Rates of Thiol–Disulfide Interchange Reactions Involving Proteins and Kinetic Measurements of Thiol pK_a Values†

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ABSTRACT: Brønsted coefficients have been determined for the rates of thiol–disulfide interchange between low molecular weight thiols and the disulfide groups of four native or modified proteins: DNase ($\beta_{nuc} \simeq 0.36$), lysozyme ($\beta_{nuc} \simeq 0.55$), adenylate kinase($SSCH_3$)$_2$ ($\beta_{nuc} \simeq 0.65$), and papain($SSCH_3$) ($\beta_{nuc} \simeq 0.45$). These values are similar to those observed for reductions of oxidized glutathione and Ellman's reagent by a similar set of thiols ($\beta_{nuc} \simeq 0.5$). Glutathione is anomalously slow in reduction of certain protein disulfide groups: although this effect may in part reflect steric hinderance to attack by the relatively large glutathione molecule at disulfides shielded by protein tertiary structure, other (presently undefined) factors appear also to be important, at least in the case of DNase. The rates of reduction of several disulfide derivatives of papain(SSR) by DTT were determined. These data provide estimates of the Brønsted coefficient for the "central" thiol in thiol–disulfide interchange: these estimates fall in the range $\beta_c \simeq -0.25$ to -0.43. Rates of reduction of protein disulfide moieties were analyzed by using a Brønsted equation developed previously [Szajewski, R. P., & Whitesides, G. M. (1980) *J. Am. Chem. Soc. 102*, 2011] to yield pK_a values for the participating thiol moieties: in particular, for papain, pK_a(Cys-25) = 8.4 at pH 9 and pK_a (Cys-25) = 4.1 at pH 6. The thiols of the structurally essential cysteine group of lysozyme seem to have p$K_a \simeq 11$. The advantages and disadvantages of this method for estimating thiol pK_a values are discussed.

Thiol–disulfide interchange reactions involving proteins are important in a number of processes, including formation and cleavage of structural cystines (Anfinsen, 1972, 1973; Wetlaufer & Ristow, 1973; Baldwin, 1975; Anfinsen & Scheraga, 1975; Liu, 1977), modification of catalytic thiols (Boyer & Segal, 1954; Boross et al., 1969; Brocklehurst & Little, 1973; Smith et al., 1975; Zannis & Kirsch, 1978), and oxidation–reduction processes requiring thiols (Jocelyn, 1972; Friedman, 1973; Torchinskii, 1974; Flaharty, 1974). Studies of base-catalyzed thiol–disulfide interchange reactions involving low molecular weight reactants have defined many features of the mechanisms of this process (Szajewski & Whitesides, 1980; Whitesides et al., 1977a; Wilson et al., 1977; Creighton, 1975; Rosenfeld et al., 1977; Freter et al., 1979). Thiolate anion attacks the disulfide bond along the sulfur–sulfur axis, in a process which places appreciable charge on all three sulfur atoms in the transition state (Rosenfeld et al., 1977) and which involves little d-orbital participation (Snyder & Carlsen, 1977) (eq 1).

$$R_{nuc}S^- + R_cS\text{–}SR_{lg} \rightarrow R_{nuc}S\text{–}SR_c + {}^-SR_{lg} \qquad (1)$$

The Brønsted coefficient characterizing the attack of thiolate nucleophiles on oxidized glutathione (GSSG)[1] and Ellman's reagent is approximately $\beta_{nuc} \simeq 0.5$. A Brønsted coefficient for the "central" sulfur atom is less well established but is approximately $\beta_c \simeq -0.3$ (Szajewski & Whitesides, 1980; Freter et al., 1979). The Brønsted coefficient of the leaving-group sulfur has not been directly measured experimentally but has been estimated as $\beta_{lg} \simeq -0.5$ to -0.7 (Szajewski & Whitesides, 1980). No intermediate is apparent along the reaction coordinate.

This paper describes an examination of the rates of thiol–disulfide interchange between low molecular weight mono- and dithiols and disulfide groups in enzymes and enzyme derivatives. This work had three related objectives: first, to establish whether the rates of thiol–disulfide interchange involving representative proteins fit the simple mechanistic pattern established for small reactants or whether steric and electrostatic interactions characteristic of protein tertiary structures led to significantly different patterns of reactivity; second, to explore the utility of thiol–disulfide interchange rates in characterizing the pK_a values and reactivities of thiol groups in proteins; third, to provide information relevant to the choice of protein antioxidants for use in organic synthetic procedures requiring enzymatic catalysis. In each instance, rates were determined by following the loss or recovery of the enzymatic activity characteristic of the native enzyme during treatment of the enzyme or enzyme derivative with thiol reducing agents.

Four proteins were examined. DNase (EC 3.1.4.5, from bovine pancreas) contains two cystine groups. The structure of DNase has not been determined by X-ray diffraction, and the locations of these groups in its tertiary structure are not known. Treatment with thiol reducing agents leads to complete loss of activity by a process in which the first splitting of a disulfide bond is believed to be rate limiting, although the identity of this bond has not been identified. Neither intramolecular nor intermolecular disulfide bonds are formed (Price et al., 1969). Lysozyme (EC 3.2.2.17) contains four cystines (X-ray structure: Blake et al., 1965; Imoto et al., 1972). Its treatment with reducing agents also leads to complete loss in activity, by a process which involves at least two of the four disulfide bonds: 6–127, 30–115, 64–80, and 76–94 (Azari, 1966). The Cys-6–Cys-127 bond can be opened without inactivation of the enzyme (Jolles et al., 1964).

Papain (EC 3.4.22.2) contains three cystines (22–63, 56–95, and 153–200) and an active-site cysteine moiety [Cys-25, Drenth et al. (1968, 1970)]. One of its three disulfide links

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[1] Abbreviations used: DNase, deoxyribonuclease; AdK, adenylate kinase; DTT, dithiothreitol; GSH, glutathione; GSSG, oxidized glutathione; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid), Ellman's reagent; Cys-Et, cystine diethyl ester; mesyl chloride, methanesulfonyl chloride; NBD-chloride, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; DTP, 1,3-dithio-2-propanol; ME, 2-mercaptoethanol; GMA, glycol dimercaptoacetate; proteins having modified thiol groups are represented by, e.g., AdK($SSCH_3$)$_2$.

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is reduced by 0.3 M 2-mercaptoethanol in 8 M urea, but complete reduction of the disulfide links can be achieved only in 6 M guanidine hydrochloride (Shapira & Arnon, 1969). The active-site thiol group of papain was modified by conversion to *S*-thioalkyl derivatives [papain(SSR)] (Smith & Kenyon, 1974; Smith et al., 1975; Lewis et al., 1976; Zannis & Kirsch, 1978): The modified proteins were enzymatically inactive, and the rates of thiolate–disulfide interchange were followed by observing the restoration of enzymatic activity on treatment of papain(SSR) with thiols. AdK (EC 2.7.4.3, porcine) contains two thiol groups (Cys-25 and Cys-187). The former is close to the active site but not functionally involved in catalysis (Schulz et al., 1974; 1974; Noda, 1973). The latter is more distant. Treatment of AdK with methyl methanethiolsulfonate gave AdK$(SSCH_3)_2$. This modified protein showed ~70% of the activity of the native enzyme (Whitesides et al., 1979).

Experimental Section

Materials and Methods. Thiols were obtained from the following sources and were recrystallized under argon or distilled under vacuum before use: dithiothreitol (DTT), oxidized (GSSG) and reduced (GSH) glutathione, *N*-acetyl-L-cysteine hydrochloride, and glycol dimercaptoacetate were obtained from Sigma Chemical Co.; 1,3-dithio-2-propanol (*caution: this material is reported to have higher toxicity than similar dithiols* (Adams et al., 1960)), 2-diethylaminoethanethiol hydrochloride, methyl thioglycolate, 2-mercaptoethanol, L-cysteine ethyl ester hydrochloride, 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent), sodium sulfide monohydrate, and methanesulfonyl chloride (mesyl chloride) were obtained from Aldrich. *N*,*N*′-Bis(2-mercaptoethyl)urea was prepared as described (Szajewski & Whitesides, 1980). 2,2,2-Trifluoroethanol and 1*H*,1*H*-pentafluoropropanol were obtained from PCR Research Chemicals Inc. Ethoxycarbonyl ethyl disulfide was prepared from ethyl chlorocarbonyl disulfide (Brois et al., 1970). Cleland's method was used to prepare *trans*-4,5-dihydroxy-1,2-dithiane (oxidized DTT) (Cleland, 1964).

Assays. Standard literature procedures were employed for the enzymes: adenylate kinase (Whitesides et al., 1977b; Rhoades & Lowenstein, 1968); deoxyribonuclease I (Kunitz, 1950); papain (Arnon, 1965; Mole & Horton, 1973); lysozyme (Shugar, 1952). Enzymes were obtained from Sigma, Boehringer-Mannheim, or Worthington in the best available purity and were used without additional purification.

Activation of Papain and AdK. Commercial papain and AdK 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: for 3.0 mg/mL papain (<0.1 unit mg^{-1}), a solution containing 0.05 M L-cysteine, 0.06 M 2-mercaptoethanol, and 0.01 M EDTA was used in a 1-h incubation at 30 °C; for 1 mg/mL AdK (340 units/mg), 0.08 M DTT was used in a 1-h incubation at 30 °C. The enzyme was separated from excess reducing agent by placing in a stirred ultrafiltration cell (Amicon Diaflo, P10 membrane) and passing 2 L (papain) or 0.6 L (AdK) of degassed 0.05 M phosphate buffer (pH 7.0, 0.1 M in KCl) through the cell at 4 °C. The resulting enzymes typically had specific activities of 3.8 units mg^{-1} (for hydrolysis of benzoyl-DL-arginine-*p*-nitroanilide) for papain and 400 units mg^{-1} for AdK.

Methyl Methanethiolsulfonate. Methyl disulfide (14.1 g, 0.16 mol) 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 °C and H_2O_2 (34.0 g of 30% solution; 10.2 g = 0.30 mol of H_2O_2) was added slowly while maintaining the temperature below 5 °C. *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 5 °C for ~1 h. After destroying the excess of peroxide by additional heating at 50 °C for 1 h and testing for peroxide with starch–iodide paper, the glacial acid was removed in vacuo. 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 the yellow oil was distilled. A colorless liquid (7.2 g, 0.057 mol) was obtained, bp 60–70 °C (0.3 torr) [lit. 54 °C (0.04 torr)] (Wijers et al., 1969). 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).

Sodium methanethiolsulfonate was prepared according to a literature method with minor modifications (Kenyon & Bruice, 1978). Sodium sulfide ($Na_2S{\cdot}9H_2O$, 216.3 g, 0.9 mol) was dissolved in 250 mL of water with careful heating to ~80 °C in a 1-L round-bottomed flask. The flask was fitted with a reflux condenser and a dropping funnel. Mesyl chloride (103.5 g, 69.9 mL, 0.9 mol) was added slowly, dropwise, maintaining vigorous stirring over a 60-min period at 0–5 °C. *Caution: Hydrogen sulfide is toxic and methanesulfonyl chloride is a potent lachrymator. This reaction is highly exothermic and liberates a dense, white, noxious cloud. A well-ventilated hood should be used.* The reaction mixture was heated at reflux for ~24 h. Most but not all of the solvent was removed, giving a yellow precipitate which was collected by filtration. The resulting yellow cake was dried for 24 h under vacuum, ground to a fine powder, and futher dried for 24 h under vacuum. The yellow powder was extracted at room temperature with 50–100-mL portions (300 mL total) of absolute ethanol and the combined ethanol solution was slowly cooled. The crystals were collected by filtration and washed with a small amount of ice-cold ethanol. The mother liquor was concentrated and filtered. The resulting combined crystalline solid was dissolved in a minimum amount of absolute ethanol at ambient temperature, filtered, taken to dryness, and recrystallized from a minimum amount of absolute ethanol, and dried under vacuum at room temperature. Sodium methanethiolsulfonate was obtained in 78% yield as large shiny flaky crystals (hygroscopic), mp 271–272 °C [lit. mp 272–273.5 °C (Kenyon & Bruice, 1978)].

Propyl Methanethiolsulfonate. Sodium methanethiolsulfonate (6 g, 0.044 mol) was placed in a 250-mL three-necked round-bottomed flask. Absolute ethanol (125 mL) was added to the flask, and the mixture was heated to 50–60 °C to dissolve the salt. A condenser and an addition funnel were fitted to the flask, and 1-bromopropane (5.58 g, 4.12 mL, 0.044 mol) was added while stirring the reaction mixture. Ice-cooled water was passed through the condenser, and the reaction mixture was heated at 60–65 °C for 24 h. The reaction mixture was cooled and filtered, and the ethanol was removed by using a rotary evaporator. A slightly yellow oil was obtained and distilled under vacuum to give a colorless liquid (49% yield), bp 95–97 °C (1 torr) [lit. 100–101 °C (1 torr) (Boldyrev et al., 1961)]. The ^1H NMR spectrum in $CDCl_3$ [δ 1.07 (t, J = 7 Hz, 3 H), 1.8 (m, J = 7 Hz, 2 H), 3.2 (t, J = 8 Hz, 2 H), 3.37 (s, 3 H)] was consistent with that expected for propyl methanethiolsulfonate.

2-Hydroxyethyl methanethiolsulfonate was prepared by using an analogous procedure. Sodium methanethiolsulfonate

(6 g, 0.044 mol) was placed in a 250-mL three-necked round-bottomed flask. The flask was equipped with a condenser and a dropping funnel. The mixture was heated to dissolve the salt, and 2-bromoethanol (5.66 g, 3.2 mL, 0.044 mol) was added while stirring. The mixture was refluxed for 24 h. Filtration and evaporation under vacuum gave a yellow oil. The oil was distilled, giving a clear viscous liquid in 53% yield: bp 116–118 °C (0.05 torr) [lit. bp 104–105 °C (0.01 torr) (Boldyrev et al., 1967)]; ^{1}H NMR ($CDCl_3$) δ 3.0 (m, 2 H), 3.2 (s, 3 H), 3.37 (t, 1 H, J = 6 Hz).

2,2,2-Trifluoroethyl methanethiolsulfonate was prepared by a similar procedure. 2,2,2-Trifluoroethyl mesylate [8.53 g, 0.044 mol, prepared by the procedure of Williams & Mosher (1954)] was added to an alcoholic solution of sodium methanethiolsulfonate (6 g, 0.044 mol) by using a procedure analogous to that described for the other alkyl methanesulfonates. A yellow oily product was obtained. Distillation yielded a colorless liquid (72%), with bp 72 °C (1 torr). The ^{1}H NMR spectrum in $CDCl_3$ showed a singlet at 3.13 ppm (3 H) and a quartet at 4.55 ppm (2 H, J = 8.0 Hz) consistent with that expected for 2,2,2-trifluoroethyl methanethiolsulfonate.

1H,1H-Pentafluoropropyl methanethiolsulfonate was prepared by an analogous procedure. It had bp 84–86 °C (1 torr) and an NMR spectrum consistent with the assigned structure ($CDCl_3$): δ 3.13 (3 H, s), 4.51 (2 H, J = 12 Hz).

Caution: All the 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. In addition, methoxycarbonylmethyl methanethiolsulfonate ($CH_3SO_2SCH_2CO_2Me$), synthesized by us but not utilized in this study, is a potent lachrymator.

Dimethyl dithiodiacetate was prepared by oxidation of methyl thioglycolate with dimethyl sulfoxide using a literature procedure (Yiannios & Karabinos, 1963) and had bp 112 °C (1.5 torr) [lit. 154 °C (14 torr) (Price & Twiss, 1908)]: NMR ($CDCl_3$) δ 3.62 (4 H, s), 3.79 (6 H, s).

L-*Cystine diethyl ester dihydrochloride* was prepared by an analogous procedure and had mp (dec) 186–188 °C after recrystallization from ethyl alcohol–water [lit. mp (dec) 187–189 °C (Abderhalden & Wybert, 1916)].

2-Mercaptoethyl Thioglycolate. Methyl thioglycolate (10 g, 0.094 mol) was placed in a 500-mL round-bottomed flask that contained 300 mL of benzene. To this solution was added 2-mercaptoethanol (10 g, 0.13 mol) and *p*-toluenesulfonic acid (100 mg). The reaction mixture was heated at 80 °C until NMR analysis indicated that no methyl thioglycolate was left (~4 days). The reaction mixture was cooled to room temperature and washed 3 times with 300-mL portions of 5% sodium bicarbonate solution. The reaction mixture was dried over sodium sulfate, and the solvent was removed on a rotary evaporator. Distillation yielded a colorless liquid (82%), with bp 55 °C (0.015 torr). The ^{1}H NMR spectrum in $CDCl_3$ showed a triplet at 1.60 ppm (1 H, J ~ 9 Hz), a triplet at 2.09 ppm (1 H, J ~ 8 Hz), a doublet of triplets at 2.80 ppm (2 H, J ~ 9 Hz, 7 Hz), a doublet at 3.32 ppm (2 H, J ~ 8 Hz), and a triplet at 4.29 ppm (3 H, J ~ 7 Hz).

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 any of the thiol agents employed in this study restored >98% of the native enzyme. A degassed solution (100 mL; 0.05 M phosphate buffer, pH 7.0, 10^{-4} M in EDTA and 0.1 M in KCl) containing 0.3 g (1.3×10^{-5} mol) of completely activated papain was treated with 35.2 mg (2.8×10^{-4} mol, 20× excess) of CH_3S-SO_2CH_3 under argon. The decrease in activity was monitored: after 1–2 h at 30 °C 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, PM 10 membrane), separating the protein from the rest of the solution, and passing 2 L of 0.1 M phosphate buffer (pH 7.0, 10^{-4} M in EDTA, 0.1 M in KCl) through the cell at 4 °C.

An analogous procedure was used for AdK, starting with treatment of 0.1 g (4.8×10^{-6} mol, 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 30 min of incubation at 30 °C. Excess $CH_3SSO_2CH_3$ was removed as described for papain($SSCH_3$).

Modifications of adenylate kinase with $EtSSCO_2Me$ and with Ellman's reagent (Ellman, 1959) were carried out by using analogous procedures. The reaction times required to reach constant activities and those activities (expressed as a percent of the specific activity of native AdK) were as follows: for AdK(SSEt)$_2$, 2 h and 49% (±3), and for AdK(SSEllman's)$_2$, 5 h and <0.1%.

Treatment of a solution of each of the modified enzymes (0.1–0.5 mg of protein/mL) with DTT (5–20× molar excess) for 1–2 h at 30 °C regenerated 98% (±2) of the activity of the native enzyme. Assay of AdK($SSCH_3$)$_2$ for residual thiol groups using Ellman's reagent indicated that less than 0.001 of the thiols present in the native enzyme remained (see below). The thiol-modified proteins were handled with appropriate precautions to avoid contact with reducing agents (*thiol antioxidants and cross-linking agents in rubber tubing rapidly regenerate unmodified protein*).

Estimation of protein sulfhydryl groups by Ellman's reagent was done by a modification of the method developed by Ellman (Ellman, 1959). Modified protein (0.8 mL of a solution containing 0.1 mg of protein/mL, free from excess blocking reagent) was incubated in a 1-cm UV cell with Ellman's reagent (DTNB, 0.2 mL, 5× molar excess) at 30 ± 0.5 °C in a phosphate buffer (pH 7.0, 10^{-4} M in EDTA) for 15 min. The appearance of 2-nitro-5-thiobenzoic acid was followed at 412 nm. An extinction coefficient of 13 700 M^{-1} cm^{-1} was used for Ellman's anion (Whitesides et al., 1977a; Riddles et al., 1979).

Kinetics of Thiol–Disulfide Interchange Reactions. One representative kinetics run will be described; others followed similar procedures. 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 (pH 7.0) and flushed with argon. Additional degassed buffer (10 mL) containing 5 mM EDTA was added, and the solution was equilibrated under argon in a 30.0 ± 0.5 °C constant temperature bath. The enzyme solution was assayed. Sufficient DTT (100 μL) was added at t = 0 to make the solution 21 mM 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 (5×10^{-4} M, 10^{-4} 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. Representative kinetic data are shown in

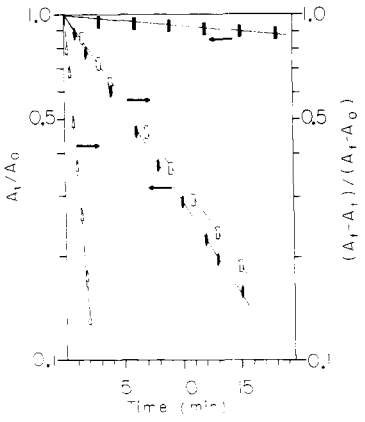

FIGURE 1: Thiol–disulfide interchange reactions involving proteins: A_t is the observed enzymatic activity at time t; A_0 is the starting activity; A_f is the activity corresponding to complete conversion to product. Lysozyme (■); AdK($SSCH_3$)$_2$ (□); DNase (●); papain-($SSCH_3$) (△) (all were treated with DTT). All reactions were carried out in 0.1 M phosphate buffer and 0.1 M KCl, pH 7.0, at 30.0 ± 0.5 °C under argon.

Figure 1. 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) which required 2–5 days to proceed to completion were carried out in a 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.

Determination of the Extinction Coefficient for trans-4,5-Dihydroxy-1,2-dithiane (Oxidized DTT). These determinations were carried out by using solutions which were 1.7×10^{-4} M in Ellman's reagent and $\sim 5.1 \times 10^{-4}$ M in oxidized DTT. Measurements were made at the extinction maximum for the cyclic disulfide and a temperature of 30 ± 0.1 °C. The following values of the extinction coefficient (M^{-1} cm^{-1}) were determined (ϵ, pH, buffer, buffer concentration in M): 274, 3.2, citric acid–Na_2HPO_4, 0.1; 279, 5.0, citric acid–Na_2HPO_4, 0.1; 275, 6.0, Na_2HPO_4–KH_2PO_4, 0.1; 275, 7.0, Na_2HPO_4–KH_2PO_4, 0.1; 274, 8.5, Tris-HCl, 0.1; 276, 9.0, Tris-HCl, 0.1; 276, 10.0, KOH–KCl, 0.1. Throughout the work described in this paper, $\epsilon = 275$ M^{-1} cm^{-1} was used. Previous determinations of ϵ for oxidized DTT are 273 in H_2O (Cleland, 1964) and 110 at 310 nm, pH 8.0, and 0.05 M phosphate buffer (Iyer & Klee, 1973).

pH–Rate Profile for the Thiol–Disulfide Interchange between Papain($SSCH_3$) and DTT. These buffers were used: KCl–HCl (pH 1–2); citric acid–Na_2HPO_4 (pH 3.0–5.5); KH_2PO_4–Na_2HPO_4 (pH 6.0–8.0); Tris-HCl (pH 8.0–9.0); Na_2CO_3–Na_2HCO_3 (pH 9.0–10.0) or borate–HCl (pH 9.0–10.0); KCl–KOH (pH 10–12). All buffers were 0.1 M in NaCl or KCl and 10^{-4} M in EDTA. Doubly distilled water was boiled under argon and used immediately. After the buffers were prepared, argon was bubbled through the solutions for 30 min. In low pH solutions (pH 3.5–6), DTT was required in 1000–2000× molar excess over papain($SSCH_3$) to give practical rates of reduction: in higher pH solutions (7.0–10.5), DTT was present in 2–40× molar excess. Reactions were carried out by using concentrations of papain-($SSCH_3$) which, when reduced, correspond to 0.3–0.6 unit/mL papain (pH 7.0, 0.1 M phosphate buffer, 0.1 M KCl). Reproducible results could not be obtained below pH 3.3 due to inactivation of the enzyme (Hinkle & Kirsch, 1970). The reductions of papain($SSCH_3$) with DTT in solutions of different pH were followed by dialyzing papain($SSCH_3$) (0.3 mg/mL) with the appropriate buffer and then placing the protein in a small polypropylene vial that had been rinsed with the same buffer. The solution (5 mL) was equilibrated in a 30.0 ± 0.5 °C constant-temperature bath, and the pH of the solution was measured. At $t = 0$, DTT (0.05–0.5 M) was added under argon with a micropipet in volumes that depended on the pH of the solution. The concentration of DTT in the reaction mixture was dependent on the pH of the solution. The concentration of DTT in the reaction mixture was determined by using Ellman's assay. Aliquots (10–50 μL) were removed every 0.25–1.0 min and added to cuvettes containing 1 mL of assay solution and analyzed immediately for the increase in the enzymatic activity.

Determination of Thiol pK_a Values. Titration curves were obtained under argon with careful exclusion of atmospheric carbon dioxide by using a 0.085 M carbonate-free potassium hydroxide solution. Dilute solutions of thiols were used (0.001 M) so that activity corrections were not required. The KOH required to neutralize the thiol was added in 20 equal portions, and the pH of the solution was measured 1 min after each addition. The pH water was standardized against pH 7.00 and 10.00 Mallinckrodt BuffAR solutions. Analysis of data followed a literature procedure (Britton, 1954; Albert & Serjeant, 1962; Lilburn, 1976). The electrode was removed from the mixture between measurements to avoid errors due to thiol adsorption (Hill & Spivey, 1974).

pH–Rate Profiles for the Thiol–Disulfide Interchange: Reactions of Dimethyl Dithiodiacetate, Oxidized Glutathione, and L-Cystine Diethyl Ester Dihydrochloride with DTT. Buffers employed were the same used for the reduction of papain($SSCH_3$). Dimethyl dithiodiacetate ($CH_3O_2CCH_2SSCH_2CO_2CH_3$, 5×10^{-5}–3×10^{-3} M), oxidized glutathione (1×10^{-4}–6×10^{-3} M), and L-cystine diethyl ester dihydrochloride (1×10^{-5}–7×10^{-4} M) solutions, all 10^{-4} M in EDTA, were treated with DTT (2–2000× molar excess). The dithiol solutions were prepared and used within 1 h. Dithiol concentrations were determined by using Ellman's reagent. The concentration of oxidized DTT was followed continuously at 283 nm. Cells with 1- or 10-cm path lengths were used, and argon was passed through the cell compartment during the kinetic runs. At time zero, DTT (0.1–0.3 M) was added rapidly with a micropipet in volumes that depend upon the pH of the solutions.

Results

Characterization of Papain(SSR) and AdK($SSCH_3$)$_2$. These modified proteins were prepared by reactions of papain with CH_3SSO_2R (R = CH_3, $CH_2CH_2CH_3$, CH_2CH_2OH, $CH_2CF_2CF_3$, and CH_2CF_3) and of adenylate kinase with $CH_3SSO_2CH_3$. For papain, two observations are sufficient to characterize the modified proteins because the modifications involve a thiol required for catalytic activity. First, the papain(SSR) derivatives were catalytically inactive (<0.1% of native activity) in the assay used for papain; second, treatment of all papain(SSR) derivatives with DTT restored 98 ± 2% of the starting activity. In addition, papain($SSCH_3$) had fewer than 0.001 free thiol groups detectable by reaction with Ellman's reagent. These results are compatible with previous

preparations of papain($SSCH_3$) (Lewis et al., 1976; Angelides & Fink, 1978, 1979). Characterization of AdK$(SSCH_3)_2$ is more complicated, because the modified protein retains ~70% of the activity of the native enzyme. Prolonged treatment with $CH_3SSO_2CH_3$ produces no further decrease in activity; full activity is regenerated following incubation with thiols. Treatment of AdK$(SH)_2$ with an excess of Ellman's reagent followed by dialysis and reactivation of the enzyme with DTT gave 2 equiv of Ellman's anion as measured spectrophotometrically at 412 nm. This experiment demonstrates that inter- or intramolecular disulfide bonds are not formed on reaction of AdK$(SH)_2$ with Ellman's reagent. The conclusion that intramolecular disulfide bonds are not formed is in accord with the crystal structure of this enzyme: the sulfur atom of Cys-187 is 13 Å from the sulfur atom of Cys-25 (Schulz et al., 1974). Analysis of the modified protein using Ellman's reagent indicated the presence of less than 0.001 thiol group per enzyme. In addition, treatment of AdK with $EtSSCO_2Me$ under conditions similar to those used to prepare AdK-$(SSCH_3)_2$ produced a different protein, AdK$(SSEt)_2$, having 49 ± 3% of the activity of AdK, while treatment with Ellman's reagent generated another modified protein previously identified (Price et al., 1975) as AdK(SSEllman's)$_2$. AdK-(SSEllman's)$_2$ has <0.1% of the activity of AdK. The magnitudes of the residual activities in these modified proteins correlate inversely with the size of the modifying groups and are compatible with the hypothesis that substitution of an $-SSCH_3$ group for –SH at Cys-25 and Cys-187 has relatively little effect on enzymatic activity but that substitution by larger groups reduces enzymatic activity.

Kinetics of Thiol–Disulfide Interchange Reactions. Reactions were followed by measuring enzymatic activity as a function of time in solutions containing a large excess of thiol. Under these conditions, the empirical rate equations, eq 2 (for

$$-[P]/dt = k^{obsd}[P][RSH]_0 = k_1[P][RS^-]_0 \quad (2)$$

$$-d[P]/dt = 2k^{obsd}[P][HSRSH]_0 = 2k_1[P][HSRS^-]_0 \quad (3)$$

monothiols, RSH) and 3 (for dithiols, HSRSH), were followed to greater than 95% completion. Figure 1 reproduces typical data. In these equations [P] is the concentration of the protein whose disulfide group(s) is (are) being reduced; $[RSH]_0$ and $[HSRSH]_0$ are the starting concentrations of thiol-containing species (both thiol and thiolate) in solution and are effectively constant throughout the course of the reaction; $[RS^-]_0$ and $[HSRS^-]_0$ are the equilibrium concentrations of thiolate anions at the solution pH. The observed enzymatic activity is assumed to be related to [P] by expressions of the form of eq 4 (de-

$$\text{activity}_t = C([P]_0 - [P]_t) \text{ or } C[P]_t \quad (4)$$

$$k_1 = (10^{pK_a - pH} + 1)k^{obsd} \quad (5)$$

pending on whether activity is increasing or decreasing with time). Assuming further that $HSRS^-$ is the only nucleophile present in solutions of dithiols (that is, assuming the concentration of $^-SRS^-$ to be small and its nucleophilicity to be similar to that of $HSRS^-$), k_1 and k^{obsd} are related by eq 5: this assumption is incorrect for pH >9 (see below). Table I summarizes the rate constants obtained by using eq 2–5.

For each protein several rate constants were explicitly examined for their dependence on thiol concentration and in all cases found to be essentially invariant to changes of factors of 2–4. Gorin had reported that the rate of reduction of lysozyme by DTT at pH 10 was zero order in DTT (Gorin et al., 1968). This result was interpreted as evidence that a conformation change in the protein was overall rate limiting in the reduction of the disulfide bond essential for maintenance

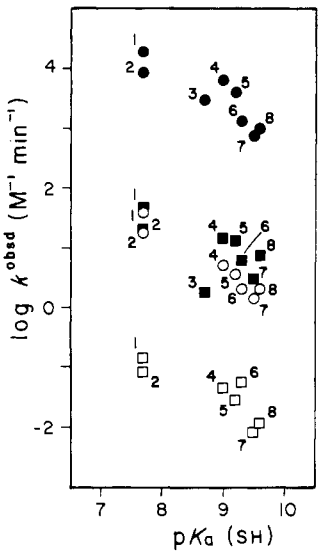

FIGURE 2: Plots of log k^{obsd} vs. thiol pK_a for thiol–disulfide interchange reactions involving proteins. Identifying numbers for the thiols refer to Table I; papain($SSCH_3$) (●); AdK$(SSCH_3)_2$ (■); DNase (○); lysozyme (□). All reactions were carried out in 0.1 M phosphate buffer and 0.1 M KCl, pH 7.0, at 30.0 ± 0.5 °C under argon.

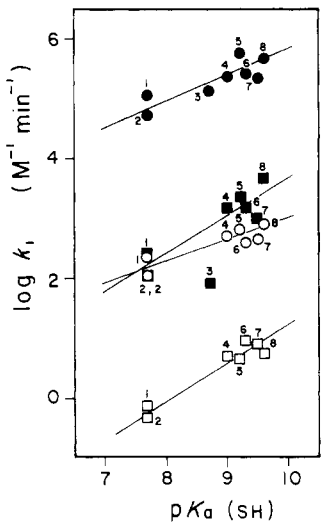

FIGURE 3: Plots of log k_1 vs. thiol pK_a for thiol–disulfide interchange reactions involving proteins. Identifying numbers for the thiols refer to Table I; papain($SSCH_3$) (●); AdK$(SSCH_3)_2$ (■); DNase (○); lysozyme (□). The rate constants (k_1) were calculated by using eq 5.

of enzymatic activity. Gorin speculated that the thiol–disulfide interchange reaction should become rate limiting at lower pH values. Our results confirm this suggestion and establish that reductions of the other proteins examined here are also bimolecular: first order in thiol(ate) and first order in protein.

Figure 2 plots log k^{obsd} vs. thiol pK_a values; Figure 3 plots log k_1 vs. thiol pK_a values. The former plot is relevant to the practical problem of choosing thiol reducing agents for protein disulfide bonds on the basis of rate. As expected, the most rapidly reducing thiols are those having pK_a values closest to the solution pH. The second is a Brønsted plot. Several conclusions follow immediately from this figure. First, the generally satisfactory Brønsted correlation observed for the rates of thiol–disulfide interchange between these low molecular weight thiols and protein disulfide bonds suggest that steric and electronic effects peculiar to the protein tertiary structures are less important, *for these thiols and these proteins*, than the electronic effects responsible for the reactivi-

Table I: Rate Constants (M^{-1} min^{-1}) for Thiol-Disulfide Interchange Reactions Involving Proteins[a]

thiol	pK_a[b]	DNase		lysozyme		papain($SSCH_3$)		AdK($SSCH_3$)$_2$		GSSG[b]	
		k^{obsd}	k_1	k^{obsd}	k_1	$10^{-3}k^{obsd}$	$10^{-3}k_1$	k^{obsd}	k_1	k^{obsd}	k_1
(1) $(CH_2OCOCH_2SH)_2$ (GMA)	7.7 (9.0)	38[c]	230	0.13[c]	0.78	18[c]	110	46[c]	270	90	490
(2) $(CH_3CH_2)_2NHCH_2CH_2SH$	7.7[d]	18	110	0.080	0.48	8.7	52	19	110		
(3) glutathione (GSH)	8.7	<0.001[e]		<0.001[e]		2.8[c]	140	1.8[c]	89		
(4) $HSCH_2CHOHCH_2SH$ (DTP)	9.0 (10.3)	4.7[c]	480	0.046[c]	4.6	2.4[c]	250	15[c]	1500	9.3	9300
(5) $HSCH_2CHOHCHOHCH_2SH$ (DTT)	9.2 (10.4)[f]	4.0[c]	640	0.026[c]	4.2	3.3[c]	530	14[c]	2200	14	2200
(6) $OC(NHCH_2CH_2SH)_2$	9.3 (10.1)	2.1	420	0.047	9.4	1.3	270	7.9	1600		
(7) $O_2CCH(NHCOCH_3)CH_2SH$	9.5	1.4	440	0.0080	2.6	0.71	230	3.1	980	5.2	1800
(8) $HOCH_2CH_2SH$ (ME)	9.6	2.0[c]	810	0.012[c]	5.3	1.2[c]	500	8.4[c]	3400	8.7	3400

[a] The reproducibility of these rate constants is ca. ±15%. All rates were determined in 0.1 M phosphate buffer and 0.1 M KCl, pH 7.0, at 30.0 ± 0.5 °C under argon. The individual rate constants are typically an average of two determinations. The rate constants for dithiols are statistically corrected. [b] From Szajewski & Whitesides (1980, and references cited therein); the rate constants involving thiol–disulfide interchange of GSSG with thiols are not statistically corrected. [c] Examined at two or more concentrations. [d] Kostyukovskii et al. (1972). [e] No reduction was detectable. [f] Measured in this study.

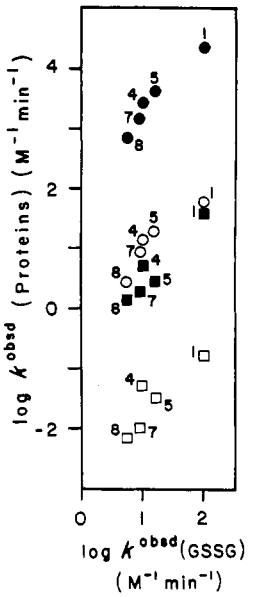

FIGURE 4: Log k^{obsd} for protein vs. log k^{obsd} for oxidized glutathione. The identifying numbers for the thiols refer to Table I; papain($SSCH_3$) (●); AdK($SSCH_3$)$_2$ (■); DNase (○); lysozyme (□). The least-squares slopes are as follows: papain $SSCH_3$ = 1.1 (r^2 = 0.94); Ad($SSCH_3$)$_2$ = 0.90 (r^2 = 0.84); DNase = 1.1 (r^2 = 0.93); lysozyme = 1.0 (r^2 = 0.77).

ty–basicity relation which underlie Brønsted correlations. The observation that charged thiols [$Et_2N^+HCH_2CH_2SH$ and $^-O_2CCH(NHCOCH_3)CH_2SH$] fall on the same correlation line as neutral thiols is noteworthy. The single thiol which falls appreciably off the lines is glutathione: the reactivity of this material is discussed in greater detail below. The Brønsted slopes derived from these plots are not very accurate, because the pK_a range spanned and the number of thiols included are limited, because the set of thiols studied would not necessarily provide a good set for Brønsted studies even in mechanistically uncomplicated cases, and because the precision of individual rate constants is only modest. Nonetheless, the values of these slopes obtained by least-squares analysis (omitting the points for glutathione) are similar to the value of $\beta \simeq 0.5$ observed for thiol–disulfide interchange with Ellman's reagent and GSSG:

$$\beta_{nuc}^{papain(SSCH_3)} = 0.45 \ (r^2 = 0.77)$$

$$\beta_{nuc}^{AdK(SSCH_3)_2} = 0.65 \ (r^2 = 0.85)$$

$$\beta_{nuc}^{DNase} = 0.36 \ (r^2 = 0.80)$$

$$\beta_{nuc}^{lysozyme} = 0.55 \ (r^2 = 0.76)$$

Second, the Brønsted lines, although roughly parallel, are significantly displaced from one another: thus, for example, the $PSSCH_3$ disulfide bond of papain($SSCH_3$) is cleaved $\sim 10^5$ times more rapidly than the cystine disulfide bond of lysozyme whose reduction is responsible for loss of its enzymatic activity.

Figure 4 correlates the rates of interchange between thiols and disulfide groups in proteins with rates involving the disulfide group of oxidized glutathione. This correlation has the feature that characteristics of the thiols which weaken the correlation between pK_a and interchange rate, and thus lead to scatter in a Brønsted plot, are suppressed: Figure 4 simply correlates the rates of several kinds of thiol–disulfide interchange reactions. The correlations are significantly better than those in Figures 2 and 3. Further, the construction of the plot emphasizes that rates of thiol–disulfide interchange with DNase and AdK($SSCH_3$)$_2$ are approximately that expected

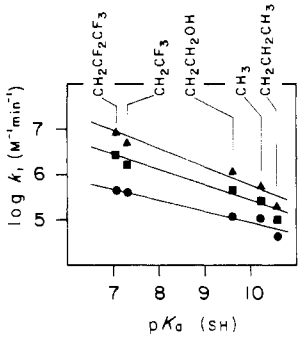

FIGURE 5: Plots of log k_1 vs. the pK_a for the central thiol for thiol–disulfide interchange between papain(SSR) and DTT (▲), DTP (■), and 2-mercaptoethyl thioglycolate (●).

by analogy with GSSG but that rates involving papain-$(SSCH_3)$ are much faster and those involving lysozyme are much slower than would be expected (see Table II).

Brønsted Coefficient for the Cysteine Sulfur of Papain-(SSR). We have examined the rate of release of papain(SH) from papain(SSR) by thiol–disulfide interchange at pH 7.0 using a selection of reducing thiols with different values of pK_a. We assume that these reactions proceed by attack at the SR center (eq 6) for two reasons. First, at pH 7.0, the papain thiol

$$R_{nuc}SH + \text{papain(SSR)} \rightarrow R_{nuc}SSR + \text{papain(SH)} \quad (6)$$

group is a stronger acid, and thus a better leaving group, than any of the RSH thiols (see below). Second, backside attack at the papain thiol center should be considerably more hindered sterically than attack at the RS center. These studies offer a direct experimental determination of the Brønsted coefficient β_c. Figure 5 gives the corresponding Brønsted plots. These Brønsted coefficients are similar to those estimated for aliphatic thiols ($\beta_c \sim -0.3$ to -0.4; Freter et al., 1979).

Thiol–Disulfide Interchange between Proteins and Glutathione. The rates of thiol–disulfide interchange reactions involving glutathione and protein disulfides are slower than would be anticipated based on simple Brønsted correlations. Glutathione is effectively unreactive toward DNase and lysozyme and approximately one-tenth as reactive toward $AdK(SSCH_3)_2$ as expected from its pK_a; only toward papain$(SSCH_3)$ is its reactivity that predicted from its acidity. The anomalously low reactivity of glutathione toward some protein disulfide groups is of obvious interest in connection with its role as a major intracellular thiol (Kosower & Kosower, 1976). We have not established the origin of this low reactivity, but preliminary observations suggest that it may be more complex than a simple steric effect. Thus, for example, glutathione (0.05 M) not only did not destroy the activity of DNase but also *prevented* the deactivation of DNase by high concentrations (0.1–0.25 M) of DTT or mercaptoethanol. (We note that certain other peptides *not* containing cysteine also protected DNase against reduction by DTT: Glu-Gly-Gly and Ala-Glu. Ala-Gly-Gly and Glu-Ala did not provide protection.)

Applications of Thiol–Disulfide Interchange Rates to the Determinations of Kinetic Acidities of Protein Thiols. The inference from this and previous work is that the rates of many thiol–disulfide interchange reactions follow a Brønsted correlation. In principle, it might therefore be possible to measure the pK_a values for cysteine thiol groups in proteins indirectly by measuring the rates of interchange reactions involving these thiols. To test this possibility, we have determined the pH–rate profile for release of enzymatically active papain from papain$(SSCH_3)$ by reduction with DTT (Figure 6). For comparison, we have also established rate–structure profiles for

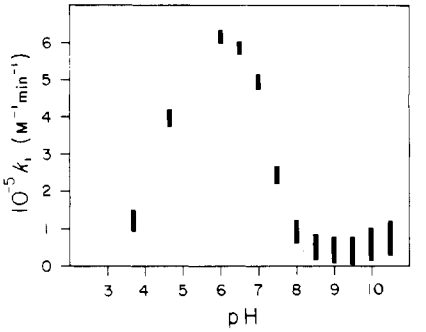

FIGURE 6: pH–rate profile for the thiol–disulfide interchange involving papain$(SSCH_3)$ and DTT. The rates [k_1 (M^{-1} min^{-1})] were calculated from k^{obsd} according to eq 5. All reactions were performed at 30 ± 0.5 °C under argon. The individual rate constants are an average of at least three determinations and the reproducibility of these rates is ca. ±15%.

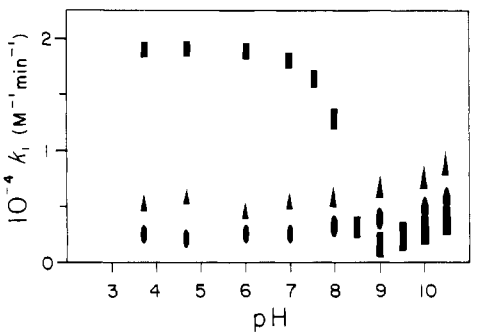

FIGURE 7: pH–rate profiles for thiol–disulfide interchange between dimethyl dithiodiacetate (▲), oxidized glutathione (●), and L-cystine diethyl ester dihydrochloride (■) with DTT. All reactions were performed at 30 ± 0.5 °C under argon. The individual rate constants are an average of at least two determinations. The reproducibility of these rates is ±5% for the pH range of 4.7–7.5 and ±15% for the pH range of 8–10.5. The rates (k_1) were calculated from the observed rates (k^{obsd}) by eq 5.

reduction of dimethyl dithiodiacetate, oxidized glutathione, and L-cystine diethyl ester (Figure 7). The rate data in Figures 6 and 7 were analyzed by using eq 7 (Szajewski &

$$\log k_1 = 7.03 + 0.5pK_a^{nuc} - 0.27pK_a^{c} - 0.73pK_a^{lg} \quad (7)$$

Whitesides, 1980) to obtain pK_a values for the thiol groups being generated in the interchange reactions.

This particular equation, and not another of the several related equations described previously (Szajewski & Whitesides, 1980), was used because it appears to provide the best empirical fit to the available range of rate data rather than because it is the most easily justified theoretically. The correctness of this equation, in which the magnitudes of β_{nuc} and β_{lg} are not equal, has not been established. Fortunately, although the best form for eq 7 remains unclear theoretically, the differences between pK_a values obtained by using proposed alternative forms for it are relatively small. Analysis of the rate data summarized in Figures 6 and 7 using alternative values of the coefficients in eq 7 (Szajewski & Whitesides, 1980) resulted in thiol pK_a values which were effectively indistinguishable from those described here: the values of the pK_a's are not particularly sensitive to the differences in coefficients in the several forms of the Brønsted equations we have developed to date.

The pH profile for dimethyl dithiodiacetate is expected to be featureless, because this substance contains no other acidic or basic groups whose state of ionization might influence pK_a^c and pK_a^{lg}. This expectation is confirmed experimentally (Figure 7). Oxidized glutathione contains both carboxylic acid

Table II: Rate Constants [$10^{-4}k^{obsd}$ or $10^{-4}k_1$ (M^{-1} min^{-1})] for Thiol-Disulfide Interchange Reactions between Papain(SSR) and Dithiols[a]

thiol	pK_a	CH_3		$CH_2CH_2CH_3$		CH_2CH_2OH		CH_2CF_3		$CH_2CF_2CF_3$		β_c	r^2
		k^{obsd}	k_1	k^{obsd}	k_1	k^{obsd}	k_1	k^{obsd}	k_1	k^{obsd}	k_1		
$HSCH_2CO_2CH_2CH_2SH$	7.5 (9.6)[b]	2.4	10	0.98	4.1	2.7	11	8.9	37	9.4	39	−0.25	0.88
$HSCH_2CHOHCH_2SH$ (DTP)	9.0 (10.3)[c]	0.24	24	0.10	11	0.36	37	1.6	170	2.1	220	−0.33	0.92
$HSCH_2CHOHCHOHCH_2SH$	9.2 (10.4)[b]	0.33	53	0.13	22	0.64	100	3.5	550	4.5	730	−0.43	0.90

[a] All rates were determined in 0.1 M phosphate buffer, pH 7.0, and 0.1 M KCl at 30 ± 0.5 °C under argon. The reproducibility of these rate constants is ca. ±15%. The individual rate constants are an average of at least two determinations. The rate constants are statistically corrected. [b] Measured in this study. [c] From Whitesides et al. (1977a).

Table III: pK_a Values Inferred from Rate Constants [k_1 (M^{-1} min^{-1})] for Thiolate-Disulfide Interchange[a]

compd	group	pH	$10^{-2}k_1$	pK_a	lit. pK_a
CH_3OCOCH_2SH	SH	4–9	52	7.9	7.91[b]
GSH (NH_3^+, CO_2H)	SH	4	13	8.5	8.72[c]
GSH (NH_3^+, CO_2^-)	SH	8	16	8.4	8.72[c]
Cys-Et (NH_3^+)	SH	4–6	190	7.4	7.3[c]
Cys-Et (NH_2)	SH	9	11	8.6	8.87[c]
Cys-Et[d]	NH_3^+	*d*		8.2	8.33[c]
papain	SH (Cys-25)	6	6200	4.1	
papain	SH (Cys-25)	9	13	8.4	
papain	H^+ (His-159)	*d*		7.5	
DNase	*e*	7	6.4	8.8	
AdK	SH (Cys-25)	7	22	7.5	
lysozyme	*e*	7	0.042	11.0	

[a] DTT is the reducing dithiol in all experiments. [b] Wilson et al. (1980). [c] Reuben & Bruice (1976). [d] From inflections in pH-rate profiles: see Figures 6 and 7. [e] The location of the thiol group(s) determining these pK_a values has not been established. The two protein cysteine thiol groups are assumed to have the same pK_a.

and amino groups whose ionization state change with pH. Other studies have, however, already established that these changes do not significantly influence the thiol pK_a (Reuben & Bruice, 1976). Thus, this compound would also be expected to display a featureless pH–rate profile. By contrast, the thiol pK_a of cysteine ethyl ester is strongly influenced by protonation of the amino moiety. Thus, the rate constant for thiolate–disulfide interchange involving oxidized cystine diethyl ester should show an inflection at the pK_a of the amino group. The experimental data (Figure 7) confirm all of these qualitative predictions. Analysis of these rates using eq 7 yields the pK_a values summarized in Table III. A pK_a for methanethiol of 10.3 (Kreevoy et al., 1964) was used in eq 7 to obtain the pK_a of the Cys-25 thiol group of papain. The agreement between the pK_a values calculated from the interchange rates and those obtained by conventional methods is satisfactory.

The relative accuracy of the data of Figure 7 (as well as that of Figure 6) in different pH regimes deserves comment. At pH <4 the accuracy of k_1 decreases, because k^{obsd} becomes small: only a small fraction of the DTT is present in the ionized form, the thiol–disulfide interchange reactions are slow, and the correction (eq 5) required to convert k^{obsd} to k_1 becomes large. At values of pH >9 the accuracy of k_1 again decreases. In this regime reactions are so rapid that dilute solutions must be used to give conveniently measurable rates by using conventional kinetic methods, and autoxidation due to adventitious dioxygen in the solution becomes an experimental problem. Moreover, we estimate the second pK_a of DTT to be 10.4, and for pH >9 the contribution to the dianion of DTT becomes significant. All of the values of k_1 in Figures 6 and 7 were calculated on the assumption that DTT was acting exclusively as a monobasic nucleophile. The upward turn in the values of k_1 observed for all of the species at pH 9.5 presumably reflects the breakdown of this assumption, as reactions of DTT dianion become significant. We have not explicitly measured a value of β_{nuc} for DTT dianion, but assuming that reactions of the second thiolate center are still adequately described by eq 7 and estimating the contribution of DTT dianion to the interchange reaction, corrected values of k_1 can be calculated for GSSG and dimethyl dithiodiacetate which are constant over the entire range of pH.

Table III also contains pK_a values inferred for the thiol groups of the several proteins examined. Papain presents an interesting problem. The details of the interactions between the active-site cysteine (Cys-25) and the array of proximate

Lewis bases (His-159, Asn-175, and Asp-158) are still a subject of active research (Zannis & Kirsch, 1978; Angelides & Fink, 1978, 1979, and references cited in each). Values of k_1 for thiolate disulfide interchange between papain($SSCH_3$) and DTT at pH 6 and pH 9.5 translate into thiol pK_a values of 4.1 and 8.4, respectively, with the inference that the pK_a of the group responsible for this change in thiol pK_a is ~7.5: these values are in accord with proposals made by Jolley & Yankeelov (1972), Shipton et al. (1975), Lewis et al. (1976), and Angelides & Fink (1979). The decrease in k_1 observed at low pH suggests another important ionizable group with a pK_a of ~4.5, but the inaccuracies in the kinetic measurements at this pH are such that this assignment should be considered tentative. Others have also suggested an important ionizable group at approximately this pH (Sluyterman & Wolthers, 1969; Sluyterman & Wijdenes, 1973; Shipton et al., 1975; Bendall & Lowe, 1976; Lewis et al., 1976; Angelides & Fink, 1979).

Discussion

The rates of reduction of the protein disulfide groups examined in this work all follow unexceptional Brønsted relation in the pK_a values of the reducing thiols. This observation is itself useful in characterizing these groups, because it indicates that their reactivity is dominated by the local (predominantly electrostatic) effects which determine acidity rather than steric effects originating in the protein tertiary structure. The magnitudes of the rate constants nonetheless vary widely: in particular, those for reduction of papain($SSCH_3$) and lysozyme differ by 10^5 at pH 7. The rapid rate of reduction of the former is satisfactorily rationalized by the high acidity of the active site thiol. The low rate of reduction of the structurally essential cystine of lysozyme apparently *also* reflects an anomalous (but in this instance unexpectedly *high*) thiol pK_a. This inference raises interesting questions about the origin of the high thermodynamic and kinetic stability of certain protein disulfide moieties. This stability is often attributed to steric shielding of the disulfide from attacking nucleophiles by surrounding groups in the protein. The observation that the relatively large reducing agent glutathione is slower in several of its reactions than would be expected from the Brønsted correlations indicates that steric effects may, in fact, influence the kinetic lability of certain protein disulfide groups (Wilson et al., 1980). The assignment of pK_a = 11 to the thiol groups of the essential disulfide group of lysozyme suggests, however, that there may be an important electrostatic contribution to unexpectedly slow rates of reduction. In the same sense that proximate positively charged groups increase the acidity of the active site thiol of papain to a value appropriate for effective catalysis at pH 6, arrays of negatively charged (or possibly hydrophobic; Hine, 1975) groups around the structurally important disulfide of lysozyme might *increase* the pK_a values of its constituent thiols to values which render reduction slow. We note that the common observation that reduction rates increase on unfolding the protein in, for example, guanidine hydrochloride does not differentiate between a steric basis and an electronic basis for the original stability.

The rates of thiol–disulfide interchange involving proteins should provide a useful method for determining kinetic acidities of thiols. Knowles (1976) has outlined the problems in obtaining structurally interpretable pK_a measurements of active-site groups from kinetic measurements as a function of pH: among these are the difficulty in defining unambiguously the elementary step which is influenced by the change in pH and the ambiguities encountered in disentangling this step from prior association equilibria. Measurement of rates of thiol–disulfide interchange rates circumvents these problems. The rate-limiting elementary step (attack of thiolate anion on the disulfide bond) is well-defined, and the observation that the rates of reaction of several thiols of different structures and charge types follow a Brønsted relation provides a convincing if qualitative demonstration that preequilibrium complex formation between the protein and the reducing thiol is probably not important in determining rates. This technique has, of course, the potential disadvantage that it is necessary to form a disulfide derivative of the protein when the protein does not naturally incorporate the thiol of interest into a disulfide. This derivatization may, in principle, induce a change in the structure of the active site or a change in the acidities of the active-site functional groups.

The application of this technique to the characterization of the active-site thiol of papain illustrates the potential utility of this method. It is clear that at low pH, this thiol behaves as a leaving group in thiol–disulfide interchange as if it had a pK_a of ~4, while at high pH it behaves as if it had a normal pK_a of ~8.5. The transition between these two regimes occurs at pH 7–8 and is attributable to protonation of a base with this pK_a in the active site. A useful feature of the thiol–disulfide interchange reaction as a method for estimating thiol pK_a values is that, unlike convential methods, it can be carried out as a function of pH. Thus, it is possible to observe changes in the thiol pK_a with changes in the state of ionization of proximate acids and bases.

These estimates of the pK_a values of the active-site functional groups of papain are in general agreement with previous proposals by others. Angelides & Fink (1979), in particular, have used kinetic data obtained by cryoenzymatic techniques to assemble a detailed proposal for the mechanism of action of papain. A key feature of this mechanism is the proposal of two conformations of the papain active site: one conformation (the UP conformation) binds substrate rapidly but is catalytically inactive, and a second (the DOWN conformation) binds substrate slowly but is catalytically active. These two conformations differ, among other features, in the orientation of His-159 and in active-site pK_a values. In the UP conformation, His-159 is hydrogen bonded to Asn-175. In this conformation, which is stable at pH >8.5, Cys-25 has pK_a ~ 8 and His-159 has pK_a ~ 4. In the DOWN conformation (stable below pH 8), His-159 is present as an imidazolium ion hydrogen bonded to the carboxylate of Asp-158, and pK_a values are Cys-25, pK_a ~ 4, and His-159, pK_a ~ 8. Our techniques offer no insight into possible conformational changes of papain but do confirm the values of Cys-25 at high and low values of pH. They suggest, moreover, that since the Cys-25 thiol is present as a $SSCH_3$ group over the entire pH range and is thus electrostatically roughly equivalent to an SH group, any conformation changes in the modified enzyme cannot be driven by ionization of the thiol group but must be determined predominantly by the interaction between His-159 and Asp-158.

The pK_a values inferred for thiol groups of DNase and adenylate kinase are compatible with previous characterizations. The disulfide group of DNase responsible for maintaining the enzyme in its active conformation (i.e., the disulfide group whose reduction is rate limiting in loss of activity) has been suggested to lie close to the protein surface (Price et al., 1969). The pK_a estimated in this work for the leaving thiol of this disulfide (pK_a = 8.8) is close to that of GSH and is compatible with this suggestion. The Cys-25 thiol group of adenylate kinase [the one closer to the enzyme active site and presumably the one whose liberation from the disulfide group

of AdK$(SSCH_3)_2$ is reflected by an increase in enzymatic activity during thiol–disulfide interchange with DTT] is abnormally acidic (pK_a = 7.5). This thiol has been reported previously to react ~40 times faster with NBD-chloride than the more distant Cys-187 (Price et al., 1975). The details of the structural factors responsible for the enhanced acidity of Cys-25 are not known.

The combined uncertainties in the experimental measurement of rates of thiol–disulfide interchange involving proteins, and in the derivation of thiol acidities from these rates using an analysis based on the Brønsted eq 7 or a related equation, are such that the pK_a values obtained by using this technique should be regarded a semiquantitative. Since the uncertainties in this method are of a quite different character than those encountered in other analyses of pH–rate profiles for enzymes, it nonetheless provides useful, independent data for characterization of protein thiol and disulfide groups.

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References

Abderhalden, E., & Wybert, E. (1916) *Chem. Ber. 16*, 2449.
Adams, E. P., Doyle, F. P., Hatt, D. L., Holland, D. O., Hunter, W. H., Mansford, K. R. L., Nayler, J. H. C., & Queen, A. (1960) *J. Chem. Soc.*, 2649.
Albert, A., & Serjeant, E. P. (1962) in *Ionization Constants of Acids and Bases*, p 52, Methuen, London.
Anfinsen, B. C. (1972) *Biochem. J. 128*, 737.
Anfinsen, B. C. (1973) *Science 181*, 223.
Anfinsen, B. C., & Scheraga, H. A. (1975) *Adv. Protein Chem. 29*, 205.
Angelides, K. Y., & Fink, A. L. (1978) *Biochemistry 17*, 2659.
Angelides, K. Y., & Fink, A. L. (1979) *Biochemistry 18*, 2355, 2363.
Arnon, R. (1965) *Immunochemistry 2*, 107.
Azari, P. (1966) *Arch. Biochem. Biophys. 155*, 230.
Baldwin, R. L. (1975) *Annu. Rev. Biochem. 44*, 453.
Bendall, M. R., & Lowe, G. (1976) *Eur. J. Biochem. 65*, 481.
Blake, C. C. F., Koening, D. F., Mair, G. A., North, A. C. T., Phillips, D. C., & Sarma, V. R. (1965) *Nature (London) 206*, 757.
Boldyrev, B. G., Golerova, C. A., & Dvorko, A. T. (1961) *Zh. Obshch. Khim. 31*, 2402.
Boldyrev, B. G., Grivnak, L. M., Kolesnikova, S. A., Kolmakova, L. E., & Volshim, G. A. (1967) *Zh. Org. Khim. 3*, 37.
Boross, L., Cseke, E., & Vas, M. (1969) *Acta Biochim. Biophys. 4 (3)*, 301.
Boyer, P. D., & Segal, H. L. (1954) in *A Symposium on the Mechanism of Enzyme Action* (McElroy, W. D., & Glass, B., Eds.) p 520, Johns Hopkins Press, Baltimore, MD.
Britton, H. T. S. (1954) in *Hydrogen Ions*, 4th ed., p 217, Chapman and Hall, London.
Brocklehurst, K., & Little, G. (1973) *Biochem. J. 133*, 67.
Brois, S. J., Pilot, J. F., & Barnum, H. H. (1970) *J. Am. Chem. Soc. 92*, 7629.
Cleland, W. W. (1964) *Biochemistry 3*, 480.
Creighton, T. E. (1975) *J. Mol. Biol. 96*, 767.
Drenth, J., Jansonius, J. N., Koekoek, R., Swen, H. M., & Wolthers, B. G. (1968) *Nature (London) 218*, 929.
Drenth, J., Jansonius, J. N., Koekoek, R., Sluyterman, L. A. E., & Wolthers, B. G. (1970) *Phil. Trans. R. Soc. London, Ser. B 257*, 231.
Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70.
Flaharty, A. L. (1974) in *The Chemistry of the Thiol Group* (Patai, S., Ed.) p 589, Wiley, New York.
Freter, R., Pohl, E. R., Wilson, J. M., & Hupe, D. J. (1979) *J. Org. Chem. 44*, 1771.
Friedman, M. (1973) *The Chemistry and Biochemistry of the Sulhydryl Group*, Pergamon Press, New York.
Gorin, G., Fulford, R., & Deonier, R. C. (1967) *Experientia 24 (1)*, 26.
Hill, D. E., & Spivey, H. O. (1974) *Anal. Biochem. 57*, 500.
Hine, J. (1975) *Structural Effects on Equilibria in Organic Chemistry*, Chapter 5, Wiley, New York.
Hinkle, P. M., & Kirsch, J. F. (1970) *Biochemistry 9*, 4633.
Imoto, T., Johnson, L. N., North, A. C. T., Phillips, D. C., & Rupley, J. A. (1972) *Enzymes, 3rd Ed. 7*, 665.
Iyer, K. S., & Klee, W. A. (1973) *J. Biol. Chem. 248*, 707.
Jocelyn, P. C. (1972) *Biochemistry of the SH Group*, Academic Press, New York.
Jolles, P., Jouregui-Adell, J., & Jolles, J. (1964) *C. R. Hebd. Seances Acad. Sci. 258*, 3926.
Jolley, C. J., & Yankeelov, J. A., Jr. (1972) *Biochemistry 11*, 164.
Kenyon, G. L., & Bruice, T. W. (1978) *Methods Enzymol. 47*, 407.
Knowles, J. R. (1976) *CRC Crit. Rev. Biochem. 3*, 165.
Kosower, N. S., & Kosower, E. M. (1976) in *Free Radicals in Biology* (Pryor, W. A., Ed.) Vol. II, Chapter 2, Academic Press, New York.
Kostyukovskii, Y. L., Bruk, Y., Kokushkima, A. V., Mirkin, B. S., Slavachevskaya, N. M., Pavlova, L. V., & Belenkaya, I. A. (1972) *Zh. Obshch. Khim. 42*, 662.
Kreevoy, M. M., Eichinger, B. E., Stary, F. E., Katz, E. A., & Sallstadt, J. H. (1964) *J. Org. Chem. 29*, 1641.
Kunitz, M. (1950) *J. Gen. Physiol. 33*, 349, 363.
Lewis, S. D., Johnson, F. A., & Shafter, J. A. (1976) *Biochemistry 15*, 5009.
Lilburn, J. E. (1976) Ph.D. Thesis, Massachusetts Institute of Technology, Cambridge, MA.
Liu, T.-Y. (1977) *Proteins (3rd Ed.) 3*, 239.
Mole, J. E., & Horton, H. R. (1973) *Biochemistry 12*, 816.
Noda, L. (1973) *Enzymes, 3rd Ed. 8*, 279–305.
Price, N. C., Cohn, M., & Schrimer, R. H. (1975) *J. Biol. Chem. 250*, 644.
Price, P. A., Stein, W. H., & Moore, S. (1969) *J. Biol. Chem. 244*, 929.
Price, T. S., & Twiss, D. F. (1908) *J. Chem. Soc.*, 1645.
Reuben, D. M. E., & Bruice, T. C. (1976) *J. Am. Chem. Soc. 98*, 114.
Rhoades, D. G., & Lowenstein, J. M. (1968) *J. Biol. Chem. 243*, 3963.
Riddles, P. W., Blakeley, R. L., & Zermer, B. (1979) *Anal. Biochem. 94*, 75.
Rosenfield, R. E., Parthasarathy, R., & Dunitz, J. D. (1977) *J. Am. Chem. Soc. 99*, 4860.
Schulz, G. E., Elzinga, M., Marx, F., & Schirmer, R. H. (1974) *Nature (London) 250*, 120.
Shapira, E., & Arnon, R. (1969) *J. Biol. Chem. 244*, 1026.
Shipton, M., Kierstan, M. P. J., Malthouse, J. P. G., Stuchburg, T., & Brocklehurst, K. (1975) *FEBS Lett. 50*, 365.
Shugar, D. (1952) *Biochim. Biophys. Acta 8*, 302.
Sluyterman, L. A. E., & Wolthers, B. G. (1969) *Proc. K. Ned. Akad. Wet., Ser. B: Palaeontol., Geol., Phys., Chem. 72*, 14.
Sluyterman, L. A. E., & Wijdenes, J. (1973) *Biochim. Biophys. Acta 302*, 95.

Smith, D. J., & Kenyon, G. L. (1974) *J. Biol. Chem. 249*, 3317.
Smith, D. J., Maggio, E. T., & Kenyon, G. L. (1975) *Biochemistry 14*, 766.
Snyder, J. P., & Carlsen, L. (1977) *J. Am. Chem. Soc. 99*, 2931.
Szajewski, R. P., & Whitesides, G. M. (1980) *J. Am. Chem. Soc. 102*, 2011.
Torchinskii, Y. M. (1974) *Sulfhydryl and Disulfide Groups in Proteins*, Plenum Press, New York.
Wetlaufer, D. B., & Ristow, S. (1973) *Annu. Rev. Biochem. 42*, 135.
Whitesides, G. M., Lilburn, J. E., & Szajewski, R. P. (1977a) *J. Org. Chem. 41*, 332.
Whitesides, G. M., Lamotte, A., Adalsteinson, O., & Colton, C. K. (1977b) *Methods Enzymol. 44*, 887.
Whitesides, G. M., Lamotte, A. L., Adalsteinson, O., Baddour, R. F., Chmurny, A. C., & Colton, C. K. (1979) *J. Mol. Catal. 6*, 177.
Wijers, H. E., Boelens, H., & Van Der Gen, A., & Brandsma, L. (1969) *Recl. Trav. Chim. Pays-Bas 88*, 519.
Williams, H. R., & Mosher, H. S. (1954) *J. Am. Chem. Soc. 76*, 2984.
Wilson, J. M., Bayer, R. J., & Hupe, D. J. (1977) *J. Am. Chem. Soc. 99*, 7922.
Wilson, J. M., Wu, D., Motin-DeGrood, R., & Hupe, D. J. (1980) *J. Am. Chem. Soc. 102*, 359.
Yiannios, C. N., & Karabinos, J. V. (1963) *J. Org. Chem. 28*, 3246.
Zannis, V. I., & Kirsch, J. F. (1978) *Biochemistry 17*, 2669.

Refolding of Reduced Short Neurotoxins: Circular Dichroism Analysis†

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ABSTRACT: The four disulfide bonds of nine homologous short curare-like polypeptides are cleaved by reduced dithiothreitol. Air oxidation renaturations of the reduced compounds are followed by far-ultraviolet circular dichroism analysis, and the kinetics of refolding thus determined are compared. They indicate that three toxins refold 4–10 times more slowly than the six others. It is shown that a significant difference between the refolding kinetics still subsists when renaturations are made in the presence of various concentrations of thiol–disulfide exchange reagents or at various pH values. From an examination of the toxin sequences, it is proposed that a single additional amino acid insertion is responsible for the difference in the observed kinetics. This proposal is supported by temperature studies of renaturation kinetics.

The refolding of reduced polypeptides is known to occur spontaneously as originally demonstrated by Anfinsen and co-workers (Sela et al., 1957; Haber & Anfinsen, 1962). Such a spontaneity strongly suggests that the required information for refolding is contained in the amino acid sequence of the polypeptide chain. Although there is no definitive explanation of the mechanism by which a reduced molecule refolds to its native structure, evidence is now accumulating which indicates that interactions between nearest-neighbor side chains play an important role in directing the refolding of a polypeptide chain (Lewis et al., 1971; Zimmerman & Scheraga, 1977; Go & Taketomi, 1978; Matheson & Scheraga, 1978). Such interactions are thought to trigger the formation of nucleation sites, as summarized by Anfinsen & Scheraga (1975). Obviously, longer range interactions are also involved in the refolding process but probably to a lesser extent than the short-range interactions (Anfinsen & Scheraga, 1975).

In order to gain a clear understanding of the refolding process, it is important to establish which amino acids define the nucleation sites. Selective modifications and the preparation of synthetic analogues of the polypeptide chain should, in principle, allow an experimental examination of the influence of the constituent amino acids upon the refolding process. For instance, in the particular case of ribonuclease, it has been shown that specific iodination of Tyr-115 prevents the refolding of the reduced molecule (Friedman et al., 1966). Unfortunately, the synthesis of protein analogues is a prohibitively difficult task. However, groups of natural polypeptides possessing homologous sequences do exist. Such protein families have the potential of being useful tools for the study of the relationships between amino acid sequence and the ability of a chain to refold.

The short neurotoxins isolated from snake venoms constitute a large group of homologous polypeptides of 60, 61, or 62 residues, cross-linked by four disulfide bonds (Lee, 1972; Yang, 1974). These molecules possess a curare-like activity, specifically blocking the nicotinic acetylcholine receptor. Extensive data are now available which highlight the close structural (Dufton & Hider, 1977; Ménez et al., 1978) and biological (Ishikawa et al., 1977) homologies of these polypeptides. In addition, the X-ray structure of one of the toxins has been elucidated in two different laboratories (Tsernoglou & Petsko, 1976; Low et al., 1976). Thus, this polypeptide group would appear to be particularly well suited for a comparative refolding study.

In the present work, nine reduced short neurotoxins are subjected to air oxidation at 37 °C and pH 7, and the kinetics of refolding, measured by the variations of optical activity, are determined. Under these conditions, it appears that the neurotoxins do not all refold at the same rate. When renaturation experiments are carried out at different pH values or in the presence of thiol–disulfide exchange reagents, a significant difference of behavior still subsists. In order to establish the origin of this phenomenon, the kinetics of the air

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