Multiple Oxidation Products of Sulfhydryl Groups near the Active Site of Thiolase I from Porcine Heart†

Elżbieta Izbicka-Dimitrijević‡ and Hiram F. Gilbert*

ABSTRACT: The inactivation of porcine heart thiolase I with the disulfide reagents 5,5′-dithiobis(2-nitrobenzoate) (DTNB) and 2,2- and 4,4-dithiopyridine in 0.2 M phosphate buffer, pH 7.5, follows second-order kinetics with rate constants of 2.2×10^2, 25×10^2, and 5.8×10^2 M^{-1} min^{-1}, respectively. Stoichiometric concentrations of the thiol-oxidizing reagent diethyl azodicarboxylate inactivate thiolase in less than 1 min at pH 7.5. The presence of saturating concentrations of the substrate acetoacetyl coenzyme A or the formation of the acetyl enzyme (a normal catalytic intermediate) results in a significant protection against the inactivation of thiolase by DTNB, 2,2-dithiopyridine, and diethyl azodicarboxylate. All five sulfhydryl residues of native thiolase react with either of the dipyridyl disulfides, but only the equivalent of 3.2 residues react with DTNB even at high concentrations and prolonged incubation times. The reaction of thiolase with DTNB leads to the formation of 1.0–1.4 mol of intrachain disulfide and 0.65 mol of mixed disulfides. After inactivation of thiolase with an equimolar concentration of diethyl azodicarboxylate, 1.2 mol of intrachain disulfide per subunit is found. No cross-linking between the subunits occurs as a result of the reaction of thiolase with DTNB or diethyl azodicarboxylate. The DTNB-inactivated enzyme can be reactivated with excess dithiothreitol while the diethyl azodicarboxylate inactivated enzyme is totally resistant to reactivation by dithiothreitol. There appear to be at least two different ways of forming inactive, oxidized enzyme products depending on the oxidant used, suggesting the possibility of multiple sulfhydryl groups at or near the active site.

The enzyme thiolase catalyzes the coenzyme A (CoA)[1]-dependent cleavage of 3-keto thiol esters of CoA into two acyl-CoA molecules (Gehring & Lynen, 1972).

$$RCOCH_2COSCoA + CoA \rightleftharpoons RCOSCoA + CH_3COSCoA \quad (1)$$

Two thiolase isozymes are found in porcine heart (Staack et al., 1978), both of which mitochondrial in origin (Middleton, 1973). While thiolase II is specific for AcAcCoA, thiolase I catalyzes the cleavage of long-chain 3-ketoacyl-CoA thiol esters, as well as AcAcCoA.

For all thiolases examined so far, the enzyme reaction proceeds chemically in two half-reactions involving an acetyl thiol enzyme intermediate (Gehring & Lynen, 1972; Clinkenbeard et al., 1973; Gilbert et al., 1981).

$$RCH_2COCH_2COSCoA + ESH \rightleftharpoons CH_3COSCoA + ESCOCH_2R \quad (2)$$

$$ESCOCH_2R + CoASH \rightleftharpoons RCH_2COSCoA + ESH \quad (3)$$

Previously, while attempting to attach a fluorescent probe to the catalytically essential sulfhydryl group, we were surprised to find that there were at least two sulfhydryl groups at or near the active site of thiolase I from porcine heart (Izbicka-Dimitrijević & Gilbert, 1982). Although one of the sulfhydryl groups clearly has a role in catalysis by the enzyme, the function or location of the other sulfhydryl group is unclear.

We were interested in determining if the two reactive sulfhydryl groups of thiolase I were located close together in the active site, or in spatially distinct regions of the protein. If the sulfhydryl groups of the protein were spatially isolated, it would be expected that the reaction of the two sulfhydryl groups with various disulfides might stop at the stage of mixed disulfides. However, if the two sulfhydryl groups are located close together, the reaction could proceed with the formation of an intramolecular disulfide. For example, the reaction of pig muscle glyceraldehyde-3-phosphate dehydrogenase with DTNB leads to the formation of an intramolecular disulfide between two nearby sulfhydryl groups (Boross, 1969). A similar observation was made for the reaction of fatty acid synthetase with DTNB (Stoops & Wakil, 1981).

It is becoming increasingly apparent that the chemistry of the sulfhydryl group with respect to thiol–disulfide exchange could be significant in protein denaturation–renaturation (Anfinsen & Haber, 1961; Bradshaw et al., 1967; Levinthal et al., 1962), protein aggregation (Nikkel & Foster, 1971), and the regulation of enzyme activity (Barron, 1951; Pontremoli et al., 1967; Forest & Kemp, 1968; Manboodiri et al., 1981; Gilbert, 1982). Since there appear to be multiple sulfhydryl residues near the active site of thiolase I, the oxidation of the enzyme into an inactive intramolecular disulfide form could function in the regulation of the enzyme activity or in targeting the protein for subsequent degradation.

Experimental Procedures

Materials. CoA was purchased from P-L Biochemicals. AcAcCoA was prepared as described previously (White & Jencks, 1976). The pyridine disulfides 2,2-dithiopyridine and 4,4-dithiopyridine were obtained from Aldrich under the trade names of Aldrithiol-2 and Aldrithiol-4, respectively. Ribonuclease and oxidized glutathione were from Sigma. Diethyl azodicarboxylate (from Aldrich) was distilled and stored

† From the Verna and Marrs McLean Department of Biochemistry, Baylor College of Medicine, Houston, Texas 77030. *Received September 14, 1983.* Supported by Grant GM-25347 from the National Institutes of Health, by Grant Q-785 from the Robert A. Welch Foundation, and by Research Career Development Award HL-01020.

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

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frozen. Stock solutions in ethanol were freshly prepared prior to use. Arsenic(III) oxide (Aldrich) was dissolved in 1 M KOH and immediately neutralized with 1 M HCl. Periodic acid (Pierce) stock solutions were freshly prepared and stored at 4 °C. Iodoacetic acid was obtained from Eastman, and Gdn·HCl was from Pierce. FMA was synthesized according to the method of Karush et al. (1964) and purified by gel filtration as described by Takeushi & Maeda (1977). All other reagents were reagent grade or better. Water was deionized and glass distilled.

Methods. All spectrophotometric measurements were made by using a Varian 634 or a Beckman DU7 spectrophotometer with the cell compartment thermostated at 25.0 ± 0.1 °C. A Corning Model 130 pH meter with a combination electrode (Radiometer GK 2321) was used for pH determination. HPLC measurements were performed by using a 0.5 × 25 cm ODS-II reversed-phase column (Custom LC, Houston, TX). Isocratic elution was accomplished with 50 mM phosphate, pH 6.6. Fluorescence measurements were performed with a Perkin-Elmer MFB 540-60 spectrofluorometer equipped with a Hitachi 650-0178 data processor and a circulating water bath thermostating the cell compartment at 25.0 ± 0.1 °C. All fluorescence measurements were done in the ratio mode. The solutions used for fluorescence experiments were filtered through a 0.45-μm Metricel membrane or Millipore filters.

Thiolase I from pig heart was prepared according to the method of Staack et al. (1978) with minor modifications. Pig hearts obtained from a local slaughterhouse were chilled to 4 °C within 30 min after death. Tissue was homogenized within 6 h after death. Thiolase activity was assayed in the direction of AcAcCoA cleavage as described previously (Gilbert et al., 1981). Protein concentrations were measured by the method of Bradford (1976). The enzyme preparation had a specific activity of 19 units/mg of protein, with the unit of activity as described previously (Gilbert et al., 1981), and was greater than 95% homogeneous on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis in the system described by Laemmli (1970). Unless stated otherwise, the enzyme (100–500 μL) was dialyzed against 2 L of argon-degassed 0.1 M phosphate buffer, pH 6.8, containing 1 mM EDTA before each experiment with no significant loss of specific activity.

The acetyl enzyme was prepared by incubating thiolase (1–2 units) with AcCoA as described by Gilbert et al. (1981) or with 0.58 mM AcAcCoA in 0.1 M phosphate buffer, pH 6.8, for 5 min and isolated by gel filtration. The acetyl enzyme was used within 5–10 min of gel filtration. Enzyme inactivation measurements were performed at 25 °C in 0.2 M phosphate buffer, pH 7.5, with thiolase concentrations of 0.5–2 units/mL. For inactivation experiments using high (above 0.5 mM) concentrations of thiol reagents, 10 mM 2-mercaptoethanol was added to the enzyme assay buffer in order to avoid depletion of the CoA substrate by reaction with the reagent in the assay mixture. Control experiments showed that addition of the thiol reagents directly into the assay mixture had no significant effect on the measured activity of the enzyme. The number of sulfhydryl groups in thiolase was determined by reaction of the protein (8-10 μM) with DTNB in 6 M Gdn·HCl as described before (Izbicka-Dimitrijević & Gilbert, 1982) or with 0.1–0.3 mM 4,4-dithiopyridine in 0.2 M phosphate buffer, pH 7.5, using an extinction coefficient at 324 nm for 4-thiopyridone of 1.98×10^4 M^{-1} cm^{-1} (Grassetti & Murray, 1967).

The stoichiometry of TNB bound to thiolase as mixed disulfides was determined after gel filtration by treating the inactive protein fraction with 0.1 mM DTT in 6 M Gdn·HCl for 30 min. A control sample of the DTNB- or 4,4-dithiopyridine-treated protein was not incubated with DTT. Both samples (20–40 μM protein) were analyzed for free DTNB or 4-thiopyridone by HPLC using the modified procedure described earlier (Izbicka-Dimitrijević & Gilbert, 1982) and following the absorbance at 412 or 324 nm, respectively. The amount of free TNB or DTNB in the control samples was generally less than 20% of that in the DTT-treated sample. DTNB and 4,4-dithiopyridine (1–10 μM) in 6 M Gdn·HCl in the presence of 0.1 mM DTT were used as standards.

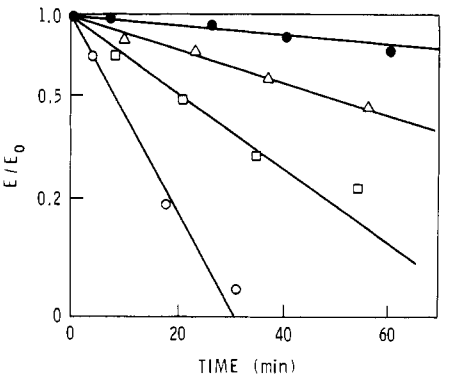


FIGURE 1: Inactivation of thiolase by various concentrations of DTNB at pH 7.5 in 0.2 M potassium phosphate buffer, 25.0 °C. E and E_0 represent enzyme activity at the indicated time and before addition of the disulfide, respectively. Thiolase (2 μM subunits) was incubated with 0.5 (○), 0.13 (□), or 0.052 mM (△) DTNB; (●) no additions.

In some cases when the enzyme concentration was in the range of 1 μM (usually after gel filtration), the HPLC method was also used to determine the concentration of sulfhydryl groups in thiolase. Native or modified enzyme was denatured in 6 M Gdn·HCl, pH 7.0, for 15 min and treated with 50 μM–0.2 mM 4,4-dithiopyridine or with 0.5 mM DTNB for 30 min, and 20–40-μL samples were analyzed for TNB or 4-thiopyridone by HPLC. A small (<15%) correction was applied for TNB or 4-thiopyridone resulting from hydrolysis of the starting disulfides in the absence of thiolase.

The number of disulfides in native and oxidized thiolase was also determined by measuring quenching of the fluorescence of 1–2 μM FMA in 1 M KOH according to the method of Karush et al. (1964). Ribonculease and oxidized glutathione were used as standards. The fluorescence excitation wavelength was 480 nm, and the emission was monitored at 520 nm. After oxidation of the enzyme with DTNB, dipyridyl disulfides, or diethyl azodicarboxylate until less than 5% enzyme activity remained, excess reagent was removed by gel filtration. The protein-containing fraction (10 μM thiolase subunits) was denatured in 6 M Gdn·HCl, pH 8.0, in the presence of 2 mM iodoacetamide for 2 h to modify residual sulfhydryl groups. The contents of cysteine residues in thiolase were determined by amino acid analysis of the enzyme hydrolyzed in 6 N HCl as described by Stoops et al. (1978).

Results

Enzyme Inactivation. Disulfides such as DTNB, 4,4-dithiopyridine, and 2,2-dithiopyridine as well as other sulfhydryl reagents inactivate thiolase. In all cases, the inactivation is first order in sulfhydryl reagent concentration. A typical time course for inactivation by DTNB is shown in Figure 1. Second-order rate constants for enzyme inactivation by several disulfides and other thiol reagents are given in Table I.

The presence of high concentrations (0.9 mM) of the substrate AcCoA causes at least a 10-fold decrease in the rate constants for enzyme inactivation by 2,2-dithiopyridine, 4,4-

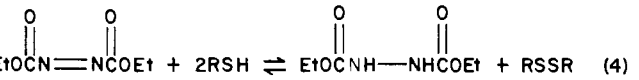
Table I: Rate Constants for Inactivation of Thiolase by Dithiol Reagents at pH 7.5 in 0.2 M Potassium Phosphate Buffer, 25.0 °C

reagent	concn range (mM)	k ($\times 10^{-2}$ M^{-1} min^{-1})
DTNB	0.05–1.0	2.2 ± 0.3
2,2-dithiopyridine	0.04–0.14	25 ± 6
4,4-dithiopyridine	0.04–0.215	5.8 ± 0.4
diethyl azodicarboxylate	0.002–0.004	$>1 \times 10^4$
cystamine	1.0–4.0	0.031
periodate[a]	0.07	17.1
iodoacetate[b]	4.4	0.45
iodoacetamide[a,c]	0.02–0.1	27

[a] pH 7.0, 20 mM Tris–acetate buffer. [b] pH 7.0, 0.1 M potassium phosphate buffer. [c] Izbicka-Dimitrijevič & Gilbert (1982).

dithiopyridine, DTNB, and all of the other reagents listed in Table I. The concentration of CoASH, as a contaminant of AcCoA (<2 mol % by HPLC), was less than 10 μM, so that reaction of the disulfides (0.5–1 mM) with free CoASH could not account for the observed protection.

Thiolase is also rapidly inactivated by incubation with stoichiometric concentrations of diethyl azodicarboxylate (Figure 2), a reagent which specifically oxidizes thiols to disulfides (Muakiyama & Takahashi, 1968; Kosower & Kosower, 1969).



Inactivation of thiolase by diethyl azodicarboxylate is inhibited greater than 100-fold in the presence of 300 μM AcAcCoA.

Reversibility of the Inactivation. Native thiolase incubated with 0.3 mM DTNB at pH 7.5 in 0.2 M potassium phosphate buffer at 25.0 °C was assayed before and after incubation with 40 mM DTT for 1 h. At short times of reaction with DTNB (<2 half-lives), most of the activity (>80%) could be recovered by reduction with DTT; however, a slow irreversible activity loss (approximately 6 times slower than the reversible activity loss) was observed. The half-life for enzyme reactivation by DTT is independent of the extent of activity loss, suggesting that the nature of the reversibly inactivated enzyme was the same at 60% and 95% inactivation (Figure 3). Inactivation by stoichiometric concentrations of diethyl azodicarboxylate was totally irreversible even after a 24-h incubation with 40 mM DTT. Reversible and irreversible inactivation by 2,2- and 4,4-dithiopyridine is similar to that observed with DTNB; however, the irreversible component to the inactivation is somewhat larger.

Thiolase oxidized with 1 mM DTNB for 1 h or treated with an equimolar concentration of diethyl azodicarboxylate migrates as a single band of a similar mobility as the native

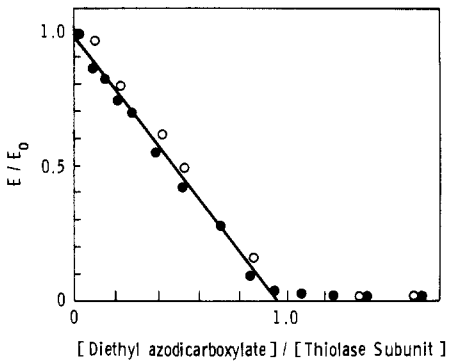


FIGURE 2: Titration of thiolase activity with diethyl azodicarboxylate. Activity measurements were made after incubation of the enzyme (2 μM subunits) with the indicated concentration of diethyl azodicarboxylate for 2 min at pH 7.5 in 0.2 M phosphate, 25.0 °C. (●) Activity of thiolase after 2-min incubation with diethyl azodicarboxylate; (○) activity of thiolase inactivated with diethyl azodicarboxylate after incubation with 80 mM DTT for 1 h. E and E_0 represent enzyme activity after incubation with diethyl azodicarboxylate and the initial activity, respectively. The thiolase concentration was based on a subunit molecular weight of 46 000 and a specific activity of 19.0 units/mg of protein.

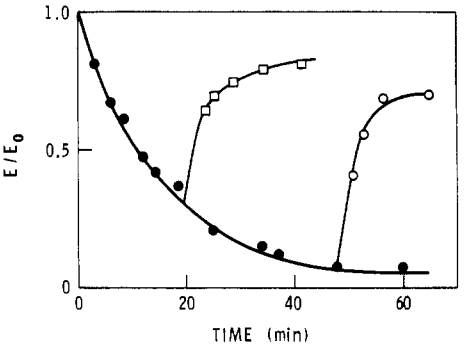


FIGURE 3: Time course for inactivation of thiolase with DTNB and the subsequent reactivation with dithiothreitol. Thiolase (2 μM subunits) was treated with 0.3 mM DTNB at pH 7.5 in 0.2 M potassium phosphate, 25.0 °C. At the indicated times, 30 mM dithiothreitol was added to an aliquot and the time course of reactivation determined. (●) Activity before the addition of dithiothreitol; (□, ○) activity after the addition of dithiothreitol.

enzyme on $NaDodSO_4$–polyacrylamide gel electrophoresis under nonreducing conditions. In contrast, inactivation of the enzyme with excess 2,2-dithiopyridine or 4,4-dithiopyridine is accompanied by extensive subunit cross-linking and the appearance of high molecular weight species on $NaDodSO_4$–polyacrylamide gel electrophoresis.

Stoichiometry. Reaction of thiolase in 6 M Gdn·HCl at pH 7.0 with excess DTNB or 4,4-dithiopyridine results in the

Table II: Stoichiometry for Reaction of Native Thiolase, Denatured Thiolase, and Acetyl Enzyme with Various Thiol-Oxidizing Reagents

treatment[a]	mol of thiolate formed/mol of subunit	mol of protein–reagent mixed disulfide/mol of subunit	mol of intramolecular disulfide/mol of subunit	
			calcd[b]	found[c]
native				<0.05
native + DTNB[d]	3.1	0.65	1.0	1.4
native + 4,4-dithiopyridine[e]	5.2			2.1
denatured with 6 M Gdn·HCl + 4,4-dithiopyridine	5.0	1.0	1.9	2.1
1:1 molar ratio of diethyl azodicarboxylate + 4,4-dithiopyridine	2.9		1.0	1.2
acetyl enzyme + 4,4-dithiopyridine	3.5	3.9	0.0	

[a] All measurements performed in 0.2 M phosphate, pH 7.5, with thiolase concentration of 5–20 μM subunits. [b] Calculated from the difference between the number of thiols which disappeared on modification (5.0 − number found) and the number of protein–reagent mixed disulfides found. [c] Determined by quenching of the fluorescence of FMA in alkali. [d] Thiolase was inactivated with 0.5 mM DTNB, gel filtered, and treated with DTT; TNB release was measured spectrophotometrically as described under Results. [e] Excess reagent was removed by gel filtration, protein incubated with DTT, and released chromophore measured by HPLC as described under Experimental Procedures.

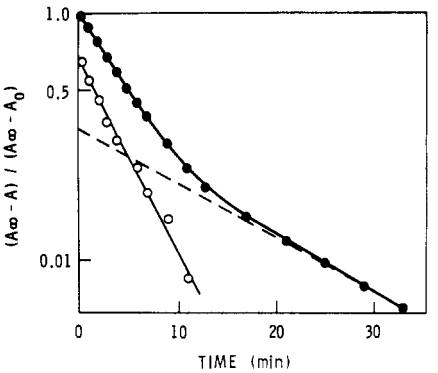


FIGURE 4: Time course for the increase in absorbance at 412 nm during the reaction of thiolase with DTNB. Thiolase (2.5 μM) was treated with 0.9 mM DTNB at pH 7.5 in 0.2 M phosphate, 25.0 °C. (○) Observed absorbance changes; (●) corrected for the slow increase in absorbance observed after completion of the initial fast reaction. The rate constant for the slow increase in absorbance is 0.051 min^{-1} as determined from the linear segment of the plot observed after completion of the faster reaction. The rate constant for the more rapid process was corrected for the absorbance change due to the slow reaction as shown by the open circles. The solid, straight line is drawn with a rate constant of 0.18 min^{-1}.

formation of 5.2 ± 0.2 equiv of thiol anion per thiolase monomer (46 000 daltons) (Table II). Amino acid analysis confirms the presence of 5.4 mol of cysteine per 46 000 g of thiolase, suggesting the absence of disulfide bonds in the native enzyme and the presence of approximately five sulfhydryl groups per enzyme subunit.

The absorbance changes at 412 nm observed upon reaction of thiolase (2.5 μM) with excess DTNB at pH 7.5 are kinetically biphasic (Figure 4). The magnitude of the absorbance changes during the two phases of the reaction indicates the rapid formation of 2 equiv of TNB anion in a single first-order process followed by a slower formation of 1 additional equiv of TNB. Even at prolonged reaction times with excess DTNB, only three sulfhydryl groups react. The rate constant for the more rapid change in absorbance at 412 nm (2.0×10^2 M^{-1} min^{-1}) is identical with that for the reversible loss of thiolase activity. The rate constant for the slow, second increase in absorbance at 412 nm, although difficult to quantitate, is similar to that observed for the irreversible loss of enzyme activity.

The formation of 3 equiv of TNB anion in the reaction of DTNB with thiolase could be due to the formation of three protein–TNB mixed disulfides or one intramolecular protein–protein disulfide and one mixed protein–TNB disulfide. The following procedure was used to distinguish between these possibilities. Thiolase (22 μM) was incubated with excess DTNB (0.5 mM) for 60 min. This time corresponds to approximately 9 half-lives of the reaction leading to the rapid formation of 2 equiv of TNB and 2 half-lives of the slower reaction forming 1 equiv of TNB. The modified enzyme was separated from excess DTNB by gel filtration and denatured by incubation for at least 30 min at 25.0 °C in 6 M Gdn·HCl. Treatment of the modified, denatured enzyme with excess (0.1 M) dithiothreitol for 60 min was employed to reduce any protein–reagent mixed disulfide. The amount of TNB anion released under these conditions was determined by HPLC or by the increase in absorbance at 412 nm. Although reaction of the native enzyme with DTNB leads to the formation of 3.1 equiv of TNB per thiolase subunit, only 0.65 equiv of TNB was found to be covalently bound to the enzyme as a mixed disulfide under these conditions.

Reactivation of DTNB-inactivated thiolase is not accompanied by the formation of TNB anion. Thiolase, inactivated to approximately 10% residual activity, was separated from excess DTNB by gel filtration. At this time of incubation, only about 20% of the enzyme activity has been lost irreversibly. After addition of 30 mM dithiothreitol, the increase in enzyme activity and the absorbance changes at 412 nm were observed in two parallel samples. Reactivation of the enzyme occurred with a half-life of approximately 3 min under these conditions with no detectable (<0.01 AU) time-dependent increase in absorbance at 412 nm. At the concentration of enzyme employed, an absorbance change of 0.04 would have been expected for the formation of 1 equiv of TNB per enzyme monomer.

Inactivation of thiolase with 1 equiv of diethyl azodicarboxylate results in the disappearance of the equivalent of two (1.8–2.2) of the five total sulfhydryl groups as determined by titration of the inactive enzyme with excess DTNB or 4,4-dithiopyridine in 6 M Gdn·HCl. Although DTT reduction of the diethyl azodicarboxylate modified enzyme does not result in the recovery of activity, the two sulfhydryl group equivalents can be recovered if the modified enzyme is first denatured and then reduced with DTT. The diethyl azodicarboxylate treated enzyme (1.0 equiv of reagent/enzyme subunit) denatured in 6 M Gdn·HCl shows 2.8 residual sulfhydryl groups titratable with 4,4-dithiopyridine (2.2 residues modified). After reduction at pH 7.0 in 6 M Gdn·HCl for 120 min with 2.0 mM DTT and addition of 25 mM arsenite to mask the excess DTT (Zahler & Cleland, 1968), 5.2 ± 0.4 sulfhydryl groups could be titrated.

The preceding stoichiometry data suggest the possibility that the reaction of thiolase with DTNB and diethyl azodicarboxylate leads to intramolecular protein disulfide formation. In order to further substantiate the formation of disulfides by both reagents, we used the method of Karush et al. (1964) to estimate the number of disulfides formed. Quenching of FMA fluorescence in alkaline solution was measured by using standards of known disulfide content to relate the concentration of disulfide in the sample to the extent of FMA quenching. Oxidized ribonuclease (0.12 μM, four disulfides per mole) quenched 45% of the FMA fluorescence (excitation, 480 nm; emission, 520 nm) while 0.5 μM oxidized glutathione quenched 29% of the fluorescence of 1 μM FMA in 1 M potassium hydroxide, in good agreement with the data of Karush et al. (1964). Denatured thiolase (0.5 μM) treated with excess iodoacetamide quenched less than 8% of the FMA fluorescence under these conditions, corresponding to less than 0.1 mol of disulfide per mol of enzyme subunit. Using this method, 1.2 ± 0.1 mol of disulfide per mol of thiolase subunit was found in the diethyl azodicarboxylate modified enzyme, and 1.4 ± 0.2 mol of disulfide was found in the DTNB-modified enzyme (thiolase treated with 0.5 mM DTNB for 6–8 half-lives of the fast reaction). Higher contents of disulfide groups (1.8–2.3) were observed in thiolase modified with either of the pyridyl disulfides.

Mutual Competition for Sulfhydryl Groups at the Active Site. The acetyl enzyme is a normal catalytic intermediate in the reaction catalyzed by thiolase (Gilbert et al., 1981). Previous results (Izbicka-Dimitrijevič & Gilbert, 1982) have shown that formation of the acetyl enzyme intermediate is accompanied by the disappearance of one sulfhydryl group in the native enzyme, presumably because the acetyl group is bound at the active site as an acetyl thiol ester.

When the acetyl enzyme, prepared by incubation with AcCoA or AcAcCoA followed by gel filtration (Izbicka-Dimitrijevič & Gilbert, 1982), is treated with a large excess of DTNB, or stoichiometric diethyl azodicarboxylate, there

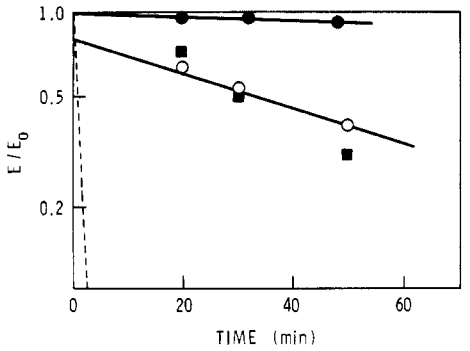


FIGURE 5: Reaction of acetyl enzyme with diethyl azodicarboxylate. The acetyl enzyme was prepared by incubation with 0.25 mM AcAcCoA at pH 6.8 in 0.1 M potassium phosphate and isolated by gel filtration. The acetyl enzyme (5 μM) was incubated with 10 μM diethyl azodicarboxylate at pH 6.8 in 0.1 M potassium phosphate and assayed for residual activity at the indicated times after gel filtration. (○) Activity of the acetyl enzyme upon incubation of diethyl azodicarboxylate; (●) activity of the acetyl enzyme without diethyl azodicarboxylate; (□) activity of the acetyl enzyme incubated with diethyl azodicarboxylate after treatment with 0.2 M DTT for 1 h. The rate constant for the inactivation is 0.022 min^{-1}. The dashed line shows the expected rate for the inactivation of the native enzyme under the same conditions.

is a significant decrease in the rate of enzyme inactivation compared to inactivation of the native enzyme. A typical experiment with diethyl azodicarboxylate is shown in Figure 5. The slow inactivation (>300-fold slower than the native enzyme) observed with diethyl azodicarboxylate occurs with approximately the same first-order rate constant as has been previously observed for spontaneous hydrolysis of the acetyl enzyme (Gilbert et al., 1981). The presence of an acetyl group bound at the active site is sufficient to protect the enzyme against inactivation by DTNB or diethyl azodicarboxylate.

Pretreatment of thiolase (15 μM) with diethyl azodicarboxylate (1.0 equiv) leaves no sulfhydryl groups available for reaction with DTNB (0.5 mM). Although a small increase in the absorbance at 412 nm was observed, the magnitude of the absorbance change (0.014 AU) and the rate constant for the absorbance change could be entirely accounted for by the presence of approximately 5% native enzyme (determined by residual activity). Reversible inactivation of thiolase with DTNB (1.0 mM for 30 min) also protects the enzyme against irreversible inactivation by diethyl azodicarboxylate. After gel filtration of the DTNB-inactivated enzyme, incubation with diethyl azodicarboxylate (1:1 ratio) for 5 min followed by quenching with excess (80 mM) dithiothreitol leads to the recovery of greater than 70% of the original enzyme activity. The native enzyme (not pretreated with DTNB) is irreversibly inactivated by reaction with diethyl azodicarboxylate under these conditions.

Previously we have shown that the fluorescent thiol reagent Dns-Cys-SHg^+ reacts specifically with two sulfhydryl groups at or near the active site of thiolase. Thiolase (2 μM subunits) pretreated with 1 equiv of diethyl azodicarboxylate per subunit and subsequently incubated with a 7-fold molar excess of Dns-Cys-SHg^+ in 0.1 M Tris–acetate buffer, pH 7.0, yields a total fluorescence change of 33% that of the native enzyme. Under the same experimental conditions, using thiolase treated with 1.0 mM DTNB for either 30 min or 3 h, the fluorescence increase due to Dns-Cys-SHg^+ binding was 72% and 78% of the control, respectively.

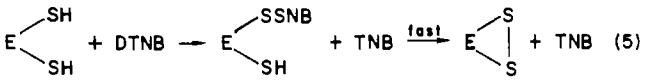
Discussion

Thiolase I from porcine heart contains an "essential" sulfhydryl group at the active site which most likely serves a catalytic role in the formation of an isolatable, chemically competent acetyl thiol ester intermediate during the course of catalysis (Raaka & Lowenstein, 1979; Gilbert et al., 1981; Izbicka-Dimitrijević & Gilbert, 1982). However, previous work has indicated the presence of at least one additional nonessential sulfhydryl group near the active site of thiolase (Izbicka-Dimitrijević & Gilbert, 1982). Substrate binding protects both the essential and the "nonessential" sulfhydryl groups against modification by Dns-Cys-SHg^+; however, the nonessential sulfhydryl group is still available for modification when the essential sulfhydryl group is blocked by formation of the acetyl enzyme intermediate or by alkylation.

Of the five sulfhydryl groups per thiolase subunit, the equivalent of two groups (65% of the total absorbance change) react with DTNB in a single first-order process with a rate constant that is identical with the rate constant for the reversible loss of enzyme activity. A subsequent slower reaction of a third sulfhydryl equivalent is also observed.

Isolation of the DTNB-modified enzyme after completion of the initial fast reaction and after 2 half-lives of the slow reaction shows that less than 1 equiv of TNB is covalently incorporated into the modified protein as a protein–reagent mixed disulfide although almost 3 equiv of TNB has been fromed. This implies the final reaction product between thiolase and DTNB contains the equivalent of one intramolecular protein disulfide and approximately one TNB–protein mixed disulfide. The absence of any significant intersubunit cross-linking of the enzyme by oxidation with DTNB requires that there is no formation of intrachain disulfides.

The presence of the substrate and the formation of the acetyl enzyme intermediate both protect against inactivation by DTNB. The reaction of DTNB occurs with active-site residues, barring the effects of an insidious conformational change induced by binding of substrate or on formation of the acetyl enzyme. The formation of 2 equiv of TNB in a single first-order kinetic process requires either that two sulfhydryl groups fortuitously react with the same rate constant or that the initial reaction of one of the sulfhydryl groups with DTNB is followed by a faster, intramolecular displacement of TNB from the mixed disulfide to form an intrachain protein disulfide.



Since the reversal of the DTNB inactivation by dithiotreitol is not associated with any appreciable formation of TNB anion and occurs at the same rate regardless of the extent of enzyme inactivation (Figure 4), the mixed disulfide intermediate of eq 5 or an initial reaction product containing two TNB–enzyme mixed disulfides must not accumulate to any appreciable extent.

In contrast to inactivation by DTNB, inactivation of thiolase by diethyl azodicarboxylate leads to totally irreversible inactivation of the enzyme and the disappearance of two of the protein sulfhydryl groups. Kosower & Kosower (1969) and Kosower et al. (1969) have shown that the azodicarboxylates are reasonably specific reagents for the oxidation of thiols to disulfides, although other reactions such as with amino groups are possible. Kornblatt & Rudney (1971) and Holland et al. (1973) have suggested a thiol imidate intermediate as a mechanism for increasing the acidity of the α-proton of AcCoA during catalysis by thiolase. This would imply the presence of an active-site lysine amino group, possibly capable of reaction with diethyl azodicarboxylate. However, unpublished results in our laboratory (E. Izbicka-Dimitrijević, unpublished results) have shown that reductive methylation of 80% of the

total lysine residues of thiolase I causes only a small activity loss, making the presence of active-site, reactive lysine residues unlikely. Diethyl azodicarboxylate inactivates the enzyme in stoichiometric concentrations and results in the disappearance of two sulfhydryl groups, which reappear on subsequent reduction under denaturing conditions. It is most likely that the reaction of diethyl azodicarboxylate with thiolase is restricted to sulfhydryl group oxidation. Inactivation by diethyl azodicarboxylate is also inhibited by substrate and by acetyl enzyme formation, again suggesting an active-site location of the two sulfhydryl group equivalents modified by this reagent. The inability to restore catalytic activity to the diethyl azodicarboxylate oxidized enzyme by reduction with excess dithiothreitol could result either from an irreversible denaturation of the oxidized protein or from the inaccessibility of the product disulfide to dithiothreitol in solution.

The reactions of DTNB and diethyl azodicarboxylate with the enzyme are mutually competitive. Enzyme modification by DTNB protects against modification by diethyl azodicarboxylate, and vice versa. In view of the mutual competition, it would at first seem likely that diethyl azodicarboxylate modification leads to the formation of the same modified enzyme as DTNB. This cannot be the case. The enzyme oxidized by reaction with DTNB can be reduced with excess dithiotreitol, restoring full enzyme activity, while the enzyme oxidized with diethyl azodicarboxylate is very resistant to reduction. Thus, the products of the reaction of thiolase with two reagents cannot be identical. In addition, incubation of the diethyl azodicarboxylate modified enzyme with Dns-Cys-SHg^+ results in about 33% of the fluorescence change observed with the native enzyme, while the DTNB-inactivated enzyme exhibits about 75% of the fluorescence of the native enzyme. Peptide mapping experiments, which will be the subject of a later report, should provide a definitive answer to the relative identity of the multiple sulfhydryl groups.

The dipyridyl disulfides behave similarly to DTNB with regard to reversible and irreversible inactivation of the enzyme, although for these compounds the rate constant for irreversible inactivation is somewhat higher than for inactivation by DTNB. While DTNB reacts rapidly with only two sulfhydryl groups (and more slowly with a third), both dipyridyl disulfides react quantitatively with all five sulfhydryl groups in native thiolase. The differences in thiol reactivity between DTNB and the dipyridyl disulfides could be attributed to charge differences between the reactive disulfide species, or to differences in hydrophobicity. Such differential reactivity has been repeatedly observed with a variety of proteins (Wilson et al., 1980; Brocklehurst, 1982). The rather nonspecific nature of the reaction of the dipyridyl disulfides with all five sulfhydryl groups of native thiolase makes interpretation of the results less straightforward.

Inactivation of thiolase with thiol reagents occurs with large differences in the rates of reactions. The reaction of DTNB with thiolase (Table I) occurs approximately 1000-fold slower than the reaction of DTNB with simple thiol compounds (Wilson et al., 1980). The dipyridyl disulfides are also rather sluggish in the reaction with thiolase (Table I), reacting 100–200 times slower with the enzyme than with small molecular weight thiols at pH 7 (Malthouse & Brocklehurst, 1980). The decrease in reactivity of protein sulfhydryl groups with these reactive disulfides is not unusual and has been observed in a number of different proteins. Decreased accessibility of protein sulfhydryl groups to these rather large, hydrophilic disulfides might actually be expected. The sulfhydryl group of thiolase is about 3 times more reactive toward the small neutral reagent iodoacetamide than a thiol of pK = 9.5 in solution (MacQuarrie & Bernhard, 1971). This could reflect either differences in chemical reactivity or differences in pK between the active-site thiol and a normal thiol in solution. For thiols in solution, iodoacetamide reacts approximately 10-fold faster than its anionic counterpart iodoacetate (MacQuarrie & Bernhard, 1971); however, iodoacetate is more than 100-fold less reactive than iodoacetamide in the reaction with thiolase, suggesting a possible discrimination against charged molecules. This may explain why thiolase is not rapidly inactivated by other reagents such as arsenite or periodate which are generally specific for vicinal dithiols such as those found at the active site of thiolase. We were surprised to find that arsenite was without any effect on thiolase I activity. Derivatives of trivalent arsenic are considered to be specific reagents for vicinal thiols (Jacoby, 1958; Peters, 1963). Lynen (1953) reported that arsenous trioxide was a strong inhibitor of thiolase II. Also, the reaction of thiolase with periodate was about 10 times slower than the data reported for several other enzymes (Rippa et al., 1981).

This reason for multiple sulfhydryl groups at the active site of thiolase I is far from clear. One sulfhydryl group can obviously function catalytically in the formation of the acetyl enzyme intermediate. There is no evidence for the chemical participation of an additional one or two sulfhydryl groups in catalysis. The enzyme glyceraldehyde-3-phosphate dehydrogenase appears to have a similar arrangement of sulfhydryl groups (at least two) at the active site. One of the sulfhydryl groups participates in covalent catalysis by the enzyme, and the two sulfhydryl groups form an intrachain disulfide on treatment with DTNB. However, in this case, DTNB inactivation is irreversible (Boross, 1969). The thiols at the active site of thiolase, capable of reversible or irreversible oxidation by thiol–disulfide exchange, could be involved in the regulation of thiolase activity (Gilbert, 1982) or in the regulation of the stability of the protein toward denaturation and proteolysis.

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Registry No. DTNB, 69-78-3; 2,2-dithiopyridine, 2127-03-9; 4,4-dithiopyridine, 2645-22-9; diethyl azodicarboxylate, 1972-28-7; thiolase I, 9029-97-4.

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Asymmetric Active Site Structures in Yeast Dicopper Dizinc Superoxide Dismutase. 1. Reconstitution of Apo-Superoxide Dismutase†

Joan C. Dunbar,* Barton Holmquist, and Jack T. Johansen‡

ABSTRACT: The configuration of the metal binding sites in yeast dicopper dizinc superoxide dismutase (Cu_2,Zn_2SOD) has been probed during metal titration of apoSOD. Cobalt can be substituted for zinc during the metal reconstitution to yield a copper-cobalt derivative that is equally as active as Cu_2,Zn_2SOD, and the incorporated cobalt provides spectral accessibility to the zinc binding site. The absorption, circular dichroism (CD), and magnetic circular dichroism (MCD) spectra of the coordinated cobalt are consistent with a tetrahedral coordination geometry. However, cobalt can be incorporated into only one of the two vacant zinc binding sites, suggesting structural differences between these sites. Analogous titration of apoSOD with zinc results in the coordination of 2 equiv of zinc, with preferential incorporation of zinc into the site to which cobalt binds. Asymmetry is also suggested for the two copper binding sites, which can be distinguished by CD and visible absorption spectra during copper titration of apoSOD and Zn_2SOD. In addition, interaction between the two active sites is indicated by the capacity of zinc incorporated at the higher affinity zinc binding site to determine the spectral properties of copper coordinated at the active site in both subunits.

Cu_2,Zn_2SOD,[1] which catalyzes the dismutation of superoxide to oxygen and hydrogen peroxide, is a dimer of 32 000 daltons (Fridovich, 1975). The two subunits have identical primary structures (Jabusch et al., 1980; Johansen et al., 1979; Lerch & Amner, 1981; Steinman et al., 1974), and each contains one copper and one zinc atom at the active site (Fridovich, 1975). Copper is the functional metal in catalysis and undergoes alternate oxidation and reduction during the dismutation reaction (Klug-Roth et al., 1973). In contrast, zinc confers structural stability to the protein and appears to direct the conformation around the active site (Cass et al., 1979; Forman & Fridovich, 1973; Lippard et al., 1977). Within each subunit, the copper and zinc lie in close proximity while in the dimeric enzyme, the two active sites are located on opposite sides of the molecule and are separated by a distance approaching 34 Å (Richardson et al., 1975). Investigations of the active site of Cu_2,Zn_2SOD have been performed almost exclusively on the enzyme isolated from

† From the Carlsberg Laboratory, Gamle Carlsberg vej 10, DK-2500 Copenhagen, Denmark (J.C.D. and J.T.J.), and the Center for Biochemical and Biophysical Sciences and Medicine, Harvard Medical School, Boston, Massachusetts 02115 (B.H.). *Received December 29, 1983.*

* Address all correspondence to this author at the Department of Biological Chemistry, California College of Medicine, University of California, Irvine, CA 92717.
‡ Present address: Carlsberg Biotechnology Ltd. A/S, Tangensvej 16, DK-2200 Copenhagen, Denmark.

[1] Abbreviations: SOD, superoxide dismutase; CD, circular dichroism; MCD, magnetic circular dichroism; OP, 1,10-phenanthroline; apoSOD, apo-superoxide dismutase; Cu_2,Zn_2SOD, superoxide dismutase containing 2 equiv of copper and zinc/mol of dimer; M_2SOD, derivative of SOD reconstituted with x equiv of metal/mol of dimer.

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