Two Sulfhydryl Groups near the Active Site of Thiolase I from Porcine Heart: Modification of Thiolase with the Fluorescent Thiol Reagent *S*-Mercurio-*N*-dansyl-L-cysteine†

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ABSTRACT: The reaction of porcine heart thiolase I with an excess of the fluorescent thiol reagent *S*-mercurio-*N*-dansyl-L-cysteine (Dns-Cys-SHg^+) leads to complete inactivation of the enzyme. The reaction occurs at pH 7.0 with a rate constant of $(1.5 \pm 0.4) \times 10^5$ M^{-1} min^{-1}. Inactivation of the enzyme is associated with a large increase in the intensity of the fluorescence emission spectrum of Dns-Cys-SHg^+ and a 55-nm blue shift of the maximum emission wavelength. The changes in fluorescence occur with a rate constant of $(1.1 \pm 0.3) \times 10^5$ M^{-1} min^{-1}, comparable to the rate constant for the loss of activity. Saturating concentrations of the substrate acetoacetyl-CoA inhibit the inactivation of the enzyme and any associated changes in the fluorescence spectrum. The fluorescent label is not removed by gel filtration and does not spontaneously dissociate from the enzyme for more than 2 h at pH 7.0. The Dns-Cys-SHg^+ can be displaced from the enzyme by excess 2-mercaptoethanol with full recovery of thiolase activity and the spectral characteristic of the native enzyme. Reaction of denatured thiolase I with Ellman's reagent shows that there are 4.9 ± 0.3 sulfhydryl groups per subunit. In the native enzyme only two sulfhydryl groups react with Ellman's reagent but with a rate constant that is 10^4 slower than the reaction of free cysteine. The reaction of Dns-Cys-SHg^+ with thiolase leads to the incorporation of 1.7 ± 0.2 mol of reagent/mol of thiolase subunit. Covalent modification of the enzyme by alkylation of only one sulfhydryl group with iodoacetamide or by acetylation of the enzyme with acetyl-CoA leads to lower but still significant amounts of incorporated Dns-Cys-SHg^+ (1.1 and 1.2 mol/mol of active sites, respectively). The intensity of the fluorescence emission spectrum of Dns-Cys-SHg^+ bound to the acetylated or alkylated enzyme is 65% and 80%, respectively, of the intensity observed on modification of the native enzyme. The acetyl group of the acetyl enzyme efficiently protects against inactivation of thiolase by Dns-Cys-SHg^+ but not against the incorporation of 1 equiv of Dns-Cys-SHg^+. Since no inactivation, label incorporation, or fluorescence change is observed in the presence of the substrate acetoacetyl-CoA, these results are consistent with the existence of two sulfhydryl groups at or near the active site of thiolase. Only one group is "essential" for activity and is probably involved in acetyl enzyme formation in the reaction catalyzed by the enzyme. Quenching of intrinsic tryptophan fluorescence by a series of ionic and nonionic quenchers in the presence and absence of substrate and Dns-Cys-SHg^+ suggests that the active site of the enzyme is hydrophobic.

Thiolase catalyzes the coenzyme A (CoA)[1] dependent cleavage of AcAcCoA into two molecules of AcCoA.

$$\text{AcAcCoA} + \text{CoASH} \rightleftarrows 2\text{AcCoA} \quad (1)$$

The enzyme from porcine heart exists in two isozyme forms, both of which are mitochondrial (Middleton, 1973). Thiolase I catalyzes the cleavage of various long chain 2-ketoacyl-CoA esters while thiolase II is specific for AcAcCoA as the substrate (Staack et al., 1978). The reaction mechanism for both thiolases I and II involves the formation of a covalent acetyl enzyme intermediate (Gehring et al., 1968; Gilbert et al., 1981).

$$\text{AcAcCoA} + \text{E} \rightleftharpoons \text{E–Ac} + \text{AcCoA} \quad (2)$$

$$\text{E–Ac} + \text{CoASH} \rightleftharpoons \text{E} + \text{AcCoA} \quad (3)$$

For thiolase II, the acetyl enzyme intermediate has been shown to be a thiol ester formed betwene the acetyl group of substrate and a cysteine residue on the enzyme (Gehring et al., 1968). Although there are significant kinetic differences between the two isozymes (Gilbert et al., 1981), thiolase I might be expected to have one reactive cysteine residue at the active site (Raaka & Lowenstein, 1979).

We were interested in determining if thiolase I contained an active-site cysteine which could be used in combination with fluorescent reagents and substrate analogues to probe the environment of the active site. In this study, we have used Dns-Cys-SHg^+ as a fluorescent sulfhydryl reagent to modify and inactivate thiolase I. This reagent has many advantageous features, of which the high specificity of the reaction with thiols is particularly important (Leavis & Lehrer, 1974). The use of Dns-Cys-SHg^+ has allowed us to correlate the loss of enzyme activity with the reaction of the fluorescent label with two sulfhydryl groups of the enzyme.

Experimental Procedures

Materials. CoA was obtained from P-L Biochemicals. AcAcCoA was prepared as described previously (Gilbert et al., 1981). Didansyl-L-cystine (free acid) [$(Dns\text{-}Cys)_2$], *N*-acetyl-L-tryptophanamide, iodoacetamide, and $Hg(NO_3)_2$ were from Sigma Chemical Co. Gdn·HCl was from Bethesda

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[1] Abbreviations: CoA, coenzyme A; AcCoA, acetyl coenzyme A; AcAcCoA, acetoacetyl coenzyme A; dansyl (Dns), 5-(dimethylamino)-naphthalene-1-sulfonic acid; Dns-Cys, dansyl-L-cysteine; $(Dns\text{-}Cys)_2$, didansyl-L-cystine; Dns-Cys-SHg^+, *S*-mercurio-*N*-dansyl-L-cysteine; DTNB, 5,5′-dithiobis(2-nitrobenzoate); EDTA, ethylenediaminetetraacetic acid; Gdn·HCl, guanidine hydrochloride; HPLC, high-performance liquid chromatography; *N*-Ac$TrpNH_2$, *N*-acetyl-2-tryptophanamide; Tris, tris(hydroxymethyl)aminomethane.

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Research Laboratories, and CsCl (ultrapure) was from Fisher Scientific. Acrylamide purchased from Bio-Rad was recrystallized from ethyl acetate, and 2,2,2-trichloroethanol was obtained from Aldrich. Preparation and characterization of Dns-Cys-SHg^+ were by the method of Leavis & Lehrer (1974). The final product moved as a single spot on Eastman 6060 silica gel plates in chloroform–methanol–acetic acid (70:25:5) as solvent system. Dns-Cys was obtained as an intermediate in the synthesis of Dns-Cys-SHg^+. The stock solutions of Dns-Cys were stored in HNO_3, pH 3. Water used in all experiments was deionized and glass distilled.

Methods. Enzyme activity and all other spectrophotometric measurements were made by using a Varian 634 double-beam recording spectrophotometer with the cell compartment thermostated at 25 °C. For pH measurements, a Corning Model 130 pH meter equipped with a Radiometer GK 2321 combination electrode was used. High-performance liquid chromatography (HPLC) was performed on a 0.5 × 25 cm ODS-I reversed phase column (Custom L C, Houston, TX). Elution was accomplished with a linear gradient of 60% methanol in 0.04 M ammonium formate to 100% methanol over 10 min at a flow rate of 1 mL/min. Fluorescence of the eluate was monitored by a FS 970 L. D. fluorometer (Schoeffel) with a GM 970 monochromator. Excitation was at 350 nm, and a 418-nm band-pass filter was used for emission measurement.

The concentrations of Dns-Cys-SHg^+ and Dns-Cys were determined from the absorbances at 350 and 292 nm (Leavis & Lehrer, 1974). The concentration of Dns-Cys was determined by the method of Ellman (1958).

Fluorescence measurements were performed with a Perkin-Elmer MFP 540-60 spectrofluorometer equipped with a Hitachi 650-0178 data processor and a circulating water bath. In addition to standard 10 × 10 mm quartz cells, 10 × 2 mm microcuvettes (NSG Precision Cells) were routinely used. The instrument was operated exclusively in the ratio mode. All measurements were done at 25 °C. The spectral bandwidth of the excitatioin was in the range of 5 nm unless specifically noted, and the emission bandwidth was never larger than 10 nm. Except for the determination of the thiolase emission spectrum, all other spectra were not corrected for the photomultiplier response. Wavelength accuracy of the excitation and emission monochromators was checked against the spectrum of the xenon lamp and found to be accurate within ±1.5 nm. All solutions used in fluorescence measurements were filtered prior to use through a 0.45-μm Metricel membrane or 0.45-μm Millipore filters.

Enzyme Preparation. Porcine heart thiolase I was purified as previously described (Gilbert et al., 1981) with minor modifications. The enzyme preparation had a specific activity of 15–20 units/mg of protein with the unit of activity defined as before (Gilbert et al., 1981). The preparation was at least 95% homogeneous on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis according to the procedure of Laemmli (1970). For all measurements, the enzyme stock solution was freshly dialyzed against argon-degassed 0.1 M phosphate buffer, pH 6.8.

Thiolase Inactivation Experiments. Thiolase activity was measured spectrophotometrically as described by Gilbert et al. (1981). For enzyme inactivation experiments, incubations were performed at 25 °C in 20 mM Tris–acetate buffer, pH 7.0, containing 0.3–1.0 unit/mL thiolase. After the starting thiolase activity in the sample was measured, aliquots of the concentrated stock solutions of appropriate inactivator were added in a volume not larger than 5% of the total sample. Dns-Cys-SHg^+, Dns-Cys, and Hg^{2+} were added as solutions in HNO_3, pH 3, without a change in the pH of the enzyme solution. $(Dns\text{-}Cys)_2$ was added in 50 mM Tris–acetate buffer, pH 8.0, and incubated with thiolase in the same buffer.

Stoichiometry of Dns-Cys-SHg^+ Incorporation. Native thiolase, the iodoacetamide-alkylated enzyme, or the acetyl enzyme (0.3–0.9 unit) in a volume of 0.3 mL of 20 mM Tris–acetate buffer, pH 7.0, was incubated with Dns-Cys-SHg^+ (15–20 μM) for 10 min. For modification of the native enzyme, this time corresponds to inactivation of approximately 95% of the original enzyme activity. The modified enzyme was applied to a 1 × 16 cm column of Sephadex G-75 equilibrated with the incubation buffer and previously calibrated with thiolase to determine the excluded volume. An aliquot of each thiolase-containing fraction was treated with 0.15 M 2-mercaptoethanol for 15 min at pH 7.0 and analyzed for free Dns-Cys by HPLC. Dns-Cys of known concentration was used as a standard. HPLC of aliquots not treated with 2-mercaptoethanol showed only low levels (<15% of treated enzyme) of Dns-Cys or Dns-Cys-SHg^+. Thiolase concentrations were determined in each aliquot by measuring the enzyme activity after treatment with 2-mercaptoethanol or by measuring the intensity of protein fluorescence in 6 M Gdn·HCl using native thiolase as a standard. Thiolase concentrations determined by both methods generally agreed within 15% for the native and acetyl enzymes.

For stoichiometry measurements with the acetyl enzyme, the acetyl enzyme was prepared by incubation of thiolase with 0.5 mM AcCoA followed by gel filtration on Sephadex G-75 (Gilbert et al., 1981). The peak protein fraction (0.4 unit) was incubated with 4.3 μM Dns-Cys-SHg^+ for 40 min and subjected to gel filtration as before. For measurements with the iodoacetamide-alkylated enzyme, thiolase (0.9 unit) was incubated with 70 μM iodoacetamide in 20 mM Tris–acetate buffer, pH 7.0, for 20 min and subsequently with 4.3 μM Dns-Cys-SHg^+ for an additional 20 min. After gel filtration, stoichiometry measurements were performed as described above.

Determination of Sulfhydryl Groups by Reaction with DTNB. The total number of sulfhydryl groups was determined by incubating thiolase (1–2 units) in a volume of 0.2 mL with 6 M Gdn·HCl and 0.2 M phosphate buffer, pH 7.5, for approximately 30 min. DTNB was added to a final concentration of 1.0 mM to the denatured enzyme and to a cell in the reference beam of the spectrophotometer containing only buffer and Gdn·HCl. The concentration of sulfhydryl groups was calculated from the observed absorbance increase at 412 nm by using an extinction coefficient of 1.36×10^4 M^{-1} cm^{-1} (Ellman, 1958).

The iodoacetamide-alkylated enzyme was prepared by incubation of thiolase (12 units/mL) with 80 μM iodoacetamide in 0.2 M phosphate buffer pH 7.5, for 40 min or with 0.8 mM iodoacetamide for 10 min. The alkylated enzyme was then denatured by incubation with 6 M Gdn·HCl for 30 min, and the total number of remaining sulfhydryl groups was determined as above.

Fluorescence Quenching Titrations. For observing intrinsic protein fluorescence, the excitation wavelength was 295 nm (slit 5 nm) and emission was at 340 nm (slit 7 nm). For quenching of Dns-Cys-SHg^+ bound to thiolase, excitation was at 350 nm with emission at 505 nm. Control titrations were performed with an *N*-AcTrpNH_2 solution in 0.1 M Tris–acetate buffer, pH 7.0, with 6 M Gdn·HCl or a Dns-Cys solution in the same solvent. Absorbance of the solutions did not exceed 0.05 unit. All data were corrected for dilution

caused by the addition of the titrant. Blank titrations were performed on identical samples without enzyme and, if necessary, subtracted from the titration curve. The corrections were less than 30% in the most quenched samples and generally in the range of 1–5%. For titrations with trichloroethanol and acrylamide, 1–5-μL aliquots of the titrant solution (10.4 and 6 M, respectively) were added to 1–2 mL of 20 mM Tris–acetate buffer, pH 7.0, containing 0.1 M KCl and about 0.15 unit/mL thiolase. The solution was stirred and the fluorescence read after 2 min. For fluorescence quenching with Cs^+ and I^-, the measurements were done in 0.1 M Tris–acetate buffer, pH 7.0, at constant ionic strength of 0.6 M maintained with KCl. The solution of KI contained 0.12 mM $Na_2S_2O_3$ to prevent formation of I_3^-. In these titrations, separate solutions were prepared for each data point. Total volume of the samples was 200 μL with about 0.15 unit/mL thiolase. There was no significant effect of trichloroethanol or acrylamide on thiolase activity during the quenching experiments at concentrations of quenchers up to 0.5 M.

For preparation of the sample of Dns-Cys-SHg^+-modified thiolase for fluorescence quenching, fast separation of excess ligand was achieved by passing the incubation mixture under low argon pressure through a 1-mL syringe filled with Sephadex G-75 and equilibrated with incubation buffer. Aliquots of 50–100 μL were applied onto the column, and the elution was accomplished by several washes with 100-μL aliquots of the incubation buffer. Collected fractions were assayed for thiolase activity after treatment with 0.15 M 2-mercaptoethanol.

Experimental data corrected for dilution effect were analyzed according to the Stern–Volmer equation (eq 4) (Stern & Volmer, 1919) and according to the modified Stern–Volmer equation (eq 5) (Lehrer, 1971):

$$F_0/F = 1 + K_{sv}[Q] \quad (4)$$

$$F_0/\Delta F = \frac{1}{f_{a(eff)}} + \frac{1}{f_{a(eff)}K_{Q(eff)}[Q]} \quad (5)$$

where F_0 and F are respectively fluorescence in the absence and presence of quencher Q, K_{sv} is the Stern–Volmer constant for the collisional quenching, ΔF is defined as $F_0 - F$, and $f_{a(eff)}$ and $K_{Q(eff)}$ are the maximum fraction of quenchable fluorescence and the effective quenching constant, respectively. The values of $f_{a(eff)}$ and $K_{Q(eff)}$ were obtained from the plot $F_0/\Delta F$ vs. $[Q]^{-1}$.

Results

Inactivation of Thiolase by Dns-Cys-SHg^+. Inactivation of thiolase (0.2 μM) by several concentrations of Dns-Cys-SHg^+ is shown in Figure 1. The second-order rate constant for inactivation determined from the slope of plots of k_{obsd} vs. Dns-Cys-SHg^+ concentration is $(1.5 \pm 0.4) \times 10^5$ M^{-1} min^{-1}. Inactivation was more than 95% at all concentrations of Dns-Cys-SHg^+. The residual activity (about 5%) observed after more than seven half-lives of the reaction at least partially represents the effect of reactivation of thiolase by free CoA present in the assay mixture in high excess over enzyme. Saturating concentrations of AcAcCoA (100 μM) provided protection against inactivation by Dns-Cys-SHg^+. With 13 μM Dns-Cys-SHg^+, the reaction in the presence of AcAcCoA is approximately 50-fold slower than in the absence of AcAcCoA. Dns-Cys and $(Dns\text{-}Cys)_2$, which might be contaminants of Dns-Cys-SHg^+ or appear in equilibrium with the compound in buffer, were found to have no effect on thiolase activity when used in the concentration range comparable to that of Dns-Cys-SHg^+ (Izbicka-Dimitrijević & Gilbert, 1982).

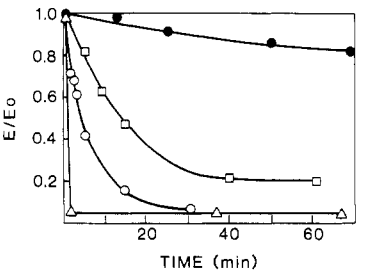

FIGURE 1: Time course of Dns-Cys-SHg^+ inactivation of thiolase. Enzyme (0.2 μM) was incubated in 20 mM Tris–acetate buffer, pH 7.0, with the following concentrations of Dns-Cys-SHg^+: (○) 1.6 μM; (△) 7 μM; (□) 13.4 μM in the presence of 100 μM AcAcCoA; (●) no additions. At indicated times, aliquots were assayed for enzyme activity as described under Experimental Procedures. E and E_0 represent activities at the given time and at zero time.

Dns-Cys-SHg^+ forms a covalent complex with thiolase. Enzyme inactivated by the reagent (until less than 8% residual activity was measured) was passed through a Sephadex G-75 column. No activity was detected in the excluded volume. Treatment of the excluded volume fractions with 0.15 M 2-mercaptoethanol at pH 7.0 for 15 min resulted in recovery of more than 90% of the original enzyme activity. Controls performed routinely on Dns-Cys-SHg^+-inactivated thiolase showed that in all cases 90–95% activity could be recovered after treatment of the inactivated enzyme with 2-mercaptoethanol. There was no spontaneous loss of the label from the enzyme, as indicated by a constant low level of activity in samples not reactivated by 2-mercaptoethanol.

Inactivation of Thiolase with Iodoacetamide. Incubation of 0.2 μM thiolase with 24–80 μM iodoacetamide in 20 mM Tris–acetate buffer, pH 7.0, results in irreversible inactivation of the enzyme. The second-order rate constant, determined from the linear plot of k_{obsd} vs. iodoacetamide concentration, is $(2.7 \pm 0.4) \times 10^3$ M^{-1} min^{-1}. The presence of 100 μM AcAcCoA provided full protection against inactivation of thiolase by 24 μM iodoacetamide. Enzyme (2.5 μM) incubated in 0.1 M phosphate buffer, pH 6.8, for 60 min with iodoacetamide at ratios of iodoacetamide to thiolase monomer in the range 0.2–5.0 shows that a ratio of iodoacetamide to thiolase subunit of 1.0 ± 0.2 is sufficient to completely inactivate the enzyme.

The reaction of iodoacetamide with thiolase previously modified with Dns-Cys-SHg^+ was also examined. Thiolase (0.2 μM), inactivated to less than 5% residual activity by reaction with 3 μM Dns-Cys-SHg^+ in 20 mM Tris–acetate, pH 7.0, was treated with 24 μM iodoacetamide. At various times an aliquot of the reaction mixture was withdrawn, and 0.1 M 2-mercaptoethanol was added to stop the reaction with iodoacetamide and to reactivate any Dns-Cys-SHg^+-inactivated enzyme. Under these conditions, a gradual loss of the ability to reactive the enzyme upon incubation with 0.1 M 2-mercaptoethanol was observed. This irreversible inactivation due to reaction with iodoacetamide was a factor of 4 slower than the rate of thiolase inactivation with iodoacetamide in the absence of Dns-Cys-SHg^+.

Reaction of the Acetyl Enzyme with Iodoacetamide and Dns-Cys-SHg^+. If the acetyl enzyme (prepared by incubation of 0.5 units of thiolase with 0.43 mM AcCoA followed by gel filtration) was mixed with 8 μM Dns-Cys-SHg^+ (12-min lapsed time since addition of AcCoA), a biphasic curve of log (percent activity) vs. time was seen (Figure 2). After an initial rapid loss of 18% of the original activity, a slow loss of enzyme activity was observed which was identical in the presence and absence of Dns-Cys-SHg^+. Under the same conditions, native

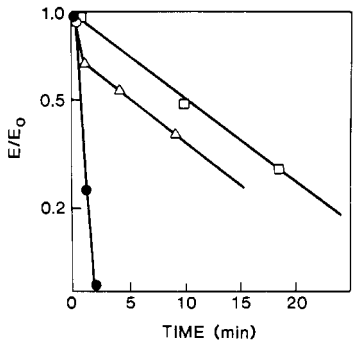

FIGURE 2: Protection against inactivation with Dns-Cys-SHg$^+$ by the acetyl group in the acetyl enzyme. Acetyl enzyme was prepared by incubation of thiolase with 0.43 mM AcCoA, followed by gel filtration. (□) Acetyl enzyme alone; (△) acetyl enzyme incubated with 8.1 μM Dns-Cys-SHg$^+$; (●) native enzyme (0.2 μM) incubated with 8.1 μM Dns-Cys-SHg$^+$. Incubations were performed in 20 mM Tris–acetate buffer, pH 7.0, at 25 °C. E and E_0 represent activities at the given time and at zero time.

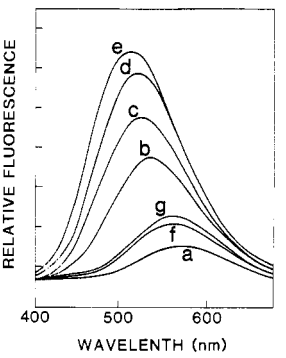

FIGURE 3: Fluorescence emission spectra of thiolase (0.37 μM) incubated with 2.8 μM Dns-Cys-SHg$^+$ in 20 mM Tris–acetate buffer, pH 7.0. (a) Dns-Cys-SHg$^+$ without thiolase; (b) Dns-Cys-SHg$^+$ after 2-min incubation with thiolase; (c) incubation 8 min; (d) incubation 12 min; (e) incubation 25 min; (f) sample after 30-min incubation treated with 80 mM 2-mercaptoethanol, spectrum recorded after 10 min, and Dns-Cys-SHg$^+$ at the same concentration treated with 80 mM 2-mercaptoethanol; (g) thiolase (0.37 μM) incubated with 2.8 μM Dns-Cys-SHg$^+$ in the presence of 0.51 mM AcAcCoA and spectrum recorded after 10 min. Fluorescence excitation wavelength was at 295 nm.

thiolase (not the acetyl enzyme) is completely inactivated in less than 3 min (filled symbols, Figure 2). The slow inactivation of the acetyl enzyme was fully reversible (more than 85%) by treatment with 0.5 M 2-mercaptoethanol for 10 min. The activity loss of the acetyl enzyme in the absence of Dns-Cys-SHg$^+$ is significantly faster than that observed for the native enzyme. This may reflect an increase rate of oxidation of the acetyl enzyme, since the spontaneous inactivation could be reversed by incubation with 2-mercaptoethanol. However, it is clear from Figure 2 that the acetyl enzyme is protected against inactivation by Dns-Cys-SHg$^+$.

Stoichiometry of Thiolase Reaction with Dns-Cys-SHg$^+$. The amount of Dns-Cys-SHg$^+$ bound to thiolase could be quantitated by isolating the modified enzyme by gel filtration and measuring the amount of Dns-Cys released from the enzyme after treatment with 2-mercaptoethanol. Thiolase (0.7 μM) was incubated with 28-fold molar excess of Dns-Cys-SHg$^+$ at pH 7.0 for 10 min (over seven half-lives for activity loss) and subjected to gel filtration. The protein-containing fractions collected from the column were treated with 2-mercaptoethanol and analyzed for free Dns-Cys by reverse-phase HPLC using a fluoresence detector. Under the experimental conditions, only low molecular weight species were resolved by HPLC, while protein was retained on the column. An average value of 1.5 ± 0.3 mol of released Dns-Cys/mol of thiolase subunit was found based on a thiolase molecular weight of 46 000 per subunit and a specific activity of 16 units/mg of protein. Samples of modified enzyme not incubated with 2-mercaptoethanol showed only a small amount of Dns-Cys or Dns-Cys-SHg$^+$ not exceeding 15% of that shown by the treated samples. When the reaction of Dns-Cys-SHg$^+$ with thiolase was performed in the presence of 0.7 mM AcAcCoA, there was no significant amount of Dns-Cys released by 2-mercaptoethanol.

The iodoacetamide-alkylated enzyme (produced by reaction of 2 units/mL thiolase with 48 μM iodoacetamide for 20 min at pH 6.8) was further modified by reaction with 4.3 μM Dns-Cys-SHg$^+$ for 10 min. When the stoichiometry of Dns-Cys-SHg$^+$ bound to the iodoacetamide-alkylated enzyme was determined, it was found that 1.1 ± 0.1 mol of Dns-Cys-SHg$^+$ had been incorporated. A similar result was obtained upon modification of the acetyl enzyme with Dns-Cys-SHg$^+$. When the acetyl enzyme (produced as before) was treated with 4.3 μM Dns-Cys-SHg$^+$ for 10 min, 1.2 ± 0.2 mol of Dns-Cys-SHg$^+$ was incorporated into the acetyl enzyme, although the acetyl enzyme at this point retained approximately 70% of the original thiolase activity.

Determination of Sulfhydryl Groups by Reaction with DTNB. When thiolase (10 units/mL) is denatured in 6 M Gdn·HCl at pH 7.5 and 0.2 M phosphate buffer and then treated with a large excess (1.0 mM) of DTNB, an instantaneous increase in the absorbance at 412 nm is observed. The magnitude of the absorbance change corresponds to the reaction of 4.89 ± 0.28 sulfhydryl groups per thiolase subunit. When the native enzyme was treated with 1.0 mM DTNB in the absence of Gdn·HCl under identical conditions, a slow increase in absorbance at 412 nm with a rate constant of 0.22 min^{-1} was observed. The magnitude of the absorbance increase showed that only two sulfhydryl groups in the native enyzme reacted with DTNB. The enzyme lost all activity under these conditions with a rate constant of 0.2 min^{-1}.

When thiolase (12 units/mL) is treated with a 5- or 50-fold excess of iodoacetamide for 40 min at pH 7.5, complete loss of activity is observed. Denaturation of these iodoacetamide-alkylated enzymes with 6 M Gdn·HCl followed by reaction with excess Ellman's reagent (1.0 mM) showed that 3.9 ± 0.2 sulfhydryl groups were still present in both cases. Under these conditions, treatment with even a 50-fold molar excess of iodoacetamide results in the alkylation of only one sulfhydryl group per subunit.

Fluorescence of Dns-Cys-SHg$^+$ Bound to Thiolase. When the fluorescence emission of the tryptophan residues of thiolase (0.2 μM) was measured in the presence of 3 μM Dns-Cys-SHg$^+$, a large quenching gradually reaching 52% of total thiolase fluorescence was observed with excitation at 295 nm and emission at 340 nm. Even more pronounced changes were seen in the emisison spectrum of Dns-Cys-SHg$^+$ with excitation at 350 nm and emission recorded above 500 nm. Figure 3 shows the changes in fluorescence of Dns-Cys-SHg$^+$ occurring after adding thiolase (0.37 μM) to a 2.8 μM solution of Dns-Cys-SHg$^+$. As the reaction progresses, there is a large increase in the emission fluorescence intensity of Dns-Cys-SHg$^+$ accompanied by a shift of maximum fluorescence intensity from 569 (free compound) to 515 nm (bound Dns-Cys-SHg$^+$). Upon the addition of 80 mM 2-mercaptoethanol to the sample, the spectrum after correction for the background fluorescence was identical with that of free label treated with 2-mercaptoethanol (see Figure 3, curve f). If 0.51 mM AcAcCoA was present in the mixture of thiolase and Dns-

Table I: Effects of Modifications on Quenching Parameters of Thiolase Fluorescence[a]

quencher	fluorophore	$K_{Q(eff)}$ (M^{-1})	$f_{a(eff)}$	K_{sv} (M^{-1})
trichloroethanol	thiolase	23.3	1.01	26.2
	thiolase + AcAcCoA	15.8	0.55	15.5
	thiolase-Dns-Cys-SHg^+ [b]	5.3	1.02	6.4
	thiolase-Dns-Cys-SHg^+ [b,c]	3.2	0.13	2.6
	thiolase-Dns-Cys-SHg^+ [b,d]	26.9	0.97	24.9
	thiolase-Dns-Cys-SHg^+ [b,c,d]	3.6	1.08	1.5
	N-AcTrpNH_2	23.1	0.99	23.5
acrylamide	thiolase	13.9	1.04	12.1
	thiolase + AcAcCoA	8.3	0.47	9.9
	thiolase-Dns-Cys-SHg^+ [b]	5.9	1.03	5.3
	thiolase-Dns-Cys-SHg^+ [b,c]	7.9	0.88	10.2
	N-AcTrpNH_2	18.8	1.02	16.8
I^-	thiolase	3.8	0.53	2.4
	thiolase + AcAcCoA	2.4	0.68	1.6
	thiolase-Dns-Cys-SHg^+ [b]	3.2	0.75	1.5
	thiolase-Dns-Cys-SHg^+ [b,c]	12.9	0.78	11.0
	N-AcTrpNH_2	11.2	0.97	9.9
Cs^+	thiolase	2.6	0.16	0.5
	thiolase + AcAcCoA	2.4	0.15	0.6
	thiolase-Dns-Cys-SHg^+ [b]	3.1	0.15	1.5
	thiolase-Dns-Cys-SHg^+ [b,c]	1.9	0.11	0.6
	N-AcTrpNH_2	1.8	0.95	1.7

[a] All studies were performed at 25 °C in 20 mM Tris-acetate and 0.1 M KCl, pH 7.0 (trichloroethanol, acrylamide), or 0.1 M Tris-acetate and 0.5 M KCl, pH 7.0, (I^-, Cs^+). Unless stated otherwise, excitation was at 295 nm and emission at 340 nm. [b] Thiolase was modified with Dns-Cys-SHg^+ and the excess reagent removed by gel filtration. [c] Excitation at 350 nm and emission at 505 nm. [d] Sample incubated with 100 mM 2-mercaptoethanol for 30 min.

Cys-SHg^+, only minor changes in the emission spectrum of the Dns-Cys-SHg^+ label were seen, suggesting limited binding in the presence of the substrate (Figure 3, curve g). In control experiments, addition of 0.1 mM AcAcCoA to 2.8 μM Dns-Cys-SHg^+ in the absence of enzyme caused no significant change in fluorescence intensity.

Changes in the fluorescence intensity at 515 or 520 nm were followed to calculate the rate constant for label incorporation at several concentrations of Dns-Cys-SHg^+. The rate constant for the observed fluorescence change is $(1.1 \pm 0.3) \times 10^5$ M^{-1} min^{-1}, identical within experimental error to the rate constant for enzyme inactivation by Dns-Cys-SHg^+.

A sample of thiolase modified with 48 μM iodoacetamide was incubated with 4.2 μM Dns-Cys-SHg^+, and the changes in the emission fluorescence intensity at 520 nm were followed for 20 min. The rate constant for the increase in fluorescence, determined in five independent runs, was $(1.2 \pm 0.2) \times 10^5$ M^{-1} min^{-1}, comparable to the rate constant for the fluorescence change observed upon reaction of native thiolase with Dns-Cys-SHg^+ (and also the rate constant for thiolase inactivation by the reagent). The final emission spectrum recorded at the end of the reaction is shown in Figure 4b. Figure 4a shows the emission spectrum of the same concentration of native thiolase treated with Dns-Cys-SHg^+.

Reaction of the acetyl enzyme, prepared as before, with Dns-Cys-SHg^+ was also accompanied by a fluorescence increase at 520 nm due to the reaction of Dns-Cys-SHg^+ with the acetyl enzyme. The rate constant for the fluorescence change at 520 nm was $(0.87 \pm 0.2) \times 10^5$ M^{-1} min^{-1}. Figure 4c shows the emission spectrum of the acetyl enzyme after reaction with 2.8 μM Dns-Cys-SHg^+. The fluorescence intensity of the modified acetyl enzyme is approximately 65% of that observed upon modification of the native enzyme.

Treatment of each of the samples with 50 mM 2-mercaptoethanol results in the appearance of a spectrum characteristic of free Dns-Cys-SHg^+ treated with 2-mercaptoethanol.

Quenching of Thiolase Fluorescence. The corrected fluorescence emission spectrum of native thiolase (excitation at 295 nm) showed a maximum at 339 ± 1 nm shifting to 348 nm when the enzyme was denatured in 6 M Gdn·HCl. The quenching of intrinsic protein fluorescence by a variety of ionic and nonionic quenches was examined. Controls were run in order to determine if the compounds used to quench thiolase fluorescence inactivate the enyzme.

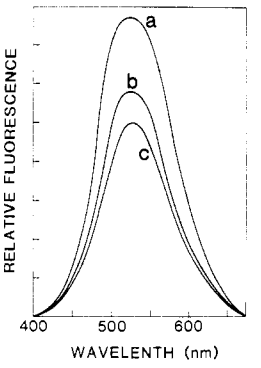

FIGURE 4: Emission spectra of Dns-Cys-SHg^+ bound to modified thiolase. (a) Thiolase treated with 4.2 μM Dns-Cys-SHg^+; (b) thiolase incubated with 48 μM iodoacetamide and then with 4.2 μM Dns-Cys-SHg^+; (c) acetyl enzyme treated at t = 10 min with 4.2 μM Dns-Cys-SHg^+. All spectra are normalized to the same enzyme concentration and are corrected for the background fluorescence of all components of the mixture without thiolase. Measurements were performed at 25 °C in 20 mM Tris-acetate buffer, pH 7.0.

Only incubations with trichloroethanol concentrations higher than 0.2 M lead to significant (50% in 20 min) thiolase inactivation. Other quenchers had little effect on the activity of thiolase over the time course of the quenching experiment. Representative data for quenching of thiolase fluorescence in the absence and presence of substrate and the quenching of enzyme-bound Dns-Cys-SHg^+ fluorescence are shown in Figure 5 and Table I. For comparison, the quenching constants showed for free *N*-acetyltryptophanamide are also included. Since the fluorescence spectrum of enzyme-bound Dns-Cys-SHg^+ is considerably blue shifted and much more intense than that of free Dns-Cys-SHg^+, it is not possible to directly compare the quenching of enzyme-bound and free

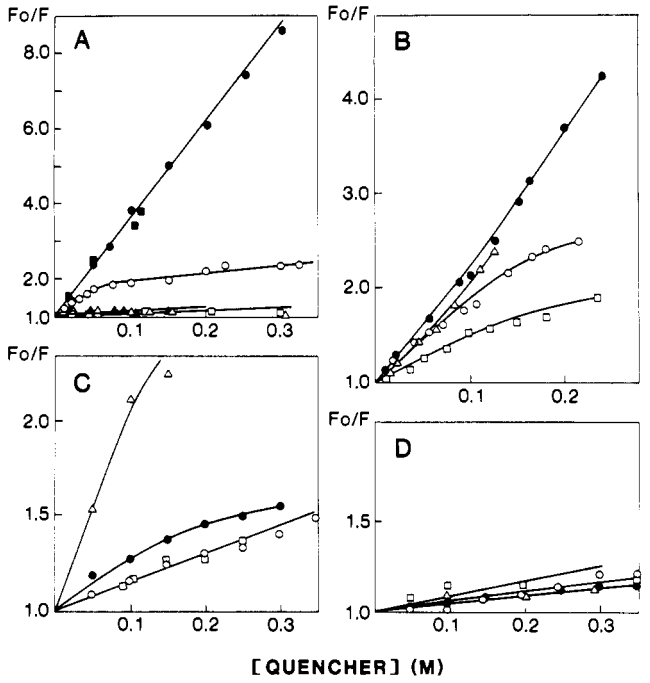

FIGURE 5: Stern–Volmer plots of quenching thiolase fluorescence by trichloroethanol (A), acrylamide (B), I^- (C), and Cs^+ (D). (●) Native thiolase; (○) native thiolase in the presence of 100 μM AcAcCoA; (□) thiolase modified with Dns-Cys-SHg^+; (△) thiolase modified by Dns-Cys-SHg^+, excitation at 340 nm and emission at 505 nm. All other fluorescence measurements were made with excitation at 295 nm and emission at 340 nm. Filled symbols (■, ▲) correspond to analogous samples represented by open symbols after treatment with excess 2-mercaptoethanol. Measurements were done at 25 °C in 20 mM Tris–acetate, pH 7.0, with 0.1 M KCl (trichloroethanol and acrylamide) or in 0.1 M Tris–acetate, pH 7.0, with 0.5 KCl (I^- and Cs^+).

Dns-Cys-SHg^+ fluorescence. Quenching titrations of free Dns-Cys-SHg^+ with acrylamide actually produced fluorescence intensity increases most likely due to changes in solvent polarity. For calculating the quenching parameters, only low concentrations (<0.1 M) of the quenchers were taken into account due to effects of static quenching occasionally observed at higher concentrations.

Discussion

The incubation of thiolase I with Dns-Cys-SHg^+ leads to rapid enzyme inactivation characterized by a second-order rate constant of $(1.5 \pm 0.4) \times 10^5$ M^{-1} min^{-1}. The inactivation of the enzyme is accompanied by an increase in fluorescence at 515 nm due to enzyme-bound dansyl fluorescence and a decrease in the fluorescence of tryptophan residues at 350 nm. Inactivation of thiolase by Dns-Cys-SHg^+ and the accompanying changes in the dansyl and tryptophan fluorescence occur with identical rate constants. The Dns-Cys-SHg–enzyme complex is covalent; however, the reagent may be easily displaced from the enyzme by incubation of the inactive enzyme with mercaptoethanol, leading to recovery of greater than 90% of the original enzyme activity and the appearance of the emission spectrum of unbound label. Inactivation of thiolase by Dns-Cys-SHg^+ must be due to the reaction of the reagent with sulfhydryl groups. This particular reagent is highly specific for sulfhydryl groups and does not react with any other amino acid side chains (Leavis & Lehrer, 1974; Harris & Stahl, 1976; O'Keeffe et al., 1980). This conclusion is also supported by the reversibility of the reaction in the presence of high concentrations of 2-mercaptoethanol.

Both the inactivation of the enzyme by Dns-Cys-SHg^+ and the associated fluorescence changes are inhibited by the presence of saturating concentrations of the substrate AcAcCoA. Thus, the sulfhydryl group(s) modified by this reagent is (are) located at or near the active site of the enzyme, or the conformation of the enzyme–substrate complex is such that the reactive residues are buried.

The reaction of Ellman's reagent with denatured thiolase shows the presence of approximately five sulfhydryl groups per enzyme subunit. However, in the native enzyme there are no sulfhydryl groups which react at a normal rate with Ellman's reagent. A typical free sulfhydryl group of $pK = 8.5$ should react with 1 mM Ellman's reagent with a rate constant of approximately 1×10^3 min^{-1} at pH 7.5 (Wilson et al., 1980). In native thiolase, two sulfhydryl groups react with 1 mM Ellman's reagent at pH 7.5 with an observed first-order rate constant of 0.2 min^{-1}. This is at least 10^3 slower than the rate constant expected for the reaction of a normal cysteine sulfhydryl group. Thus, native thiolase has no sulfhydryl groups which are freely accessible to Ellman's reagent. In contrast, the reaction of one sulfhydryl group of the enzyme with stoichiometric concentrations of iodoacetamide is relatively rapid. The observed second-order rate constant for iodoacetamide inactivation is 2.7×10^3 M^{-1} min^{-1} which is considerably faster than the observed rate constant of 42 M^{-1} min^{-1} for inactivation of the sulfhydryl group at the active site of ficin (Whitaker, 1969). The reason for the large difference in reactivity of the enzyme with DTNB and iodoacetamide is not known; however, it could be related to the difference in size or charge between the two reagents.

Previously, we have shown that incubation of thiolase I with AcCoA results in the nearly stoichiometric formation of a relatively stable acetyl enzyme (Gilbert et al., 1981). This acetyl enzyme is chemically competent and presumably is a normal reaction intermediate. Acetylation of the enzyme by AcCoA is sufficient to protect the enzyme from inactivation by Dns-Cys-SHg^+ or iodoacetamide. Furthermore, alkylation of the enzyme with iodoacetamide prevents acetylation of the enzyme by [^{14}C]acetyl-CoA (R. Cappel and H. F. Gilbert, unpublished observations), and the reversible inactivation of the enzyme by Dns-Cys-SHg^+ also protects the enzyme against irreversible inactivation by iodoacetamide. These results would suggest that iodoacetamide, AcCoA, and Dns-Cys-SHg^+ react with the same group and that the reaction of any one of these compounds with the enzyme prevents the reaction of the others.

Since Dns-Cys-SHg^+ did not inactivate the acetyl enzyme and since reactions with Dns-Cys-SHg^+, AcCoA, and iodoacetamide are all mutually competitive for the same single sulfhydryl group, we were surprised to find that reaction of native thiolase with Dns-Cys-SHg^+ was accompanied by the incorporation of approximately 2 equiv of reagent per active site and that reaction of the reagent with the iodoacetamide-alkylated and acetyl enzymes resulted in the incorporation of 1 equiv of Dns-Cys-SHg^+. This additional incorporation of Dns-Cys-SHg^+ could be due to nonspecific reaction of a nonessential sulfhydryl group with the reagent; however, in the presence of AcAcCoA there is no sulfhydryl group modification or fluorescence change on reaction of the enzyme with Dns-Cys-SHg^+.

The magnitude of the increase in dansyl fluorescence observed with the iodoacetamide-alkylated or acetyl enzymes is about 80% and 65%, respectively, of that observed upon reaction of the native enzyme with Dns-Cys-SHg^+. This could mean that modification of the nonessential sulfhydryl group contributes most of the increase in dansyl fluorescence. However, it is possible that acetylation or alkylation of one of the sulfhydryl groups could significantly alter the envi-

ronment of the other sulfhydryl group and affect the fluorescence intensity of the enzyme-bound Dns-Cys-SHg^+.

The rate constant for thiolase inactivation by Dns-Cys-SHg^+ is identical within the experimental error to the rate constant observed for the accompanying changes in the dansyl fluorescence intensity. Although two sulfhydryl groups are modified, biphasic kinetics are not observed for enzyme inactivation or for fluorescence changes. In addition, the rate constant observed fluorometrically for the reaction of Dns-Cys-SHg^+ with the nonessential sulfhydryl group of the acetyl enzyme is identical with the rate constant for modification of the native enzyme. Either the two sulfhydryl groups react with Dns-Cys-SHg^+ with very similar rate constants or the modification of one of the two sulfhydryl groups by Dns-Cys-SHg^+ greatly accelerates the reaction of the second sulfhydryl with the reagent. Similar behavior is observed for the reaction of the enzyme with Ellman's reagent. The determination of the relative topography and reactivity of the sulfhydryl groups at the active site of thiolase I will be the subject of future experiments.

Our finding of two sulfhydryl groups at the active site of thiolase I is not the first report of this kind. Earlier work of Gehring & Lynen (1972) showed the presence of two cysteine residues in peptides isolated from the vicinity of the active site of thiolase II. Recently two essential cysteine residues have been found at the active site of muscle pyruvate kinase (Tomich et al., 1981) and chicken liver fatty acid synthetase (Stoops & Wakil, 1981). In general, two sulfhydryl groups may participate in a possible regulatory mechanism of enzyme activity by thiol–disulfide exchange (Tschesche & Macartney, 1981; Gilbert, 1982). The occurrence of two thiols at enzyme active sites could be a widespread phenomenon.

Additional information concerning the environment of the active site may be obtained by determining the effects of small molecule quenchers on the intrinsic tryptophan fluorescence and on the fluorescence of the dansyl probe bound to the enzyme. The emission maximum of the tryptophan fluorescence of thiolase I at 339 nm is indicative of only partially exposed tryptophan residues (Chen, 1973; Eftink & Ghiron, 1976; Omar & Schleich, 1981). Some heterogeneity in thiolase tryptophan emission is also suggested by the upward curvature in the Stern–Volmer plots (Eftink & Ghiron, 1981).

Charged quenchers, I^- and Cs^+, are less efficient in quenching thiolase fluorescence than that of free *N*-acetyltryptophanamide, while uncharged quenchers quench all the tryptophan fluorescence in thiolase and *N*-acetyltryptophanamide with approximately the same efficiency. This would suggest that the majority of the tryptophan residues which fluoresce are in an environment which is not accessible to charged quenchers.

Binding of AcAcCoA or Dns-Cys-SHg^+ to thiolase results in the quenching of half the intrinsic protein fluorescence due either to a changed conformation of the enzyme or to the fact that the quenched tryptophan residues are near the binding site for the substrate and/or Dns-Cys-SHg^+. Although the extent of quenching of the residual fluorescence in the enzyme–AcAcCoA complex with all quenchers is low, the residual tryptophan fluorescence of the Dns-Cys-SHg^+ enzyme complex can be entirely quenched by uncharged quenchers. Binding of AcAcCoA to the enzyme shields a fraction of the tryptophan residues from contact with quenchers in solution either by a direct steric exclusion or by an induced conformation change while reaction with Dns-Cys-SHg^+ does not. The fluorescence of Dns-Cys-SHg^+ when bound to the enzyme is not efficiently quenched by trichloroethanol but almost entirely quenched by acrylamide. The same quenchers are not efficient in quenching the fluorescence of the free Dns-Cys-SHg^+ in solution (which under these conditions has much lower fluorescence than the bound label). The observed quenching the enzyme-bound Dns-Cys-SHg^+ by only nonpolar quenchers and the 55-nm blue shift of enzyme-bound dansyl fluorescence suggest that the environment of the dansyl probe is nonpolar (Gilbert, 1981).

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