Fluorescence Energy Transfer Measurements of Spatial Relationships between Sulfhydryl Groups of Thiolase I from Porcine Heart†

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ABSTRACT: Mitochondrial thiolase I from pig heart has been found to have at least two and possibly three reactive sulfhydryl residues at or near the active site [Izbicka-Dimitrijević, E., & Gilbert, H. F. (1982) *Biochemistry 21*, 6112–6118; Izbicka-Dimitrijević, E., & Gilbert, H. F. (1984) *Biochemistry 23*, 4318–4324]. In the native enzyme, fluorescein mercuric acetate reacts with two of the sulfhydryl groups and inactivates the enzyme with a rate constant of 1.6×10^{-4} M^{-1} s^{-1} in 0.1 M Tris–acetate, pH 7.0. The presence of saturating (250 μM) concentrations of acetoacetyl coenzyme A protects against both modification and inactivation. The acetyl enzyme, a normal intermediate in the reaction catalyzed by thiolase, is not inactivated by fluorescein mercuric acetate although one sulfhydryl group out of five per mole of thiolase subunit is still available for reaction with the reagent. Fluorescein mercuric acetate and *S*-mercurio-*N*-dansyl-L-cysteine (Dns-Cys-SHg^+) have been used to differentially label two of the sulfhydryl groups in thiolase. The distance between Dns-Cys-SHg^+ (donor) and fluorescein mercuric acetate (acceptor) determined by the fluorescence energy transfer is less than 14 Å. The fluorescent analogue of coenzyme A, 1,N^6-etheno coenzyme A, is recognized by thiolase as a substrate (K_m = 21 μM); however, substrate inhibition and equilibrium dialysis show that the affinity of the free enzyme for CoA is quite low (K_i = 100 μM). The quantum yield of the fluorescence of the three thiolase tryptophan residues is low (0.024), corresponding to about 12% of the fluorescence expected from equivalent concentrations of tryptophan.

Recent experiments with thiolase I from porcine heart have suggested the presence of at least two (Izbicka-Dimitrijević & Gilbert, 1982) and possibly three sulfhydryl groups (Izbicka-Dimitrijević & Gilbert, 1984), located in the vicinity of the active site. One of these sulfhydryl groups is "essential" for thiolase activity (Izbicka-Dimitrijević & Gilbert, 1982) and is most likely involved in the formation of an acetyl thiol ester enzyme intermediate during catalysis (Gilbert et al., 1981). When modification of the essential sulfhydryl group is blocked by formation of the acetyl enzyme, one sulfhydryl group can still be modified with only a small effect on enzyme activity (Izbicka-Dimitrijević & Gilbert, 1982). In the presence of substrate, there are no available, reactive sulfhydryl groups. At least two sulfhydryl groups in the thiolase monomer are close enough together to form an intrachain disulfide on oxidation of the enzyme with DTNB or diethyl azodicarboxylate (Izbicka-Dimitrijević & Gilbert, 1984).

The catalytic mechanism usually written for thiolase does not require or suggest the need for chemical participation of more than one sulfhydryl group. The function of two or more nearby sulfhydryl groups in or near the active site of the enzyme is not known. We wanted to observe the relative location of the reactive sulfhydryl groups and other binding domains in order to determine if the sulfhydryl groups were close together, near the site of acetyl enzyme formation, or were more distant. In the work presented here, fluorescence resonance energy transfer has been used to "map" the active site in the neighborhood of the two sulfhydryl residues. The method, based on the theory of Förster (1948), can be used as a spectroscopic ruler in the 10–60-Å range to reveal proximity relationships in biological macromolecules (Stryer & Haugland, 1967; Stryer, 1978). A differential, covalent labeling of two of the sulfhydryl groups at the active site has been achieved with two reagents of high specificity for thiols: *S*-mercurio-*N*-dansyl-L-cysteine (Dns-Cys-SHg^+) (Leavis & Lehrer, 1974) and fluorescein mercuric acetate (FMA) (Karush et al., 1964).[1]

Experimental Procedures

Materials. CoA was obtained from P-L Biochemicals. 3-Hydroxyacyl-CoA dehydrogenase and 1,N^6-etheno coenzyme A (ϵ-CoA) were purchased from Sigma. [1-^{14}C]AcCoA was from New England Nuclear. All other reagents were at least reagent grade. Mercaptoethanesulfonic acid and tubes for amino acid hydrolysis were from Pierce. AcAcCoA was prepared and assayed as described previously (Gilbert et al., 1981).

Dns-Cys-SHg^+ was synthesized by the method of Leavis & Lehrer (1974) and characterized as previously described (Izbicka-Dimitrijević & Gilbert, 1982). FMA was either synthesized according to the procedure of Karush et al. (1964) and purified by the method of Takeushi & Maeda (1977) or purchased from Regis Chemical Co. Both batches of FMA had the absorbance maximum at 499 nm in 1 N KOH. The extinction coefficient of 7.8×10^4 M^{-1} cm^{-1} (Karush et al., 1964) was used to determine the concentration of FMA. Stock solutions of ϵ-CoA prepared in 1 mM HCl were purified by HPLC by isocratic elution with 25% methanol in 40 mM ammonium formate, pH 5, using a 0.9 × 25 cm C18 reversed-phase column (5 μm; Custom LC, Houston, TX). Under these conditions a contaminant (about 20%, based on absorbance at 260 nm) was separated from the ϵ-CoA fraction. The peak of ϵ-CoA was collected, the pH adjusted to 3 with 1 M HCl, and the solution purged with argon and stored in the dark at −20 °C. The concentration of the stock solution was measured by the Ellman's procedure (Ellman, 1958).

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[1] Abbreviations: AcCoA, acetyl coenzyme A; AcAcCoA, acetoacetyl coenzyme A; CoA, coenzyme A; Dns-Cys-SHg^+, *S*-mercurio-*N*-dansyl-L-cysteine; DTNB, 5,5′-dithiobis(2-nitrobenzoate); DTT, dithiothreitol; ϵ-CoA, 1,N^6-etheno coenzyme A; FMA, fluorescein mercuric acetate; HPLC, high-performance liquid chromatography; Gdn·HCl, guanidine hydrochloride; *N*-AcTrpNH_2, *N*-acetyl-2-tryptophanamide; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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Thiolase was prepared from porcine heart obtained freshly from a local slaughterhouse and assayed as previously described (Gilbert et al., 1981; Izbicka-Dimitrijević & Gilbert, 1982). The enzyme was greater than 95% homogeneous on $NaDodSO_4$–polyacrylamide gel electrophoresis. The specific activity of thiolase was 20–22 units/mg. Protein concentration was measured by the method of Bradford (1976). A molecular weight of 46 000 per thiolase subunit (Staack et al., 1978) was assumed in all calculations.

Methods. Spectrophotometric measurements were made either on a Varian 634 double-beam recording spectrophotometer or on a Beckman DU7 spectrophotometer interfaced with a Cromemco System One Microcomputer. The cell compartments of both spectrophotometers were thermostated at 25.0 °C. A Corning Model 130 pH meter and a Radiometer GK 2321 combination electrode were used for pH measurements. Liquid scintillation counting was performed with a Beckman LS 6800 spectrometer in 10 mL of Hydroscint cocktail (ICN). For high-performance liquid chromatography a 0.5 × 25 cm ODS-I reverse-phase column (Custom LC, Houston, TX) or a cation-exchange column (Alpert, 1983) was used.

For measurements of thiolase inactivation (native thiolase or the acetyl enzyme) by FMA and/or Dns-Cys-SHg^+, the enzyme (2–5 μM subunits) was preincubated with the given reagent in 0.1 M Tris–acetate, pH 7.0, and 5–10-μL aliquots were assayed for thiolase activity after different times of incubation.

Initial velocity enzyme kinetics were performed at pH 8.3 in 0.1 M Tris-HCl, containing 25 mM $MgSO_4$. After thermal equilibration of the assay mixture, the reaction was initiated by the addition of a small aliquot of thiolase (20 μL). Initial velocities were measured in duplicate for each of the substrate concentrations. The initial velocity data were fitted to eq 1

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_{AcAc}}{V_{max}}\frac{1}{[AcAc]}\left(1 + \frac{[CoA]}{K_{i,CoA}}\right) + \frac{K_{CoA}}{V_{max}}\frac{1}{[CoA]}\left(1 + \frac{[AcAc]}{K_{i,AcAc}}\right) \quad (1)$$

(Cleland, 1963) where K_{AcAc} and K_{CoA} are the Michaelis constants for AcAcCoA and CoA, $K_{i,CoA}$ and $K_{i,AcAc}$ are the inhibition constants for CoA and AcAcCoA, and V_{max} is the maximum velocity.

Differential Modification of Thiolase with Dns-Cys-SHg^+ and FMA. Thiolase was modified with Dns-Cys-SHg^+ by first protecting the essential thiol by formation of the acetyl enzyme intermediate (Izbicka-Dimitrijević & Gilbert, 1982). The acetyl enzyme (4 μM) was treated with Dns-Cys-SHg^+ (8–10 μM) at pH 7.0 in 0.1 M Tris–acetate buffer at 25 °C for 0.5 h followed by gel filtration to remove excess reagent. The singly modified enzyme was incubated for 1.3 h to allow for hydrolysis of the acetyl enzyme followed by treatment for 2 h with an approximately equimolar concentration of FMA (0.8 μM). A similar procedure was used for the preparation of FMA-monosubstituted thiolase. The acetyl enzyme (2–4 units) was incubated with a 2–5-fold molar excess of FMA in 0.1 M Tris–acetate, pH 7.0, for 1 h followed by removal of the excess reagent by gel filtration on Sephadex G-25. The singly modified enzyme was then incubated for 1 h with an equimolar ratio of Dns-Cys-SHg^+ (based on protein concentration) and used immediately. The concentration of the remaining sulfhydryl groups was measured with DTNB (Ellman, 1958) or with 4,4′-dithiopyridine (Grassetti & Murray, 1967; Izbicka-Dimitrijević & Gilbert, 1982) after denaturation of the modified enzyme in 6 M Gdn·HCl. All modifications of thiolase were performed with minimum exposure to light.

Determination of the Extent of Hydrolysis of the Acetyl Enzyme in Dns-Cys-SHg^+-Modified Thiolase. The acetyl enzyme was prepared by incubating 2.2 units of thiolase in a volume of 0.1 mL with [^{14}C]AcCoA (0.04 mM, 57 mCi/mmol) for 1 min followed by the addition of 0.8 mM unlabeled AcCoA and further incubation for 4 min. The mixture was gel filtered on a 1-mL Sephadex G-25 column equilibrated with 0.1 M Tris–acetate buffer, pH 7.0. The peak protein fraction, containing about 0.5 μM subunit (50 000 cpm), was treated with 0.8 μM Dns-Cys-SHg^+ 15 min after the preparation of the labeled acetyl enzyme and with 6 μM FMA at 36 min. The hydrolysis of the acetyl enzyme was followed by measuring the release of trichloroacetic acid soluble radioactivity as described by Gilbert et al. (1981).

Steady-State Fluorescence Measurements. All steady-state fluorescence measurements were performed with a Perkin-Elmer MFP 540-60 spectrofluorometer equipped with a Hitachi 650-0178 data processor. The measurements were carried out at 25 °C in 2 × 10 mm microcuvettes (NSG Precision Cells). The instrument was operated exclusively in the ratio mode. All measured spectra were corrected for the instrument response using a standard solution of Rhodamine B (Weber & Teale, 1957). In all titration experiments the data were corrected for a small background fluorescence of the titrant in the absence of protein and for dilution effects. To avoid inner filter effects, the final absorbances of the titrated samples were less than 0.05–0.08 absorbance unit at the excitation wavelength. The quantum yield of tryptophan in native thiolase was measured in 0.1 M Tris–acetate, pH 7.0 or pH 7.5, by using an isoabsorbing solution of tryptophan in the same buffer or quinine sulfate in 0.1 H_2SO_4. The quantum yields of the dansyl label in Dns-Cys-SHg^+-modified thiolase were determined in 0.1 M Tris–acetate, pH 7.0 buffer with quinine sulfate solution in 0.1 N H_2SO_4 as the standard. Quantum yields were calculated by using eq 2 where subscripts

$$Q_1/Q_2 = (F_1/F_2)/(A_2/A_1) \quad (2)$$

1 and 2 refer to the standard and the sample, respectively, Q is the quantum yield, F is the area under the corrected emission spectrum, and A is the absorbance at the excitation wavelength. The areas of the corrected emission spectra were determined by cutting out and weighing the recorded spectrum. A value of 0.2 was used for the quantum yield of the tryptophan standard (Kronman & Holmes, 1971), and for quinine sulfate the value of 0.7 was used (Scott et al., 1970).

Fluorescence Energy Transfer Measurements. Energy transfer was determined from the quenching of the fluorescence of the donor molecule by using eq 3 where E is the

$$E = 1 - F_{d,a}/F_d \quad (3)$$

efficiency of transfer and $F_{d,a}$ and F_d are the donor fluorescence intensities at a given wavelength in the presence and the absence of the energy acceptor. Similarly, the donor lifetime in the presence and the absence of the acceptor ($t_{d,a}$ and t_d, respectively) was used to measure the efficiency of transfer according to eq 4. In some cases the efficiency of transfer

$$E = 1 - t_{d,a}/t_d \quad (4)$$

was also determined from the enhancement of acceptor fluorescence using the method of Stryer (1978) or from the enhancement of acceptor emission as described by Freifelder (1976). The values of R_0, a critical transfer distance (at which the efficiency is 0.5), were calculated according to eq 5

(Matsumoto & Hammes, 1975) where Q_0 is the donor

$$R_0 = (9.79 \times 10^3 \text{ Å})(JQ_0\kappa^2/n^{-4})^{1/6} \quad (5)$$

quantum yield in the absence of energy acceptor, n is the refractive index of the medium, J is the integral of the spectral overlap, and κ^2 is the dipole orientation factor. The refractive index of water ($n = 1.4$) was used in all calculations. The value of the orientation factor (κ^2) was assumed to be equal to 0.66 unless stated otherwise. The overlap integral was approximated by eq 6. $F_d(\lambda)$ is the corrected fluorescence of the donor,

$$J = \frac{\sum_{\lambda} F_d(\lambda)\epsilon_a(\lambda)\lambda^2\Delta\lambda}{\sum_{\lambda} F_d(\lambda)\Delta\lambda} \quad (6)$$

and $\epsilon_a(\lambda)$ is the excitation coefficient of the acceptor. The terms were summed over 5-nm intervals. The distances between the donor and acceptor were calculated from the relationship of eq 7.

$$E = (R_0/R)^6/[1 + (R_0/R)^6] \quad (7)$$

Fluorescence Lifetime Measurements. Lifetimes were measured with a phase/modulation spectrofluorometer (SLM Instruments, Urbana, IL, Model 4800) interfaced to a Hewlett-Packard 9810 calculator. For measurements of tryptophan lifetimes with 10-MHz modulation frequency, the excitation was at 295 nm (Corning filter 7-54), and the emission above 300 nm was followed with the filter 7-60. For the lifetime measurements of thiolase-bound Dns-Cys-SHg$^+$, excitation was at 355 nm (filter 7-54), and the emission filter was 3-144. Modulation frequencies were 10 and 30 MHz.

Amino Acid Analysis. Amino acid analysis was done with a Beckman 120C amino acid analyzer equipped with a Glenco 56 absorbance monitor. The protein, dialyzed against 50 mM ammonium bicarbonate, was hydrolyzed with 3 M mercaptoethanesulfonic acid (Penke et al., 1974) in sealed hydrolysis tubes at 105 °C for 24 and 48 h. The conditions of the analysis were as described by Stoops et al. (1978). For calculation of the contents of tryptophans in thiolase, the protein concentration was determined by the method of Bradford (1976), and the molecular weight of the subunit was taken as 46 000 (Staack et al., 1978).

Fluorescence Determination of Tryptophan in Thiolase. A calibration curve was prepared by measuring the fluorescence emission at 340 nm (excitation at 295 nm) of 0.1–1.5 μM *N*-AcTrpNH$_2$ solution in 6 M Gdn·HCl (Eftink & Ghiron, 1976). The fluorescence intensity of the enzyme was measured in 6 M Gdn·HCl. The corresponding concentration of tryptophan was determined from the calibration curve. Thiolase concentrations were estimated by taking an average of triplicate measurements of activity (assays performed immediately prior to fluorescence measurements). For calculating the number of tryptophan residues, the specific activity of thiolase was taken as 21 units/mg of protein, and the molecular weight of the subunit was taken as 46 000 (Staack et al., 1978).

Results

Kinetics and Stoichiometry of the Reaction of Thiolase with FMA. The activity of native thiolase is rapidly lost upon exposure to low concentrations of FMA (6–15 μM). The second-order rate constant for enzyme inactivation measured in 0.1 M Tris-HCl, pH 7.0, is $(1.6 \pm 0.2) \times 10^4$ M^{-1} min^{-1}. After loss of greater than 90% of the initial activity, most of the thiolase activity (>80%) can be recovered by incubation with 0.1 M DTT for 30 min. Prolonged incubation of the enzyme with FMA (>30 times the half-life for inactivation) leads to an increased extent of irreversible loss of activity so that about 25% of the initial activity can be recovered with 0.1 M DTT. A control sample not treated with FMA loses no (<10%) activity under these conditions.

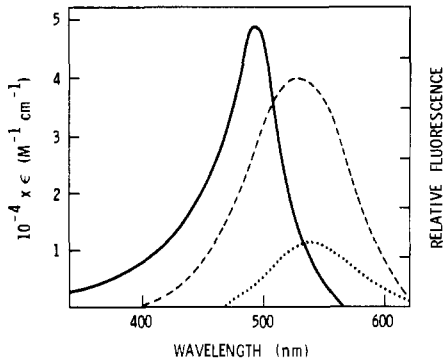

FIGURE 1: Fluorescence spectra of modified thiolase. (---) Emission spectrum of Dns-Cys-SHg$^+$-modified thiolase, excitation at 328 nm; (—) extinction coefficient of FMA-modified thiolase; (···) emission spectrum of doubly dansyl/FMA-modified thiolase, excitation at 328 nm. All spectra were measured at pH 7.0 in 0.1 M Tris-HCl buffer and have been normalized to an enzyme concentration of 1 M.

The presence of saturating concentrations of AcAcCoA (250 μM) results in a 25-fold decrease in the rate of inactivation of thiolase by FMA at pH 7.0 (Figure 1). The acetyl enzyme, a normal catalytic intermediate in which the essential sulfhydryl group is acetylated by preincubation with excess AcCoA followed by gel filtration (Gilbert et al., 1981; Izbicka-Dimitrijevič & Gilbert, 1982, 1984), is inactivated very slowly ($k < 8 \times 10^2$ M^{-1} min^{-1}) by FMA.

The stoichiometry of FMA binding to the native and the acetyl enzyme was determined by measuring the number of remaining sulfhydryl groups titratable with 4,4′-dithiopyridine which reacts with all five free sulfhydryl groups in the native enzyme (Izbicka-Dimitrijevič & Gilbert, 1984). Inactivation of native thiolase with an 8-fold molar excess of FMA leads to a loss of 1.7–2.2 mol of free sulfhydryl groups/mol of enzyme subunit. Modification of the acetyl enzyme (3 μM) by treatment with 22.6 μM FMA for 0.5 h results in the disappearance of 2.3 titratable sulfhydryl groups (the acetyl thiol ester and approximately one additional group) when compared to the native enzyme. If the FMA-modified acetyl enzyme (incubated with 22.6 μM FMA for 0.5 h) is gel filtered to remove excess FMA, then allowed to incubate for approximately 1.5 h at pH 7.0 to allow for spontaneous deacylation of the acetyl enzyme, the number of modified sulfhydryl groups decreases from 2.1 to approximately 1.3, suggesting that deacylation of the modified acetyl enzyme leads to the appearance of an additional free sulfhydryl group. The error associated with the determination of the number of titratable sulfhydryl groups in the acetyl enzyme is approximately ±20%.

Energy Transfer between Dns-Cys-SHg$^+$ and FMA Bound to Thiolase. Selective modification of one of the active site sulfhydryl groups was achieved by using the acetyl group of the acetyl enzyme intermediate as a protective agent for the essential thiol. We have earlier shown that Dns-Cys-SHg$^+$ modified two sulfhydryl residues per thiolase subunit in the native enzyme but only one "nonessential" sulfhydryl group in the acetyl enzyme (Izbicka-Dimitrijevič & Gilbert, 1982). The acetyl enzyme (2–4 μM) was incubated with a 2–4-fold molar excess of Dns-Cys-SHg$^+$ for 5 or 30 min at pH 7.0. After gel filtration, the Dns-Cys-SHg$^+$-modified enzyme was further treated with 2 μM FMA, for an additional 1 h. The product, having about 10–15% residual thiolase activity, ex-

Table I: Fluorescence Energy Transfer Parameters[a]

donor	acceptor	Q_d	E	R_0 (Å)	R (Å)
Dns-Cys-SHg$^+$	FMA	0.06	0.92[b]	20.1	13
			0.80[b,c]		15
tryptophan	Dns-Cys-SHg$^+$	0.05	0.82[d]	24.6	19.0

[a] All measurements were performed at pH 7.0 in 0.1 M Tris-acetate at 25.0 °C. [b] Calculated from quenching of donor emission. [c] Calculated from donor fluorescence lifetimes. [d] Calculated from enhancement of acceptor fluorescence.

hibited spectral characteristics on both labels and had 2.8–3.1 mol of sulfhydryl groups titratable with 4,4′-dithiopyridine (1.9–2.2 groups modified).

To ensure that under the conditions of differential modification the spontaneous hydrolysis of the acetyl enzyme still occurred, the acetyl enzyme was prepared by incubation with [^{14}C]AcCoA followed by gel filtration as described under Experimental Procedures. Subsequent additions of Dns-Cys-SHg$^+$ (1.6-fold molar excess over thiolase subunits) and FMA (12-fold molar excess) did not interfere with the spontaneous hydrolysis of the acetyl enzyme—85 min after the preparation of the acetyl enzyme, 93% of the total counts were found in the Cl_3CCOOH-soluble fraction. The apparent half-life for the hydrolysis is about 30 min, similar to that observed for spontaneous deacylation of the native acetyl enzyme (Gilbert et al., 1981).

The overlap between the fluorescence emission of Dns-Cys-SHg$^+$–thiolase (donor) and the absorption spectrum of FMA–thiolase (acceptor) is shown in Figure 1. The absorption spectrum of FMA–thiolase was measured after incubation of the native enzyme with 1 equiv of FMA for 2 h. The observed absorption spectrum was essentially identical with that of free FMA except for a decrease in the extinction coefficient. At pH 7.0 in Tris-HCl buffer, the extinction coefficient for free FMA is 7.4×10^5 M^{-1} cm^{-1} in good agreement with the value of 7.8×10^5 M^{-1} cm^{-1} reported by Karush et al. (1964) in 0.1 M KOH. Addition of equimolar thiolase results in a time-dependent decrease in absorbance at 499 nm. The final extinction coefficient of the thiolase–FMA complex is 4.9×10^5 M^{-1} cm^{-1} (Figure 1). These data were used to calculate J, the overlap integral, by using the value of 0.063 ± 0.02 for the donor quantum yield (determined as described under Experimental Procedures).

The efficiency of fluorescence energy transfer was measured by two independent methods for the differentially modified enzyme in which the Dns-Cys-SHg$^+$ label was introduced onto the "nonessential" sulfhydryl group(s). The fluorescence lifetime of thiolase-bound Dns-Cys-SHg$^+$ showed an average value of 14.2 (phase) and 17.1 ns (modulation) in the absence of FMA and 2.5 (phase) and 3.8 ns (modulation) in the presence of FMA. These values correspond respectively to efficiencies of transfer of 0.78 and 0.83 (Table I). The efficiency measured from quenching of Dns-Cys-SHg$^+$–thiolase fluorescence by FMA bound to the modified enzyme (excitation 328 nm, emission 490 nm) was 0.92.

Attempts to reverse the order of addition of FMA and Dns-Cys-SHg$^+$ met with limited success. The second-order rate constant for the reaction of thiolase with FMA is approximately 10-fold slower than that for reaction with Dns-Cys-SHg$^+$. The reaction of the acetyl enzyme with a small molar excess (1–2 equiv) of FMA could not be carried out fast enough to specifically label the available nonessential thiol before the hydrolysis of the acetyl group took place. A large increase in the FMA concentration (to 20 μM) led to the modification of approximately 2.6 sulfhydryl groups. The

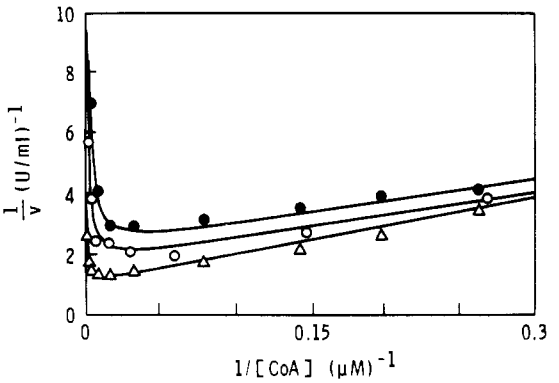

FIGURE 2: Substrate inhibition to thiolase by CoA. Measurements were performed at pH 8.3 in 0.1 M Tris-HCl and 25 mM $MgSO_4$, 25.0 °C, in the presence of (●) 3.9, (○) 6.3, and (△) 38.6 μM AcAcCoA. The curves are calculated from eq 1 by using the values of K_{CoA} = 10.3 μM, K_{AcAc} = 6.7 μM, $K_{i,CoA}$ = 112 μM, $K_{i,AcAc}$ = 300 μM, and V_{max} = 1.23 units/mL.

intensity of Dns-Cys-SHg$^+$ fluorescence in the FMA-modified enzyme after treatment with Dns-Cys-SHg$^+$ was approximately 10% that of an identical sample of acetyl enzyme treated with the same concentration of Dns-Cys-SHg$^+$–thiolase in the absence of FMA, suggesting an efficiency of transfer of approximately 0.9. However, the decreased dansyl flourescence could have resulted from a decreased extent of dansyl label incorporation. The results of reverse introduction of labels, while inconclusive, are consistent with a close proximity of the donor/acceptor pair.

Experiments in which native thiolase (not the acetyl enzyme) was modified with a stoichiometric ratio of Dns-Cys-SHg$^+$ (3–5 μM) for 1 h followed by incubation with excess FMA also gave variable but high efficiencies of energy transfer (0.6–0.9), as determined by the quenching of dansyl fluorescence upon addition of FMA.

Fluorescent Probes of Other Active Site Domains. The fluorescent analogue of CoA, ε-CoA, can frequently be used as a convenient probe for structural studies of CoA binding sites (Stryer, 1978). The binding of the fluorescent analogue of CoA, ε-CoA (0–20 μM), to native enzyme or to Dns-Cys-SHg$^+$-modified or FMA-modified thiolase could not be observed by quenching of tryptophan fluorescence, by the enhancement or quenching of ε-CoA fluorescence, or by energy transfer between bound ε-CoA and the dansyl or FMA labels at pH 7.0 and pH 8.0. However, ε-CoA is a good substrate for thiolase. In the standard assay system (0.1 M Tris-HCl, 25 mM $MgSO_4$, pH 8.3, and 30 μM AcAcCoA), the measured values of K_m and V_{max}, respectively, for ε-CoA were 20.8 ± 0.6 μM and 9.9 units/mg. For comparison, the corresponding values for CoA are 14 ± 2 μM and 23 units/mg, in good agreement with those reported earlier (Gilbert et al., 1981). Although the K_m for CoA and ε-CoA is reasonably low, the interaction of species with the enzyme occurs productively during catalysis only with the acetyl enzyme. The formation of an abortive dead-end complex between the free enzyme and CoA (and the acetyl enzyme and AcAcCoA) results in the double-competitive substrate inhibition (Stewart & Rudney, 1966) shown in Figure 2. A nonlinear least-squares fit to eq 1 shows that the dissociation of CoA from the free enzyme is 100 ± 10 μM. Thus, the absence of change in the fluorescence properties of the free and modified enzymes is most consistent with a lack of significant binding of CoA (and presumably ε-CoA) to the free enzyme.

Energy Transfer between Thiolase Tryptophans and Fluorescent Probes. In thiolase most of Trp fluorescence is

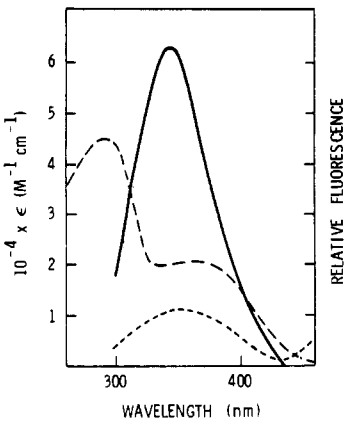

FIGURE 3: Fluorescence spectra of tryptophan in native and Dns-Cys-SHg^+-modified thiolase. (--) Extinction coefficient of Dns-Cys-SHg^+-modified thiolase; (—) fluorescence spectrum of native thiolase, excitation at 295 nm; (---) fluorescence spectrum of Dns-Cys-SHg^+-modified thiolase, excitation at 295 nM. All measurements were performed at pH 7.0 in 0.1 M Tris–acetate. Enzyme concentrations have been normalized in 1 M in all spectra.

already quenched as suggested by a very low quantum yield of 0.024 ± 0.005, corresponding to about 12% of free Trp in solution (assuming an average value of 0.2; Matsumoto & Hammes, 1975). Heterogeneity of tryptophan fluorescence emission is also suggested by lifetime measurements (3.9 ± 0.1 ns by phase and 0.9 ± 0.1 nm by modulation, 10 MHz). Thiolase contains approximately three Trp residues per subunit (2.8 ± 0.4 from fluorometric determination with *N*-AcTrpNH_2 as a standard and 2.9 ± 0.2 from amino acid analysis). For the measurements of energy transfer between tryptophan and Dns-Cys-SHg^+, the acetyl enzyme was used as an intermediate in order to modify only one sulfhydryl group. Since there is a significant overlap of tryptophan emission and the lowest energy absorbance band of thiolase-bound Dns-Cys-SHg^+ (see Figure 3), there is a possibility of energy transfer between these probes. The efficiency of transfer can be measured either from the intensity of thiolase tryptophan fluorescence in the absence and the presence of bound Dns-Cys-SHg^+ (excitation 295 nm, emission 340 nm) or from the enhancement of the acceptor fluorescence (excitation 295 nm, emission 520 nm). The large energy-transfer peak (around 520 nm) disappears after addition of 50 mM 2-mercaptoethanol, which shows that the enhancement of the fluorescence is actually due to the energy transfer. The efficiencies and calculated distances are shown in Table I.

Discussion

The theory of fluorescence energy transfer applied to protein–ligand interactions requires several starting assumptions, of which the specificity of the modification reaction, the certainty that the donor quenching results only from the energy transfer to the acceptor, and the knowledge of the dipole orientation factor, κ, are the most important.

The specificity of binding of the covalent probes, FMA and Dns-Cys-SHg^+, is well documented. The reaction of these compounds with amino acid residues other than cysteine is very unlikely (Torchinskii, 1974). The number of titratable sulfhydryl residues in doubly modified thiolase (about three SH left out of a total of five per mole of thiolase subunit) as well as the limited reactivity of Dns-Cys-SHg^+ and FMA with the acetyl enzyme (in both cases only one sulfhydryl residue is modified) indicates that the labeling does not involve more than a total of two sulfhydryl groups. Substrate protection against both modification and inactivation suggests an active site location of the modified sulfhydryl groups.

We have shown previously (Izbicka-Dimitrijević & Gilbert, 1984) that there are at least two ways of forming inactive, intramolecular disulfides at or near the active site of thiolase. Reaction of native thiolase with either DTNB or diethylazodicarboxylate leads to the oxidation of two different "active site" sulfhydryl groups to an intramolecular, intrachain protein disulfides. Because two different active site disulfides can be formed, there must be at least three and possibly four sulfhydryl groups that display the characteristics of an active site location.

In view of the potential multiplicity of active site sulfhydryl groups, the possible effects of heterogeneity in the structure of the differentially modified enzyme must be considered. In the native enzyme, both Dns-Cys-SHg^+ and FMA react with only two of the five sulfhydryl groups per subunit even under conditions of reagent excess. Modification of the acetyl enzyme with an excess of either reagent occurs with the loss of only one additional sulfhydryl group. Although this may represent the modification of either of two active site sulfhydryls, the extent of modification must be restricted to one dansyl probe per active site which could be localized on one specific sulfhydryl group or could be distributed between two groups. Subsequent hydrolysis and modification of the dansylated acetyl enzyme causes a loss of activity with the incorporation of the fluorescein label and the disappearance of one sulfhydryl group. The absence of any sulfhydryl group modification or inactivation in the presence of substrates in conjunction with the stoichiometry of the modification of the native and acetyl enzymes demonstrates that, at most, a total of two sulfhydryl groups per active site may be modified by either of the fluorescent probes.

Fluorescence lifetime measurements of the Dns-Cys-SHg-modified enzyme in the absence and presence of the FMA probe suggest that there is little heterogeneity in the fluorescence lifetimes at least at a frequency of 10 and 50 MHz. The lifetime measurements made by both phase and modulation techniques show significant agreement which would not be expected if multiply modified species of significantly different fluorescent lifetimes were present.

The efficiency of fluorescence energy transfer in the case of multiple donor–acceptor pairs is the sum of the fractional contributions from individual donor–acceptor pairs (Cantley & Hammes, 1975; Hammes, 1981). The donors at very small distances from the acceptors will contribute more significantly than those more distant. If multiply modified species are involved, at least two of the modified sulfhydryl groups must be closer than the observed value of 13 Å. Therefore, the calculated distance of 13 ± 2 Å between the Dns-Cys-SHg^+ and FMA represents an upper limit of the true distance, and two of the active site sulfhydryl groups must be at least this close. Since the sum of the molecular radii of the dansyl and fluorescein probes is close to the measured distance between the probes, introduction of two different fluorescent probes on the sulfhydryl residues may cause some distortion of the native structure of the enzyme.

The ability to covalently cross-link two of the active site sulfhydryl groups by oxidation to the disulfide suggests that at least two of the sulfhydryl groups are within 2–3 Å. If the distance of 13 ± 2 Å measured by fluorescence energy transfer is complicated by probe-induced distortions of the native structure, the distortion must be reversible since complete activity can be restored by excess thiols.

Some uncertainty in estimating the efficiency of energy transfer is due to the uncertainty in the orientation factor, κ^2. The calculated distance can only be approximated with an

accuracy depending on the assumed value of κ^2. When κ^2 is allowed to vary between 0.66 and 4 (limiting values; Stryer, 1978), the calculated distance for the pair Dns-Cys-SHg^+ to FMA changes from 13 to 18 Å. Higher values of κ^2 are usually taken for the ligands that are not freely rotating. It is reasonable to assume that the bulky fluorophores would be at least partly restricted in free rotation. On the other hand, since the measured distances between Dns-Cys-SHg^+ and FMA bound to thiolase are comparable to the molecular radii of those fluorescent labels, the sulfhydryl groups modified by these reagents are in close proximity.

The uncertainty in the orientation factor can be further narrowed by using several different donor–acceptor pairs at the same site (Stryer, 1978) or by reversing the location of the donor and acceptor. Although there is some uncertainty in the exact stoichiometry of label incorporation when the order of label introduction is FMA followed by Dns-Cys-SHg^+, the results obtained are consistent with a high efficiency of energy transfer, which is independent of the order of label addition.

The measurement of fluorescence energy transfer between intrinsic tryptophan donor(s) and chemical-introduced extrinsic probes is usually difficult to interpret in that some model must be assumed to calculate the distances between the sites in the case of multiple donor (or acceptor) sites (Cantley & Hammes, 1975). In spite of apparent difficulties, reports on energy transfer from two tryptophan residues to anilinonaphthalenesulfonate or pyridoxamine phosphate in the catalytic subunit of aspartate transcarbamylase (Matsumoto & Hammes, 1975) and from two tryptophans of α-lytic protease or three tryptophans of lysozyme to a ruthenium(III)–$(NH_3)_5$–histidine complex (Recchia et al., 1982) have appeared.

The fluorescence of the three tryptophan residues in thiolase is already partly quenched, and at least one residue is located in a hydrophobic environment in the vicinity of the active site (Izbicka-Dimitrijevič & Gilbert, 1982). Our values for the tryptophan quantum yield in thiolase are very low and approach the lower limit of those reported for various proteins (Kronman & Holmes, 1971). Covalent ligands such as Dns-Cys-SHg^+ and FMA specifically bind at the active site and quench the majority of the residual intrinsic tryptophan fluorescence. In addition, a significant enhancement of acceptor fluorescence with specific excitation of the donor tryptophan(s) is observed, which suggests that fluorescence quenching is due to energy transfer between the tryptophan and the dansyl probe. The distance between the tryptophan residues and the site of sulfhydryl groups modified by Dns-Cys-SHg^+ is calculated to be 19 Å, consistent with a tryptophan residue close to the active site of the enzyme.

Previous work (Izbicka-Dimitrijevič & Gilbert, 1984) has suggested that thiolase contains at least one pair of sulfhydryl groups in a close spatial arrangement that allows the thiols to participate in the formation of an intramolecular disulfide bond. However, as with all chemical cross-linking experiments, the ability to cross-link two residues in a protein demonstrates only that the two groups are capable of juxtaposition during the time of the chemical reaction—not that they are necessarily close together in the ground-state native structure. The fluorescence energy transfer observed in the experiments reported here provides additional evidence by an independent method that at least two sulfhydryl groups at the active site of thiolase are located in close proximity.

The function of proximal sulfhydryl groups located at the active site of thiolase is presently unknown. The sulfhydryl pairs may function catalytically in promoting acyl transfer reactions at the active site such that acylation of the enzyme by AcCoA or AcAcCoA could potentially involve multiple acyl transfers within the active site. Alternatively, these sulfhydryls groups could provide a mechanism of enzyme activity control in response to changes in the mitochondrial thiol/disulfide status (Kosower, 1978; Gilbert, 1982) via reversible or irreversible sulfhydryl group oxidation.

Registry No. FMA, 3570-80-7; Dns-Cys-SHg^+, 53509-71-0; CoA, 85-61-0; ϵ-CoA, 60037-59-4; AcAcCoA, 1420-36-6; thiolase I, 9029-97-4.

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