) through the cell at 4°. The resulting enzymes typically had specific activities of 3.8 units/mg (for hydrolysis of benzoyl-DL-arginine *p*-nitroanilide) for papain and 400 units/mg for AdK.

Methyl Methanethiolsulfonate. Methyl disulfide (14.1 g, 160 mmol) was dissolved in 60 ml of glacial acetic acid in a 250-ml three-necked flask fitted with a reflux condenser and a 125-ml dropping funnel. The flask was cooled to 0°, and H_2O_2 (34 g of 30% solution; 10.2 g = 0.30 mol of H_2O_2) was added slowly while maintaining the temperature below 5°. *Caution:* Mixtures of H_2O_2 and organic solvents are potentially hazardous and should be manipulated behind a shield. The solution was stirred for 30 min at room temperature, and the flask was slowly warmed to 50° for about 1 hr. After destroying the excess of peroxide by additional heating at 50° for 1 hr and testing for peroxide with starch–iodide paper, the glacial acetic acid was removed under reduced pressure. The residual oil was treated with 50 ml of saturated $NaHCO_3$ solution to neutralize residual acid. The oil was separated and diluted with chloroform. After drying over anhydrous $MgSO_4$, the chloroform was removed and yellow oil was distilled. A colorless liquid (7.2 g, 57 mmol) was obtained, bp 60–70° (0.3 torr) [lit. 54° (0.04 torr)].[23] The yield was 36%. The ^1H-NMR spectrum ($CDCl_3$) showed peaks at δ 2.65 (*s*, 3 H) and 3.22 (*s*, 3 H).

[23] H. E. Wijers, H. Boelens, A. van der Gen, and L. Brandsma, *Recl. Trav. Chim. Pays-Bas* **88**, 519 (1969).

Caution: Alkyl methanethiolsulfonates should be synthesized and handled with care in the hood! They all have a very unpleasant odor and some cause dizziness and headache.

Preparation of Papain(SSR) and AdK(SSR)$_2$. The following papain derivatives of the general structure papain(SSR) were prepared: papain($SSCH_3$), papain($SSCH_2CH_2CH_3$), papain($SSCH_2CH_2OH$), papain($SSCH_2CF_3$), and papain($SSCH_2CF_2CF_3$). The preparation of papain($SSCH_3$) is presented in detail; the others were prepared by analogous procedures. Treatment of all the papain(SSR) and AdK(SSR)$_2$ derivatives with a small excess of typical thiol agents restored more than 98% of the native enzyme. A degassed solution (100 ml; 50 m*M* phosphate at pH 7.0, 100 μM in EDTA and 0.1 *M* in KCl) containing 0.3 g (13 μmol) of completely activated papain was treated with 35.2 mg (280 μmol, a 20× excess) of $CH_3SSO_2CH_3$ under argon. The decrease in activity was monitored: after 1–2 hr at 30° no residual enzymatic activity (<0.1%) was observed. Excess $CH_3SSO_2CH_3$ was separated by placing the reaction mixture in an ultrafiltration cell (Amicon Diaflow, PM10 membrane), separating the protein from the rest of the solution, and passing 2 liters of 0.1 *M* phosphate at pH 7.0 (100 μM in EDTA, 0.1 *M* in KCl) through the cell at 4°.

An analogous procedure was used for adenylate kinase, starting with treatment of 0.1 g (4.8 μmol, 400 units/mg) of protein in 100 ml of degassed solution with 12 mg (20× molar excess) of $CH_3SSO_2CH_3$. This mixture reached a constant activity corresponding to 70% (±3%) of the activity of the native enzyme after a 30-min incubation at 30°. Excess $CH_3SSO_2CH_3$ was removed as described for papain($SSCH_3$).

Rates of Reduction of DNase, Lysozyme, Papain-SSR, and AdK($SSCH_3$)$_2$. Reduction of enzymes by several low molecular mass thiols were measured by the loss or recovery of the native enzymatic activity. These rates follow a Brønsted relationship with slopes (β_{nuc}) ranging from 0.36 (DNase) to 0.65 [AdK($SSCH_3$)$_2$].

A representative procedure is that for DNase. DNase (2000 Kunitz units, ~1 mg of electrophoretically purified protein) was transferred to a small polypropylene vial which had been rinsed with 0.1 *M* phosphate buffer at pH 7.0 and flushed with argon. Additional degassed buffer (10 ml) containing 5 m*M* EDTA was added, and the solution was equilibrated under argon in a 30 ± 0.5° constant-temperature bath. The enzyme solution was assayed. Sufficient DTT (100 μl) was added at $t = 0$ to make the solution 21 m*M* in DTT, and an initial aliquot (20 μl) was removed and used to check the concentration of thiol groups in solution by using Ellman's reagent (0.5 m*M*, 0.1 m*M* in EDTA). Aliquots (10 μl) were removed every 1–5 min and added to cuvettes containing 1 ml of assay

solution. These solutions were analyzed immediately for residual enzymatic activity. Manipulations of all solutions containing thiols were conducted under a static argon atmosphere, maintained by inserting a hypodermic needle connected to an argon line through the top of the polypropylene reaction vial. This procedure provided adequate protection against autoxidation for all but the most slowly reacting solutions. At the end of each run, the thiol concentration was checked again by using Ellman's assay.

Those reactions (lysozyme with 2-mercaptoethanol, *N*-acetyl-L-cysteine, and others), that required 2–5 days to proceed to completion were carried out in an S-40325-50 Sargent-Welch glovebox under argon, and higher than usual concentrations of thiols were used (0.05–2.5 *M*). Dimethyl sulfoxide (1–3% of the solution) was used to ensure complete solubility of the less-soluble thiols at these high concentrations. The activity of lysozyme is not influenced by these dimethyl sulfoxide concentrations.

Satisfactory Brønsted correlations observed for the rates of SH/S_2 interchange reactions between low molecular mass thiols and protein disulfide bonds suggest that for these thiols and proteins, steric and electronic effects characteristic of protein tertiary structures are less important than the electronic effects responsible for the Brønsted reactivity–basicity correlations. The rates of SH/S_2 interchange reactions involving glutathione and protein disulfides are, however, slower than would be anticipated based on correlations with lower molecular mass species.

Use of Eq. (10) and rates of reduction of papain($SSCH_3$) by DTT at various values of pH (4–9) give the pK_a of Cys-25 of papain as 4.1 (pH 6) and 8.4 (pH 9). The pK_a of Cys-25 of papain is abnormally low at pH 6 due to the interaction of Cys-25 thiolate with imidazolium ion of His-159, which has a pK_a of about 7.5.[17] The pK_a of Cys-25 of AdK is 7.5 (at pH 7). Assuming that the values of pK_a of the two cysteine thiol groups generated on reduction of the cystine moieties of DNase and lysozyme are the same, we estimate these values of pK_a to be 8.8 and 11.0, respectively (at pH 7).

This kinetic method of determination of values of thiol pK_a has the useful feature that it can be carried out as a function of pH. It has two disadvantages: First, that it is necessary to convert protein thiols into disulfides when the thiol of interest is not naturally present as a disulfide; this derivatization might, in principle, change the conformation of the active site. Second, that the particular protein thiol (disulfide) involved in reduction may not be obvious in proteins having more than one. The values of pK_a obtained by this method should be regarded as semiquanti-

tative due to the combined uncertainties in measurements of rates of SH/S_2 interchange and in the analysis based on Eq. (10). Since these uncertainties are quite different from those encountered in other methods for evaluating thiol pK_a, this method provides a useful, independent method for characterization of protein thiol and disulfide groups.

Determination of Values of Thiol Equilibrium Constants

Equilibration of a symmetrical disulfide, ESSE, to a thiol, ESH, with concomitant oxidation of a reducing thiol, RSH, to a disulfide, RSSR, occurs in two steps via an unsymmetrical disulfide [Eqs. (11)–(13)]. Both thiol and thiolate anion may be present in appreciable concentration in

$$(RSH + RS^-) + ESSE \underset{}{\overset{K_1}{\rightleftharpoons}} RSSE + (ESH + ES^-) \quad (11)$$

$$(RSH + RS^-) + RSSE \underset{}{\overset{K_2}{\rightleftharpoons}} RSSR + (ESH + ES^-) \quad (12)$$

$$2(RSH + RS^-) + ESSE \underset{}{\overset{K^{obsd}}{\rightleftharpoons}} RSSR + 2(ESH + ES^-) \quad (13)$$

$$K^{obsd} = K_1K_2 = [RSSR][ESH + ES^-]^2/[ESSE][RSH + RS^-]^2 \quad (14)$$

solution, and measured equilibrium constants will contain terms for the concentration of both species [Eq. (14)]. In these equations, and subsequently, an equilibrium constant referring to an interchange reaction involving a mixture of thiol and thiolate species will be denoted by the superscript "obsd."

A large contribution to SH/S_2 interchange equilibrium constants can, in certain circumstances, be attributed to the relative values of pK_a of the reducing thiol and the thiol derived from the disulfide. This contribution is dependent on the pH of the solution and is proportional to the difference between the values of thiol pK_a. It is largest when both thiols are present entirely as thiolate anion (i.e., when the equilibrium considered is thiol*ate*–disulfide interchange). It is this difference in pK_a that provides the driving force for the ability of Ellman's reagent to oxidize aliphatic thiols: values of pK_a for aryl thiols are typically 5 to 6, while values of pK_a for alkyl thiols are 9 to 10.

Equilibrium constants for SH/S_2 interchange of monothiols with similar values of pK_a are often close to unity. Many dithiols capable of forming cyclic disulfides are, however, more strongly reducing than are the corresponding monothiols. This effect is attributable in major part to a much higher forward rate for the second step of Eq. (15) than for that of Eq. (16); its origin is, in large part, entropic. This principle is, of course, the structural basis for the well-established value of dithiothreitol (DTT) as a reducing agent.[24] There is a clear correlation between the reducing

[24] W. W. Cleland, *Biochemistry* **3**, 480 (1964).

$$HSRSH + ESSE \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} HSRSSE + ESH \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \overset{\frown}{SRS} + 2\,HSE \quad (15)$$

$$2\,RSH + ESSE \underset{k'_{-1}}{\overset{k_1}{\rightleftharpoons}} RSH + RSSE + ESH \underset{k'_{-2}}{\overset{k_2}{\rightleftharpoons}} RSSR + 2\,HSE \quad (16)$$

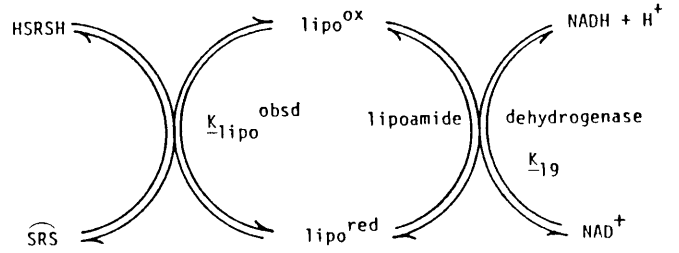
potential of an α,ω-dithiol and the size of the ring formed on its oxidation. The maximum equilibrium advantage is observed for dithiols capable of forming six-membered rings, although five- and seven-membered rings can have good stability as well.[10]

The characterization of dilute solutions of disulfides and thiols under equilibrating conditions can present substantial difficulties. The SS and SH groups are not themselves intrinsically readily detected, and differentiation between different disulfides is especially problematic. Rigorous determination of values of equilibrium constants requires a knowledge of the values of pK_a of the thiols and of the concentration of all thiol and disulfide species present in solution at equilibrium. Snyder *et al.*[20] have measured values of K^{obsd} for the reaction between *N,N'*-diacetylcystine (AA) and a cysteine-containing peptide (B). Mixtures of AA + B and BB + A were equilibrated in sealed vials. Reactions were quenched with acid (acidification converts thiolate anion to thiol and slows thiol–disulfide interchange by orders of magnitude). The components of the mixture were separated by gel filtration chromatography and their concentrations determined by UV absorption.

Separation and identification of all thiol and disulfide species is the most straightforward way to determine the concentrations of solution components at equilibrium. Such methods are, however, time consuming, and it is not always clear that the quantities of each species found after separation are truly those present at equilibrium. Rabenstein and Theriault have used an NMR method to determine equilibrium constants for captopril, penicillamine[25] (RSH), and glutathione disulfide (GSSG).[25,26] The high-field ^{1}H-NMR spectra of RSH, RSSR, and RSSG are sufficiently different to allow the relative concentrations of each species to be determined by integration. This method allows analysis of the thiol–disulfide mixture *at* equilibrium, but is not general in that the NMR spectra of many thiols are not substantially different from the spectra of the corresponding disulfides.

Equilibrium constants for reduction of lipoamide disulfide by thiols[10] can also be determined using an enzymatic procedure first described by Cleland.[24] A representative equilibration with an α,ω-dithiol (HSRSH) is presented in Eqs. (17)–(20). The lipoamide dehydrogenase-mediated reaction between lipoamide and NAD^+ was used as a shuttle to obtain reduc-

[25] D. L. Rabenstein and Y. Theriault, *Can. J. Chem.* **63**, 33 (1985).
[26] D. L. Rabenstein and Y. Theriault, *Can. J. Chem.* **62**, 1672 (1984).



(17)

$$K_{lipo}^{obsd} = [SRS][lipo^{red}]/[HSRSH][lipo^{ox}] \quad (18)$$

$$K_{19} = [lipo^{ox}][NADH]/[lipo^{red}][NAD^+] \quad (19)$$

$$K_{NAD}^{obsd} = [SRS][NADH]/[HSRSH][NAD^+] = K_{lipo}^{obsd}K_{19} \quad (20)$$

tion potentials of dithiols relative to NADH. The concentration of NADH at equilibrium was measured, and straightforward calculations based on known initial concentrations of α,ω-dithiol, lipoamide disulfide, and NAD^+ yielded the concentrations required to calculate K_{lipo}^{obsd}. This experimental procedure measures directly only the concentration of NADH and, by inference, that of reduced lipoamide; it does not identify the structures or concentrations of other thiols and disulfides present. The procedure does, however, provide a straightforward method of linking the reducing ability of a wide range of thiols to the biochemically relevant reducing agent NADH. Equilibrium constants for several thiols determined using this procedure are as follows (pK_{a1}, pK_{a2}, K_{lipo}^{obsd}): 1,2-dithio-3-propanol, 8.6, 10.5, 5.8 M^{-1}; 1,3-dithio-2-propanol, 9.0, 10.3, 0.51; dithiothreitol, 9.2, 10.1, 15; mercaptoethanol, 9.6, —, 2.0×10^{-3} M^{-1}; glutathione, 8.7, —, 1.8×10^{-3} M^{-1}.[10] These values are in good agreement with literature values.[10]

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[22] Cysteine and Cystine: High-Performance Liquid Chromatography of o-Phthalaldehyde Derivatives

By JOHN D. H. COOPER and D. C. TURNELL

Introduction

The selectivity and sensitivity of separation techniques are increased by using either pre- or postcolumn derivatization. Unlike many other amino acid derivatizing reagents, *o*-phthalaldehyde reacts rapidly with amino acids at normal ambient temperatures under aqueous conditions to yield intensely fluorescent derivatives. The relatively low polarity of these derivatives is ideally suited to separation by reversed-phase HPLC[1-3] with a sensitivity of 1 pmol for each amino acid fluorophore.[1]

The active derivatization reagent is prepared by treating *o*-phthalaldehyde with an excess of a thiol compound, e.g., 2-mercaptoethanol, to form a thiol adduct.[4] Primary amines react with the adduct to form 1-alkylthio-2-alkyl-substituted isoindoles.[5] The rapid fluorescence decay of some amino acid isoindoles[1] is not a technical limitation since the derivatives are stable once injected onto the reversed-phase column.[6]

Due to the excess thiol present in the prepared *o*-phthalaldehyde reagent, the derivative of a sulfhydryl amino acid is the reduced amino acid isoindole. Thus, cystine or cysteine will yield the same isoindole derivative. The fluorescence intensity of cysteine isoindole can be as little as 2% of that of other amino acid isoindoles.[4,7] Methods used to increase the fluorescence yield of the cysteine isoindole have included the oxidation of cysteine to cysteic acid with performic acid[8] and the reaction of the sulfhydryl of cysteine with 4-vinylpyridine,[9] ethyleneimine,[10] or iodoacetate.[11] Cysteine derivatives formed with these reagents subsequently yield isoindoles that have approximately the same fluorescence intensity as

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