Simple Alkanethiol Groups for Temporary Blocking of Sulfhydryl Groups of Enzymes†

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ABSTRACT: New reagents for the temporary blocking of active or accessible sulfhydryl groups of enzymes have been developed. These reagents, which are either alkyl alkanethiolsulfonates or alkoxycarbonylalkyl disulfides, rapidly and quantitatively place various RS- groups on the sulfhydryls to generate mixed disulfides. In all cases native enzymes can be regenerated with either dithiothreitol or β-mercaptoethanol. In general the temporary blocking groups also afford total protection against normally inhibitory thiol blocking agents. When RS- groups were attached to rabbit muscle creatine kinase (EC 2.7.3.2), a trend toward lower residual activities with increasing bulk was observed. Treatment of the native creatine kinase with $^{14}CH_3HgCl$ led to incorporation of greater than 1 equiv of CH_3Hg- group per subunit. This CH_3Hg-blocked enzyme was fully active, and the blocking group afforded no protection against iodoacetamide. These results suggest that CH_3Hg- and the RS- groups are modifying two different sulfhydryl groups on the enzyme. When papain (EC 3.4.4.10) was treated with excess methyl methanethiolsulfonate, complete and rapid inhibition was observed, and 1 equiv of CH_3S- was incorporated/mol of active enzyme. Complete protection against normally inhibitory 5,5′-dithiobis(2-nitrobenzoic acid) was afforded by the temporary blocking group. When rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) was titrated with methyl methanethiolsulfonate, two sulfhydryl groups per subunit were found to be modified, one much more rapidly than the other. If one extrapolates the initial slope of the titration curve, the inactivation of the enzyme would be complete after modification of a single cysteinyl residue per subunit.

Few existing sulfhydryl blocking reagents for enzymes deliver rapidly removable protecting groups. Those which do, *e.g.*, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB[1]) (Ellman, 1958) and tetrathionate (Pihl and Lange, 1962), are generally either bulky, charged, or both. Earlier, we reported that the introduction of the relatively small, non-hydrogen bonding methanethio (CH_3S-) group onto the active sulfhydryl group of rabbit muscle creatine kinase led to the formation of a mixed disulfide on the enzyme (Smith and Kenyon, 1974). In this paper we describe various reagents capable of attaching simple RS- groups onto active or accessible sulfhydryl groups of proteins under conditions mild enough to preserve other aspects of enzyme's structure. We also give details of the syntheses of our previously reported blocking agents, methyl methanethiolsulfonate and methoxycarbonylmethyl disulfide (Smith and Kenyon, 1974).

The fundamental reactions involved are given below.

$$\text{Enz–SH} + \text{RSSO}_2\text{R}' \longrightarrow \text{Enz–S–SR} + \text{R}'\text{–SO}_2\text{H} \quad (1)$$

$$\text{Enz–SH} + \text{R}'\text{–O–}\overset{\overset{\text{O}}{\|}}{\text{C}}\text{–SR} \longrightarrow \text{Enz–S–SR} + \text{COS} + \text{R}'\text{OH} \quad (2)$$

These RS-blocking groups have been developed with several potential uses in mind. First, if a sulfhydryl group is intimately involved in the catalytic mechanism of a particular enzyme, then blocking this sulfhydryl will obliterate enzymatic activity, a result which will tend to confirm the sulfhydryl group's essentiality. Conversely, if blocking the sulfhydryl group either does not diminish enzymatic activity, or only partially diminishes this activity, then one can conclude that the free sulfhydryl group is not essential after all.

Second, since sulfhydryl groups are such effective attacking groups, they tend to react preferentially with reagents sent in to modify other types of side-chain groups of enzymes. By temporarily blocking the -SH groups, this tendency may be circumvented. Afterwards, the RS- group may be rapidly, selectively, and completely removed under mild conditions by treatment with available thiol reagents (*e.g.*, β-mercaptoethanol or dithiothreitol).

RS- groups incorporating a variety of functionalities can be introduced onto enzymes by the procedures described here. For example, introduction of the CF_3CH_2S- group opens up the possibility of examining the environment of given modified sulfhydryl groups by means of fluorine-19 nuclear magnetic resonance (nmr) spectroscopy.

Materials and Methods

Materials used were obtained from the following sources: ATP, L-cysteine, DTNB, D,L-glyceraldehyde-3-phosphoric acid (Sigma), creatine, glycine, β-mercaptoethanol, methanesulfonyl chloride, trichloromethanesulfenyl chloride, *p*-toluenesulfonyl chloride, methanethiol, ethanethiol, propyl disulfide, and ethyl disulfide (Eastman), dithiothreitol, iodoacetamide, and NAD^+ (Calbiochem), 2,2,2-trifluoroethanol and *N*-α-benzoyl-L-arginine ethyl ester hydrochloride (Aldrich), bovine serum albumin (Armour), $^{14}CH_3Br$ (1.39 Ci/mol), $^{14}CH_3HgCl$ (3.36 Ci/mol), [^{14}C]NaCN (9.63 Ci/mol), and Aquasol (New England Nuclear). Papain was purchased from Worthington (16 IU/mg) and rabbit muscle glyceraldehyde-3-phosphate dehy-

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[1] Abbreviations used are: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); MeS-CK, *S*-(methanethio)-creatine kinase; MeHg-CK, *S*-(methylmercuri)-creatine kinase; PCMB, *p*-chloromercuribenzoate.

drogenase from Sigma (77 IU/mg). Rabbit muscle creatine kinase was purchased from Calbiochem with specific activity of 100–110 mequiv of ATP per min per mg. Sephadex gels were purchased from Pharmacia. All other reagents were either Baker or Mallinckrodt Analytical grade.

Creatine kinase assays were carried out on a Radiometer TTT2 pH-Stat at 30° (Mahowald *et al.*, 1962), and the protein concentrations were determined from the absorption at 280 nm using the relationship $A_{280\,nm} = 7.1 \times 10^4\ M^{-1}\ cm^{-1}$ (Noda *et al.*, 1954). Papain activity was determined by an assay based on the procedure of Whitaker and Bender (1965). Papain concentrations were measured by monitoring the absorbance at 280 nm and assuming a value of $E_{1\,cm}(1\%) = 24.7$ (Glazer and Smith, 1961; Bender *et al.*, 1966) and a molecular weight of 23,406 (Mitchell *et al.*, 1970). Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase was assayed by the procedure of Velick (1955). Protein concentrations in this latter case were determined by using the relationship $A_{276\,nm} = 1.52 \times 10^5\ M^{-1}\ cm^{-1}$.

Spectrophotometric assays were carried out on a Gilford 2220 spectrophotometer equipped with a Beckman monochrometer, Model 2400. The cell compartment was equipped with thermospacers and thermostated at 25° with a Haake water bath. Proteins were concentrated using an Amicon ultrafiltration cell, Model 12, with PM10 membranes. Radioactive samples in Aquasol were counted in a Nuclear-Chicago Mark I scintillation counter. Nuclear magnetic resonance spectra were measured on a Varian A-60A spectrometer. Unless otherwise noted all ^1H nmr spectra were taken in $CDCl_3$. Melting points (corrected) were determined on a Thomas capillary melting point apparatus, and all elemental analyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley, Calif.

Chemical Syntheses

Methyl methanethiolsulfonate was prepared by a modification of the method of Backer (1953). Thus, methyl disulfide (28.2 g, 0.3 mol) was dissolved in 90 ml of glacial acetic acid in a 500-ml three-necked flask fitted with both a reflux condenser and a 100-ml dropping funnel. The reaction flask was first cooled to 0°, and with vigorous stirring 30% H_2O_2 (68.1 g, 0.6 mol) was added slowly through the dropping funnel while maintaining the temperature below 10°. Initially the reaction mixture existed as two layers. After addition of the H_2O_2, the solution was stirred for 20 min, and the flask was then slowly warmed to 60° for about 1 hr (*caution*: care must be taken to warm the reaction slowly since otherwise the reaction becomes extremely exothermic). The solution, now homogeneous, was tested for peroxide with starch paper before work-up. After tests for peroxide became negative, the glacial acid was removed *in vacuo*, and the yellow oil was treated with 50 ml of saturated $NaHCO_3$ solution to neutralize all residual acid. The oil was separated, diluted with $CHCl_3$, and dried over anhydrous Na_2SO_4. The aqueous phase was extracted with three 25-ml portions of $CHCl_3$, and the organic phase was combined with the yellow oil. After drying, the $CHCl_3$ was removed and the clear, yellow oil distilled. A colorless oil (10.5 g, 0.083 mol) was obtained, bp 69–71° (0.4 mm); lit. (Boldyrev *et al.*, 1966) 67–68° (0.3 mm). The overall yield of pure product was 28%. The ^1H nmr spectrum showed peaks at δ 2.65 (3 H, s) and 3.22 (3 H, s).

[^{14}C]*Methyl methanethiolsulfonate* was more conveniently prepared by a modification of the method of Dunbar and Rogers (1966). First, potassium methanethiolsulfonate was prepared by the following procedure based on the methods of Boldyrev and Zakharchuk (1954) and Johnston and Gallagher (1961). Potassium hydrosulfide (Rule, 1911) (7.0 g, 0.097 mol, carefully dried over P_4O_{10}) was suspended in dry tetrahydrofuran in a three-necked flask equipped with a reflux condenser and a drying tube. Methanesulfonyl chloride (20.0 g, 0.175 mol) was added with stirring. The mixture was heated at reflux for 5 hr and then taken to dryness under reduced pressure. The residue was extracted with warm *N,N*-dimethylformamide, and this extract was evaporated to dryness. The residue was then washed with isopropyl alcohol to leave colorless crystals which were dried over P_4O_{10} at reduced pressure. The yield was 11.3 g (75%), mp 202–203°; lit. (Boldyrev and Zakharchuk, 1954) 203°. A 0.30-g (2.0 mmol) portion of the dry potassium methanethiolsulfonate was then suspended in 5 ml of anhydrous ethanol in a three-necked 50-ml flask equipped with a Dry Ice–acetone condenser and a drying tube. Through a septum attached to a side arm was added $^{14}CH_3Br$ (68.8 mg, 0.72 mmol). After this addition, the septum was removed and replaced with a stopper. The reaction flask was stirred at room temperature for 12 hr and the condenser was kept cold. The solution was then again frozen and the excess unlabeled CH_3Br (120 mg, 1.28 mmol) was added. After warming to room temperature, the reaction flask was then set aside for an additional 6 hr. The solution was then cooled to 0° and filtered. The white residue was washed with 2 ml of cold, anhydrous ethanol, and these washings were combined with the filtrate. Solvent was removed under low vacuum to yield a clear, yellow oil. This oil was then transferred to a micro-molecular distillation apparatus and distilled. A clear, colorless oil was obtained in 39% yield (95 mg). The ^1H nmr spectrum was identical with that of an authentic sample of methyl methanethiolsulfonate. The sample was then diluted with an equivalent amount of unlabeled methyl methanethiolsulfonate to give product with a specific activity of 1.15×10^{-2} Ci/mol.

Ethyl methanethiolsulfonate was prepared by the method used to prepare [^{14}C]methyl methanethiolsulfonate, except that the reaction was performed on a larger scale. The oily product was distilled under high vacuum to give a colorless oil (52% yield), bp 99–100° (0.1 mm); lit. (Boldyrev *et al.*, 1966) bp 101° (0.4 mm). The ^1H nmr spectrum was identical with that reported previously (Bentley *et al.*, 1972).

Propyl propanethiolsulfonate was prepared under analogous conditions to those used to prepare unlabeled methyl methanethiolsulfonate, starting with propyl disulfide. The product was isolated in 32% yield as an oil, bp 73–75° (0.3 mm); lit. (Boldyrev *et al.*, 1966) bp 112–114° (1–2 mm). The ^1H nmr spectrum showed peaks at δ 1.02 (6 H, m), 1.76 (4 H, m), and 3.52 (4 H, m).

Trichloromethyl methanethiolsulfonate was prepared by modifying the method of Boldyrev *et al.* (1961). Finely ground, dry sodium methanesulfonate (Boldyrev *et al.*, 1961) was suspended in 10 ml of dry benzene. To this solution was added with stirring trichloromethanesulfenyl chloride (18.6 g. 0.1 mol), and the mixture was heated for 5 hr on a steam bath. After filtration of NaCl, the solvent was removed *in vacuo* to give a yellow oil. Upon cooling under high vacuum the product crystallized as colorless needles. After recrystallization from anhydrous ethanol, 75 g (32% yield) of product was obtained, mp 54–55°; lit. (Boldyrev *et al.*, 1961) mp 56°.

Alkoxycarbonylalkyl disulfides were prepared following the methods of Zumach and Kühle (1970) and Brois *et al.* (1970). The preparation of the methoxycarbonylmethyl disulfide, described as follows, is typical. Freshly distilled methoxycarbonylsulfenyl chloride (Zumach and Kühle, 1970) (35 g, 0.275 mol) was dissolved in 25 ml of anhydrous methanol, and the solution was cooled to 0°. Methanethiol (14.4 g, 0.30 mol) was added, and the solution was stirred for 30 min. The solution turned from yellow to colorless as the reaction proceeded. The solvent was removed under low vacuum to yield a slightly yellow oil. After vacuum distillation, 32 g (83% yield) of analytically pure, colorless product was obtained, bp 30–33° (0.6 mm). The ^{1}H nmr spectrum showed peaks at δ 2.46 (3 H, s) and 3.84 (3 H, s). *Anal.* Calcd for $C_3H_6O_2S_2$: C, 26.08; H, 4.34; S, 46.42. Found: C, 25.99; H, 4.55; S, 46.38.

Similarly, *ethoxycarbonylethyl disulfide* was obtained in 91% yield, bp 45° (0.1 mm); lit. (Brois *et al.*, 1970) 74–75° (0.5 mm). The ^{1}H nmr spectrum showed the following peaks: δ 1.26 (6 H, t, J = 4.0 Hz), 2.77 (3 H, q, J = 4.0 Hz), and 4.28 (3 H, q, J = 4.0 Hz). *Anal.* Calcd for $C_5H_{10}O_2S_2$: C, 36.12; H, 6.05; S, 38.57. Found: C, 36.14; H, 6.17; S, 38.34.

Methoxycarbonyl-2,2,2-trifluoroethyl disulfide was prepared in 56% yield, bp 42–43° (0.7 mm). The ^{1}H nmr spectrum showed peaks at δ 3.33 (2 H, q, J = 9 Hz). *Anal.* Calcd for $C_3H_5F_3O_2S_2$: C, 23.30; H, 2.43; S, 31.10. Found: C, 23.09; H, 2.53; S, 31.38.

Stock Solutions of Inhibitors

Iodoacetamide was recrystallized from hot water before use. The colorless crystals were then washed with hexane and dried. Fresh 1.54 mM stock solutions were prepared daily to eliminate the possibility of iodine contamination. Aqueous stock solutions (0.1 M) of synthetic inhibitors, including most of the thiolsulfonates and alkoxycarbonylalkyl disulfides, were prepared and stored at 4°. The relatively water-insoluble inhibitors, methoxycarbonyl-2,2,2-trifluoroethyl disulfide and propyl propanethiolsulfonate, were instead dissolved in either methanol or ethanol and also stored at 4°.

Model Reactions

(1) Alkanethiolation of L-*Cysteine with Methyl Methanethiolsulfonate.* L-Cysteine (0.605 g, 5 mmol) was dissolved in 25 ml of water, and the resulting solution was cooled to 0°. To this stirred solution was added slowly methyl methanethiolsulfonate (0.732 g, 6 mmol) in 10 ml of ethanol. During the addition a white precipitate quickly formed. The reaction was allowed to proceed for 10 min after addition, and then the product was isolated by filtration. The colorless product was washed with two 10-ml volumes of cold ethanol and then with 10 ml of cold water. The crystals were dried *in vacuo* over P_4O_{10} to yield 0.61 g (73%) of *S*-(methanethio)-L-cysteine, mp 187–191° dec. *Anal.* Calcd for $C_4H_9NO_2S_2$: C, 28.72; H, 5.42; N, 8.37; S, 38.34. Found: C, 28.69; H, 5.62; N, 8.51; S, 38.38. A small sample was acidified with HCl, the solvent was removed, and the resulting hydrochloride salt was redissolved in D_2O for measurement of the ^{1}H nmr spectrum. The spectrum had the following peaks: δ 2.48 (3 H, s), 3.34 (2 H, t, J = 3 Hz), 4.50 (1 H, q, J = 3 Hz).

(2) Alkanethiolation of L-*Cysteine with Ethoxycarbonylethyl Disulfide.* L-Cysteine (0.70 g, 4.6 mmol) was dissolved in 50 ml of water and cooled to 0°. To the stirred solution was added ethoxycarbonylethyl disulfide (0.83 g, 5 mmol) in 10 ml of ethanol. Two drops of triethylamine was added to catalyze the reaction. Two phases were present initially, but as the reaction proceeded the solution first became homogeneous and then a white precipitate formed. The white precipitate was isolated by filtration after 1 hr and then washed successively with 10 ml of water and 20 ml of ethanol. The *S*-(ethanethio)-L-cysteine was dried under vacuum over P_4O_{10} to give an isolated yield of 0.70 g (85%), mp 185–193° dec; lit. (Small *et al.*, 1949) mp 196° dec. *Anal.* Calcd for $C_5H_{11}NO_2S_2$: C, 33.14; H, 6.07; S, 35.39. Found: C, 33.08; H, 5.78; S, 35.48. A small sample was treated with HCl, dried, and redissolved in D_2O for ^{1}H nmr analysis. The spectrum gave peaks at δ 1.38 (3 H, t, J = 4 Hz), 2.89 (2 H, q, J = 4 Hz), 3.35 (2 H, t, J = 2 Hz), 4.50 (1 H, q, J = 2 Hz).

Protein Sulfhydryl Alkanethiolation: Methanethiolation of Rabbit Muscle Creatine Kinase with [^{14}C]Methyl Methanethiolsulfonate. The following detailed procedures are typical of methods used in the alkanethiolations of enzymes. To 100–110 μM solutions of rabbit muscle creatine kinase in 1.0 ml of ice-cold 0.01 M glycine–NaOH buffer (pH 7.8) were added various concentrations of 0.106 M [^{14}C]methyl methanethiolsulfonate. Molar ratios used in inhibition titrations varied from 0.5 to 4.08 (inhibitor:enzyme). The samples were incubated in closed vials at 4° for 30 min. Samples were then passed through a Sephadex G-25 column (0.9 × 23 cm, all Teflon connections) which had been previously equilibrated with ice-cold 0.01 M glycine–NaOH buffer (pH 7.8). Column eluents were monitored at 280 nm using a Buchler fracto-scan. Protein-containing fractions (5 ml) were collected directly in a cold Amicon ultrafiltration cell. The excess methyl methanethiolsulfonate could also be detected at 280 nm, and typically emerged from the column well after the protein band (*ca.* 1.5 void volumes). The protein sample was concentrated to a final volume of 1.5 ml by applying 40 psi N_2 pressure while stirring and cooling at 4°. Final protein concentrations were determined spectrophotometrically at 280 nm. A 1.0-ml sample was pipetted into a scintillation vial with 9.0 ml of Aquasol and counted. The remainder of the sample was assayed for enzymatic activity. When the enzyme was modified with DTNB, the extent of reaction was followed by monitoring the release of the thionitrobenzoate anion spectrophotometrically (Ellman, 1959; Blumberg *et al.*, 1970).

It is extremely important to avoid spurious contamination by thiols in these assays. That is, any equipment that has had any contact with thiols (for example, syringes and electrodes) should be thoroughly washed before use. Even brief contact of blocked protein with rubber or plastic connections should be avoided. There are preliminary indications that enzymes used in coupled assay procedures may also lead to removal of these sulfhydryl blocking groups.

Demonstration of Sulfhydryl Protection by Alkanethiolation: Iodoacetamide Treatment of S-(Methanethio)-Creatine Kinase. *S*-(Methanethio)-creatine kinase (8.74×10^{-2} μmol, prepared as above) was exposed to 6.15 μmol (140 molar excess) of iodoacetamide for 20 min at 23°. The solution was then passed through Sephadex G-25, concentrated, and assayed for enzymatic activity. A small sample was treated with excess β-mercaptoethanol for 15 min at 0° and then assayed for activity. As a control a sample of unblocked enzyme was treated under the same conditions.

Treatment of Creatine Kinase with $^{14}CH_3HgCl$. To 3.7×10^{-5} mmol of rabbit muscle creatine kinase dissolved in

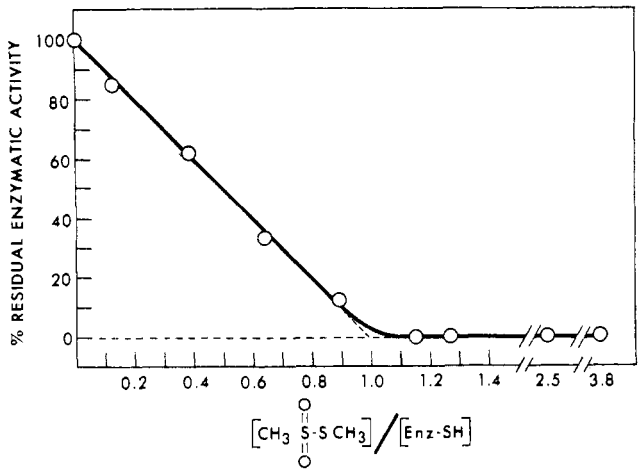


FIGURE 1: Titration of papain with methyl methanethiolsulfonate. Aliquots of papain (5 ml of 9.8 μM enzyme in 0.01 M glycine buffer containing 5 mM EDTA) were titrated with various amounts of 20 μM methyl methanethiolsulfonate at pH 8.0. Samples were incubated for 30 min at 0° and then assayed as described under Materials and Methods. The papain used in this titration was activated with 30 mM β-mercaptoethanol–25 mM EDTA (pH 4.5). Thiol was removed prior to titration by eluting the enzyme from a Sephadex G-25 column with titration buffer. The active sulfhydryl content of papain was 47% as determined by DTNB titration.

1.0 ml of 0.01 M glycine (pH 8.0) was added 1.85×10^{-4} mmol of $^{14}CH_3HgCl$, and the mixture was incubated for 1 hr at 4°. The sample was passed through a Sephadex G-25 column and then assayed for both enzymatic activity and extent of ^{14}C incorporation. The blocked enzyme was then treated with a 160 molar excess of iodoacetamide for 20 min at 25° and again assayed for activity.

Results

When papain was treated at pH 8.0 with excess [^{14}C]methyl methanethiolsulfonate, rapid and complete inhibition of activity was observed, and 1.0 equiv of $^{14}CH_3S$– was incorporated/mol of active enzyme. A titration curve, shown in Figure 1, demonstrates a 1:1 inverse correspondence between residual enzymatic activity and CH_3S– incorporation. Complete and extremely rapid inhibition was also observed when papain was treated with excess methoxycarbonylmethyl disulfide. The *S*-(methanethio)-papain was completely protected against normally inhibitory DTNB. Full activity was restored by treatment with excess β-mercaptoethanol.

Previous results on the titration of rabbit muscle creatine kinase had shown that introduction of the relatively small CH_3S– temporary blocking group onto the "active" sulfhydryl group leads to enzyme with substantial (18 ± 2%) residual enzymatic activity (Smith and Kenyon, 1974). In order to examine the effect of another relatively small, neutral sulfhydryl blocking agent on creatine kinase, the enzyme was treated with a fivefold molar excess of $^{14}CH_3HgCl$; 3 equiv of $^{14}CH_3Hg$– were incorporated/mol of enzyme (dimer). No loss of activity was observed. Treatment of the MeHg–CK with a large excess of iodoacetamide resulted in complete loss of activity.

When native creatine kinase was treated with DTNB, complete loss of activity was observed when 1.0 equiv of the blocking group was attached. Blocked enzyme which had been isolated by passing the sample through Sephadex G-25 was then treated with [^{14}C]NaCN following the procedures of Vanaman and Stark (1970). Release of the thionitrobenzoate anion was followed spectrophotometrically, and, after

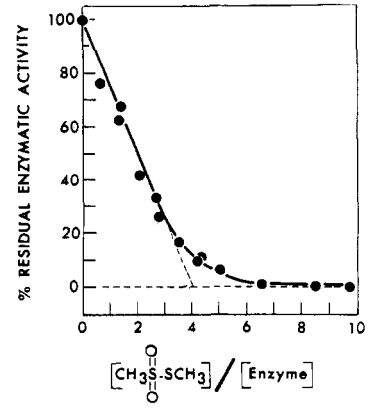


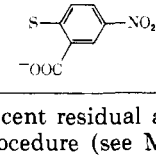
FIGURE 2: Titration of glyceraldehyde-3-phosphate dehydrogenase with methyl methanethiolsulfonate. A 6.0-ml aliquot of 6.14 μM glyceraldehyde-3-phosphate dehydrogenase was titrated with 0.50 mM methyl methanethiolsulfonate in 15 mM sodium pyrophosphate–30 mM sodium arsenate–1 mM EDTA buffer (pH 8.5). Samples were incubated for 15 min at 0° and assayed as described under Materials and Methods. The enzyme used in this titration was activated with 8 mM dithiothreitol–1.0 mM EDTA–pyrophosphate–arsenate buffer (pH 8.5) for 30 min at 0°. Thiol was removed prior to titration by gel chromatography on Sephadex G-25.

all of this anion had been released, the isolated enzyme was observed to have incorporated only a small amount of radioactivity (~15 mol %). The protein had *ca.* 30% of the original enzymatic activity; this activity was lost when the enzyme was treated with excess iodoacetamide.

The effect of increasing bulk of the attached RS– group was examined for rabbit muscle creatine kinase. The results in Table I show a trend toward lower residual activity with increasing bulk.

When glyceraldehyde-3-phosphate dehydrogenase was titrated with methyl methanethiolsulfonate, the results in Figure 2 were obtained. This enzyme has been shown to be

Table I: Effect of the Chemical Nature of Attached RS– Substituent on Per Cent Residual Activity of Rabbit Muscle Creatine Kinase.[a]

Enz-S-R, where R =	% Residual Activity (±2%)
H	(100)
SCH_3[b,c]	19
SCH_2CH_3[b]	11
SCH_2CF_3[c]	~6[d]
S—CH_2—CH_2CH_3[b]	8
S—CCl_3[b]	1.4
S—SO_3^-	0[e]
	0

[a] Per cent residual activities were measured by the pH-Stat procedure (see Materials and Methods) under V_{max} conditions. In each case the attached RS– group was observed to provide complete protection against normally inhibitory iodoacetamide. Also, each of the RS– groups could be removed by treatment with β-mercaptoethanol, leading to complete restoration of enzymatic activity. [b] Delivered as its alkyl alkanethiolsulfonate (see Materials and Methods). [c] Delivered as its methoxycarbonylalkyl disulfide (see Materials and Methods). [d] Owing to a greater instability than was observed for other attached RS– groups, this value is somewhat less reliable. [e] Kassab *et al.* (1968). [f] Delivered using DTNB.

a tetramer composed of four identical subunits (Harris and Perham, 1965). The results indicate that one of the two reactive sulfhydryl groups per subunit is modified faster than the other. When the enzyme was treated with a 100 molar excess of [^{14}C]methyl methanethiolsulfonate for 100 min at 4°, 8.18 equiv of $^{14}CH_3S$- group was bound/mol of enzyme. This fully blocked enzyme showed less than 0.3% residual activity. Treatment with either dithiothreitol or β-mercaptoethanol led to full restoration of enzymatic activity.

Discussion

We have recently observed that stoichiometric incorporation of a CH_3S- group onto the active sulfhydryl group of rabbit muscle creatine kinase led to enzyme with 18 ± 2% residual catalytic activity (Smith and Kenyon, 1974). In contrast, others have reported essentially complete inhibition of enzymatic activity with stoichiometric incorporation of a wide variety of common thiol blocking reagents (Watts, 1973). The results described here indicate that the chemical nature (*e.g.*, bulk, charge, hydrophobicity, hydrogen-bonding ability) of the blocking group not only can lead to different amounts of residual activity of the modified enzyme, but also the nature of the reagent can determine the site of reaction. Results shown in Table I indicate, in general, an inverse correspondence between size of the attached group on the active sulfhydryl and residual enzymatic activity. Sasa and Noda (1964) and later Cohn (1970) postulated that modification of the active sulfhydryl group of creatine kinase causes conformational changes which interfere with the interactions between creatine and nucleotide binding sites. Since bulkier substituents would be expected to bring about greater conformational changes on the enzyme surface, the results in Table I are consistent with the hypothesis of Noda and Cohn.

Incorporation of a negative charge appears to be especially detrimental, since iodoacetate (Ennor and Rosenburg, 1954, O'Sullivan and Cohn, 1966) delivers the completely inhibitory carboxymethyl group to the active sulfhydryl of creatine kinase, and this carboxymethyl group is sterically smaller than the $CH_3CH_2CH_2S$- group for which we observed 8% residual activity. Similarly, tetrathionate, also delivering a negatively charged group, likwise led to complete inhibition of creatine kinase (Kassab *et al.*, 1968). Iodoacetamide, which delivers a neutral group of about the same size as the carboxymethyl group, also completely inhibits creatine kinase (Ennor and Rosenburg, 1954; O'Sullivan *et al.*, 1966), presumably owing to its ability to behave as a hydrogen bonding donor-acceptor.

Peptide sequencing studies of Mahowald (1965) and Thomson *et al.* (1964) have established that both iodoacetate and 2,4-dinitrofluorobenzene modify the same, active cysteinyl residue on the enzyme (which we shall call cys-I). Owing both to its structural similarity to iodoacetate and to its behavioral similarity to iodoacetate in several studies, iodoacetamide is generally assumed to modify cys-I also (O'Sullivan *et al.*, 1966; O'Sullivan and Cohn, 1966; Watts, 1973).

The most convincing piece of evidence that we are indeed blocking cys-I (which is commonly called the "essential" cysteine residue) is the fact that we observed complete protection of MeS-CK against inhibition by iodoacetamide. Recently, treatment of creatine kinase with 2-chloromercuri-4-nitrophenol was found to cause selective modification of another thiol group (which we shall call cys-II) (Quiocho and Thomson, 1973; Quiocho and Olson, 1974). With 1:1 incorporation per subunit they observed no loss of enzymatic activity. In contrast to our findings with the MeS-CK, they found that enzyme blocked with 2-chloromercuri-4-nitrophenol showed complete loss of activity upon treatment with excess iodoacetamide. Earlier, Sasa and Noda (1964) had shown that treatment of creatine kinase with equimolar amount of *p*-chloromercuribenzoate (PCMB) at 0° for 30 min at pH 9 substantially diminished the inherent ATPase activity of the enzyme (Sasa and Noda, 1964), but had no effect on the creatine kinase activity. Ennor and Rosenberg (1954) previously had shown that larger excesses of PCMB totally inhibited the enzyme. These results of Sasa and Noda and Ennor and Rosenberg suggest that at low concentrations PCMB is modifying only cys-II and that at higher concentrations cys-I is being modified.

Our results strongly indicate that yet a third organomercurial, CH_3HgCl, is also selectively modifying cys-II since the resulting MeHg-CK was fully active and the MeHg-CK could in turn be completely inhibited upon treatment with excess iodoacetamide. The covalent radius of the mercury atom is 1.44 Å whereas the corresponding value for the sulfur atom is 1.04 Å (Dean, 1973). If both blocking groups were attached to cys-I, it is unlikely that the CH_3S- group would lead to 81% reduction in enzymatic activity whereas the bulkier CH_3Hg- group would have no effect. Furthermore, if the CH_3S- and the CH_3Hg- groups were attached to the same sulfhydryl (cys-I or cys-II), one would not expect to see such a dramatic difference in the susceptibility of the blocked enzyme toward iodoacetamide inhibition. At this time, it is not known why the three organomercurials, in contrast to the other sulfhydryl reagents, prefer to react with cys-II rather than cys-I.

In an attempt to block cys-I with the even smaller NC- group, we treated DTNB-inactivated enzyme with [^{14}C]NaCN, following procedures used by Vanaman and Stark (1970) for the cyanylation of aspartate transcarbamylase. After release of a stoichiometric amount of thionitrobenzoate anion, only a relatively small amount of [^{14}C]NC- was incorporated. The reason for the incomplete substitution is not readily apparent to us, but the results serve to point out the necessity of using radioactively labeled cyanide when attempting to use enzyme-5-thiol-2-nitrobenzoate mixed disulfides as intermediates for cyanylation of sulfhydryl groups of enzymes.

The results with creatine kinase indicated that both methyl methanethiolsulfonate and methoxycarbonylmethyl disulfide were reacting rapidly, selectively, and quantitatively with "active" sulfhydryl group. In order to examine the generality of the use of the CH_3S- group in the selective modification of sulfhydryl groups of other enzymes, we have investigated two additional enzyme systems which have "essential" sulfhydryl groups, namely, papain and rabbit muscle glyceraldehyde-3-phosphate dehydrogenase.

Papain contains only one free sulfhydryl group per molecule, and this sulfhydryl group has been postulated to be required for enzymatic activity by a number of workers (Glazer and Smith, 1971). Degani *et al.* (1970) showed that even when papain's sulfhydryl group was modified with the relatively small NC- group, all activity was lost. Our results show that with clean 1:1 incorporation of a CH_3S- group, total inhibition of enzymatic activity was observed. This confirms the selectivity of our reagents for the modification of active or accessible sulfhydryl groups in the presence of

other amino acid side chain residues, and also confirms the finding of Degani *et al.* (1970).

Of the eight reactive sulfhydryl groups per mole of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (two per subunit) (Moore and Fenselau, 1972), four (one per subunit) have been found to be generally more reactive toward sulfhydryl blocking reagents. The more reactive sulfhydryl group, Cys-149, has been postulated (Perham and Harris, 1963) to be intimately involved in the catalytic mechanism. Even though our reagent eventually blocked all eight sulfhydryl groups, four groups react faster than the others. If one extrapolates the initial slope of the titration curve shown in Figure 2, it can be seen that after modification of 4.0 sulfhydryl groups/mol of enzyme, essentially complete enzymatic activity would be lost. The results are consistent with the idea that our reagent modifies Cys-149 much more rapidly than Cys-153. The results are also consistent with the fact that Cys-149 is essential to the catalytic mechanism. The postulate that Cys-149 is essential to the catalytic mechanism of lobster glyceraldehyde-3-phosphate dehydrogenase was recently strengthened by the observation of Olsen *et al.* (1974) that in the crystalline state this thiol group is located very close to the nicotinamide ring of the bound NAD^+.

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