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The sulfhydryl content of L-threonine dehydrogenase from *Escherichia coli* K-12: relation to catalytic activity and Mn^{2+} activation

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When oxidized to cysteic acid by performic acid or converted to carboxymethylcysteine by alkylation of the reduced enzyme with iodoacetate, a total of six half-cystine residues/subunit are found in L-threonine dehydrogenase (L-threonine:NAD^+ oxidoreductase, EC 1.1.1.103; L-threonine + NAD^+ → 2-amino-3-oxobutyrate + NADH) from *Escherichia coli* K-12. Of this total, two exist in disulfide linkage, whereas four are titratable under denaturing conditions by dithiodipyridine, 5,5′-dithiobis(2-nitrobenzoic acid), or *p*-mercuribenzoate. The kinetics of enzyme inactivation and of modification by the latter two reagents indicate that threonine dehydrogenase has no free thiols that selectively react with bulky compounds. While incubation of the enzyme with a large excess of iodoacetamide causes less than 10% loss of activity, the native dehydrogenase is uniquely reactive with and completely inactivated by iodoacetate. The rate of carboxymethylation by iodoacetate of one -SH group/subunit is identical with the rate of inactivation and the carboxymethylated enzyme is no longer able to bind Mn^{2+}. NADH (0.5 mM) provides 40% protection against this inactivation; 60 to 70% protection is seen in the presence of saturating levels of NADH plus L-threonine. Such results coupled with an analysis of the kinetics of inactivation caused by iodoacetate are interpreted as indicating the inhibitor first forms a reversible complex with a positively charged moiety in or near the microenvironment of a reactive -SH group in the enzyme before irreversible alkylation occurs. Specific alkylation of one -SH group/enzyme subunit apparently causes protein conformational changes that entail a loss of catalytic activity and the ability to bind Mn^{2+}.

Introduction

Threonine dehydrogenase (L-threonine : NAD^+ oxidoreductase, EC 1.1.1.103) catalyzes the conversion of L-threonine to the putative unstable intermediate, 2-amino-3-oxobutyrate, which can either spontaneously decarboxylate liberating aminoacetone plus CO_2 or be converted to acetylCoA plus glycine (hence serine) by the action of 2-amino-3-oxobutyrate glycine-lyase (CoA-acetylating) (EC 2.3.1.29), the second enzyme in the pathway. This is the primary route for threonine utilization in both eukaryotes [1] and prokaryotes [2,3]; it accounts for 87% of the L-threonine degraded in the liver of normally fed rats [4], it is the only pathway for L-threonine degradation that is detected in chicken liver [5], and it is a highly efficient alternate pathway for serine biosynthesis in *Escherichia coli* [6]. Some details of this pathway, however, still remain to be elucidated. For example, humans normally excrete about 0.4 mg of aminoacetone/day [7]; the physiological significance of this observation is unknown. Likewise, although the pathway first suggested by Neuberger and Tait [8] – i.e., L-threonine → aminoacetone → D-1-amino-2-propanol, which compound is a part of Vitamin B-12 – has been demonstrated with enzymes of *E. coli* [9], studies must yet be done to determine whether it actually operates in corrinoid biosynthesis.

Threonine dehydrogenase has been purified to homogeneity from extracts of chicken liver [5], goat liver [10] and *E. coli* [11]. Whereas the enzyme from chicken liver

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Abbreviations: Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); Nbs^{2-}, thionitrobenzoate anion; $NTSB^{2-}$, 2-nitro-5-thiosulfobenzoate anion; CMCys, *S*-carboxymethylcysteine.

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is a single polypeptide chain and is inhibited 50% by 1.0 mM Mn^{2+}, *E. coli* threonine dehydrogenase is a tetramer manifesting a basal level of activity in the absence of added metal ions that is stimulated approximately 10-fold by either Mn^{2+} or Cd^{2+} [12,13].

Protein sulfhydryl groups have been shown to play a central role in catalysis and/or metal ion binding by several NAD^+-dependent dehydrogenases. One example is pig muscle glyceraldehyde-3-phosphate dehydrogenase where cysteine residue 149 participates in an active-site thioester mechanism [14,15]. Another example is horse liver alcohol dehydrogenase where the catalytically-essential Zn^{2+} atom is liganded to the sulfhydryl groups of Cys-46 and Cys-174 [16]. In first studies with pure preparations of threonine dehydrogenase of *E. coli,* we found that the basal level of enzymatic activity (in the absence of added Mn^{2+}) was blocked by low concentrations of a thiol-reacting reagent such as *p*-mercuribenzoate or 5,5′-dithiobis(2-nitrobenzoic acid) [11]. We also showed that the observed 10-fold stimulation of threonine dehydrogenase activity by Mn^{2+} was dependent on the presence of an added thiol [12]. Such observations prompted us to determine the free sulfhydryl/disulfide groups of the enzyme and their possible role in structure–function interrelationships. Among other findings, the results reported here show that carboxymethylation of one free sulfhydryl group (out of 4 total/subunit of *E. coli* threonine dehydrogenase) results in complete loss of activity and the ability of the enzyme to bind Mn^{2+}.

Materials and Methods

Chemicals

L-Threonine, NAD^+ and NADH were purchased from U.S. Biochemical Corp. DEAE-Sephadex (*A*50–120), Sepharose 4B, Sephadex G-25 (fine), Bistris, Trizma base, 2-mercaptoethanol and EDTA (disodium salt) were obtained from Sigma Chemical Co.; manganese (II) chloride and cadmium chloride were products of J.T. Baker Chemical Co.; Chelex-100 (200–400 mesh) was from Bio-Rad Laboratories.

S-Carboxymethyl-L-cysteine and iodoacetic acid were purchased from ICN Biomedicals, Inc.; the iodoacetic acid was recrystallized from ligroin and stored dessicated in the dark at 4 °C. Iodo[1-^{14}C]acetic acid (13.8 mCi/mmol) was also purchased from ICN Biomedicals, Inc. Safety-Solve was obtained from Research Products International Corp. $^{54}MnCl_2$ in 0.5 M HCl (> 40 Ci/g) and a 0.1 μCi $^{54}Mn^{2+}$ reference standard were obtained from New England Nuclear.

Ninhydrin, thiodiglycol, octanoic acid, stannous chloride dihydrate, amino acid Standard H, ethylene glycol monomethyl ether and 5,5′-dithiobis(2-nitrobenzoic acid) were obtained from Pierce Chemical Co. Methyl methanethiosulfonate was purchased from Aldrich Chemical Co. and *p*-mercuribenzoate from Calbiochem. All other chemicals were reagent grade.

Enzymes and enzyme assays

Homogeneous preparations of threonine dehydrogenase were obtained from extracts of a mutant strain of *E. coli* K-12 and freed of exogenous metal ions by procedures described previously [12]. Dehydrogenase activity was measured by following the formation of NADH at 340 nm [11]. Protein concentrations of threonine dehydrogenase were determined from the absorbance at 280 nm using the previously reported absorption coefficient of 1.106 for a 1 mg/ml solution [12].

Standardization of Mn^{2+} and Cd^{2+} solutions

All plasticware, glassware, cuvettes, dialysis tubing, and reagent solutions were carefully treated to remove metal ions as described before [12]. As outlined in our earlier paper [13], we standardized stock solutions of Mn^{2+} and Cd^{2+} by the methods of Schwarzenbach and Flaschka [17,18] using EDTA (disodium salt) as the primary standard.

Binding of Mn^{2+} by threonine dehydrogenase

Co-elution of enzymatic activity and $^{54}Mn^{2+}$ from a gel-filtration column was used to examine the effect of carboxymethylation on the ability of the dehydrogenase to bind Mn^{2+}. Samples of the enzyme having their sulfhydryl groups modified to varying extents were incubated with $^{54}Mn^{2+}$ in 50 mM Tris-HCl buffer (pH 8.4) containing 2-mercaptoethanol. The incubation mixtures were then applied to and eluted from a column of Sephadex G-25 (fine) at a flow rate of 10–12 ml/h. Fractions (1 ml) were collected and assayed to determine the levels of protein, dehydrogenase activity and radioactivity.

Measurement of radioactivity

Levels of ^{14}C radioactivity were determined using a Packard Tri-Carb, Model 3300, liquid scintillation spectrometer with Safety-Solve as the scintillation fluid; counting efficiency was determined by the channel ratios method. Aqueous samples containing $^{54}Mn^{2+}$ were measured directly using a Tracor Analytic Gamma-Trac 1191 counter with the window set at 735–935 keV. A counting efficiency of 13.9% was determined with a 0.1 μCi standard of $^{54}Mn^{2+}$.

Amino acid analysis

Homogeneous samples (~ 1 mg) of threonine dehydrogenase were first dialyzed exhaustively against several changes of distilled water. They were then lyophilized to dryness, the residues dissolved in 6 M HCl, and these samples hydrolyzed for 45–90 min at 150 °C in vacuum sealed tubes. Automated amino acid analyses were per-

formed on a Beckman Model 120C analyzer. Ninhydrin-positive peaks were identified and quantified by comparison with the standard H amino acid mixture obtained from Pierce Chemical Co. The content of cysteine and its disulfide was determined either as cysteic acid after samples were oxidized with performic acid [19] or as carboxymethylcysteine following alkylation with iodoacetic acid. When hydrolyzed samples of threonine dehydrogenase containing radioactivity were fractionated and assayed on the amino acid analyzer, duplicate samples were run; the first was analyzed for its amino acid content whereas fractions (2 min) of the effluent of the second sample were collected directly without ninhydrin reaction and their levels of radioactivity determined by liquid scintillation counting.

Kinetics of enzyme inactivation by iodoacetic acid

In studies where the dehydrogenase was alkylated with iodoacetic acid, all solutions were routinely saturated with argon prior to reaction and all reactions were carried out in the dark. Dehydrogenase activity was measured by the spectrophotometric assay described previously [12], except that the alkylated enzyme was incubated for 30 min at 37 °C in 200 μmol of Tris-HCl buffer (pH 8.4) containing 2.5 μmol of 2-mercaptoethanol in order to quench unreacted iodoacetate before threonine and NAD^+ were added to initiate the assay.

Detection of disulfide bonds by the 2-nitro-5-thiosulfobenzoate assay

Thannhauser et al. [20] developed an assay for determining disulfide bonds present in proteins in their native (nondenatured) state. In this method, the disulfide bond is cleaved by excess sodium sulfite.

$$RSSR' + SO_3^{2-} \rightleftharpoons RSSO_3^- + R'S^- \quad (1)$$

Since this reaction is reversible at pH 9.0, a large excess of sulfite is required to shift the equilibrium to the right. Nbs_2 reacts with sulfite forming the thionitrobenzoate anion, so Nbs_2 cannot be used here to estimate the $R'S^-$ concentration. The NTSB anion, on the other hand, reacts only with free thiols (see below).

$$R'S^- + NTSB^{2-} \rightarrow R'SSO_3^- + Nbs^{2-} \quad (2)$$

Hence, with a protein having both free sulfhydryl groups and disulfide bonds, reaction with the NTSB reagent in the presence of excess sulfite yields one equivalent of Nbs^{2-} for each free sulfhydryl group and one equivalent of Nbs^{2-} per disulfide bond (the other half of the disulfide bond forms a sulfite adduct, see Eqn. 1).

The NTSB reagent was prepared by dissolving 0.1 g of Nbs_2 in 10 ml of 1 M Na_2SO_3 at 38 °C (pH 7.5). Oxygen was bubbled through this mixture, until the intense yellow color disappeared, to regenerate Nbs_2 from the Nbs^{2-} produced by the reaction. This stock solution was stable when stored at −20 °C and was used without further purification. The 'NTSB assay solution' was made by diluting the stock solution 100-fold with 0.2 M Tris base (pH 9.5) containing 0.1 M Na_2SO_3 plus 3 mM EDTA (2 M guanidine thiocyanate was also added as a denaturant for samples containing proteins). Reproducible results were obtained when the NTSB assay solution was prepared fresh daily. In a typical determination, 0.02 to 0.2 ml of a solution containing either a free thiol, a compound having a disulfide bond, or a protein was pipetted into 2.8 ml of 'NTSB assay solution' (containing 250 μM NTSB) and the absorbance was recorded at 412 nm against a blank containing 2.8 ml of the assay reagent plus 0.2 ml of water. The reaction was complete with oxidized glutathione in 3 min at 37 °C; reaction with protein sulfhydryl groups and disulfide bonds was complete in 25 min at 37 °C. The assay was linear up to 67 μM of oxidized glutathione.

With oxidized glutathione as a standard, the absorption coefficient for the thionitrobenzoate anion is 13 600 $M^{-1} \cdot cm^{-1}$ in the absence of guanidine thiocyanate; this coefficient decreased to 12 100 $M^{-1} \cdot cm^{-1}$ in the presence of 2 M guanidine thiocyanate. The second value was used to calculate the total disulfide content of threonine dehydrogenase titrated in the presence of 2 M guanidine thiocyanate.

Results

Free thiol and disulfide content of E. coli threonine dehydrogenase

The total Cys/2 content of threonine dehydrogenase was determined by two methods. A value of 6.01 Cys/2 per subunit (determined as cysteic acid) was found by performic acid oxidation [19], confirming the result reported previously [11]. Similarly, reduction of the enzyme with dithiothreitol followed by alkylation with iodoacetate gave a value of 5.59 Cys/2 per subunit.

The number of free sulfhydryl groups in each subunit was determined by spectrophotometric titrations under denaturing conditions with three reagents. A total of four thiols per subunit was seen with Nbs_2 [21]. Values of 3.97 and 3.89 thiols per subunit were obtained with dithiodipyridine [22] and *p*-mercuribenzoate [23], respectively. Titration with Nbs_2 in the presence of 2% SDS was used routinely to confirm the thiol content of all dehydrogenase preparations.

From the foregoing values, the presence of a single disulfide bond per subunit of threonine dehydrogenase was predicted. The disulfide content of the enzyme was actually determined by two methods: (a) selective modification of free sulfhydryl groups and disulfide linkages with unlabeled and iodo[1-^{14}C]acetic acid, respectively,

and (b) the 2-nitro-5-thiosulfobenzoate assay of Thannhauser et al. [20]. Selective labeling studies with iodoacetic acid were based on the method of Hausinger and Howard [24]. The enzyme was first treated with unlabeled iodoacetate to alkylate the free thiols; the disulfide bond was then reduced with a 10-fold excess of dithiothreitol and alkylation subsequently carried out with a 10-fold excess (over the total thiol content of the reaction mixture) of iodo[1-^{14}C]acetate. The total CMCys content per dehydrogenase subunit was determined by standard amino acid analyses and the [1-^{14}C]CMCys content by fractionation on the amino acid analyzer followed by scintillation counting as described in Materials and Methods. Analysis of two different dehydrogenase preparations by this selective labeling method gave values of 1.71 and 1.94 equivalents of ^{14}CMCys per enzyme subunit, demonstrating the presence of 0.86 and 0.97 (or essentially 1.0) disulfide bond per subunit. Two controls were run in these studies. With one, the enzyme was first alkylated with unlabeled iodoacetate to react with the free sulfhydryl groups and then immediately treated with iodo[1-^{14}C]acetate without adding any dithiothreitol. For the second control, the enzyme was first reduced with excess dithiothreitol, then alkylated with unlabeled iodoacetate, and finally exposed to an excess of iodo[1-^{14}C]acetate. It was found with controls 1 and 2 that 0.03 and 0.09 of an equivalent of ^{14}CMCys, respectively, was formed per enzyme subunit, indicating that radioactive labeling resulted only by alkylation of cysteine residues in threonine dehydrogenase that had been generated by reduction of the disulfide bond with dithiothreitol. Furthermore, amino acid analyses of all enzyme samples showed no detectable modification of any other amino acid residues. The presence of a single disulfide bond per enzyme subunit was confirmed by the 2-nitro-5-thiosulfobenzoate assay (see Materials and Methods); a value of 1.11 disulfide bond/threonine dehydrogenase subunit was obtained by this method.

Correlation of threonine dehydrogenase activity with modification of free thiols by various reagents

The effect of several specific thiol-reacting reagents of varying size and charge on threonine dehydrogenase activity was examined. For this purpose, the enzyme was incubated for 10 min with varying concentrations of a given reagent before being assayed for activity. Complete inactivation of dehydrogenase activity was observed with 400 μM methyl methanethiosulfonate (a 350-fold molar excess over total enzyme sulfhydryl groups) while incubation with 3.2 mM iodoacetamide caused less than a 10% loss. Reaction with a 40-fold molar excess of Nbs_2 under non-denaturing conditions caused a gradual 99% loss of dehydrogenase activity as 3.44 thiols per enzyme subunit were modified. The rate of modification ($k_{mod} = 0.012\ min^{-1}$) was 1.8-times faster than the rate of inactivation ($k_{inact} = 0.0065\ min^{-1}$), suggesting that the observed loss of activity was most likely due to modification of some but not all of the thiols. Semi-logarithmic plots of both rates were linear; neither plot provided any evidence for the presence of a sulfhydryl group that was uniquely reactive toward Nbs_2. Attempts to examine the kinetics of threonine dehydrogenase inactivation by a 10-fold excess of *p*-mercuribenzoate were unsuccessful; inactivation occurred so rapidly that a rate could not be followed. The rapid, stoichiometric reaction of *p*-mercuribenzoate with threonine dehydrogenase was confirmed in titration experiments performed under non-denaturing conditions [23]. After each limited addition of *p*-mercuribenzoate, an aliquot of the titration mixture was removed, diluted 25-fold and assayed for dehydrogenase activity. Titration of the enzyme with this reagent showed a linear decline to complete inactivation as 3.59 mol of *p*-mercuribenzoate reacted per mol of enzyme subunit. Collectively, the results suggest that threonine dehydrogenase has no sulfhydryl groups which are uniquely reactive with bulky thiol-modifying reagents; rather, all such groups appear to be equally accessible to and reactive with Nbs_2 and *p*-mercuribenzoate.

Effect of thiol modification by iodoacetate on Mn^{2+} interaction with E. coli threonine dehydrogenase

The thiol-dependence of threonine dehydrogenase activation by Mn^{2+} [12] prompted us to examine whether a relationship existed between thiol modification and Mn^{2+} binding. Threonine dehydrogenase which had been modified with *p*-mercuribenzoate showed, by the column chromatography method, a significant loss in the ability to bind Mn^{2+}. However, attempts to quantify these results were not possible, since the addition of 2-mercaptoethanol that is required for complete enzyme activation by Mn^{2+} also reversed the modification by *p*-mercuribenzoate. We turned, therefore, to the use of iodoacetic acid since adducts formed with this reagent are not reversed by subsequent addition of an exogenous thiol. For this purpose, a large quantity of 'Mn^{2+}-free' threonine dehydrogenase was reacted with iodo[1-^{14}C]acetate in a single reaction vessel; aliquots were withdrawn when approx. 25, 50 and 75% of the dehydrogenase activity had been lost. These enzyme samples were then assayed for their ability to bind Mn^{2+}, their Mn^{2+}-independent enzyme activity, and the extent to which Mn^{2+} stimulated their activity. Carboxymethylation of approx. 1.0 equivalent of cysteine per enzyme subunit resulted in complete loss of both 'Mn^{2+}-free' dehydrogenase activity (Fig. 1A) and the ability of the enzyme to bind Mn^{2+} (Fig. 1B). Furthermore, the kinetics of Mn^{2+} activation of partially carboxymethylated enzyme showed that the Mn^{2+}-dependent velocity decreased linearly with carboxymethylation of 1.02 equivalents of cysteine per

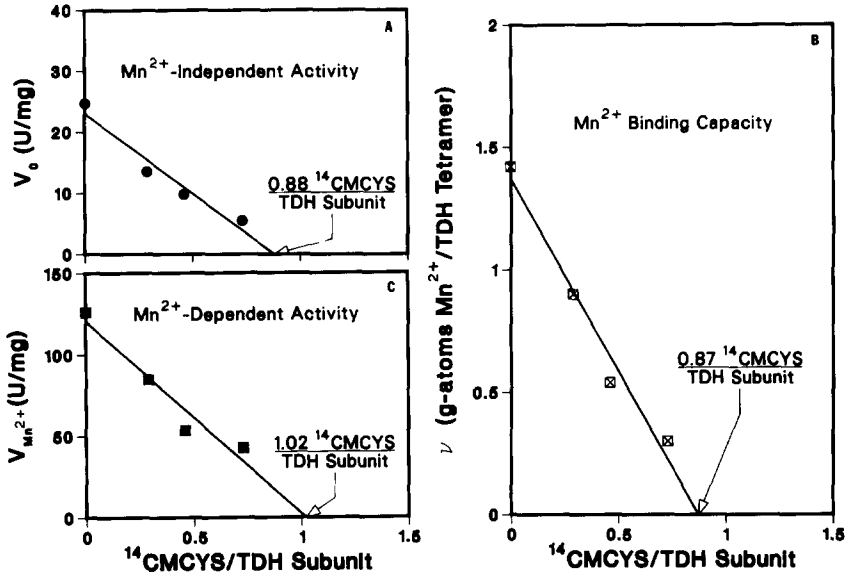

Fig. 1. Effect of carboxymethylation by iodoacetate on the activity and the Mn^{2+}-binding capacity of threonine dehydrogenase. 'Mn^{2+}-free' enzyme (8.3 mg) was first incubated at 4° C in 45 mM Tris-HCl buffer (pH 8.4) for 15 min after which time an aliquot (2 mg) was removed as the control. A 40-fold excess of iodo[1-^{14}C]acetate (1150 dpm/nmol) was then added and aliquots (20 μl) withdrawn periodically for assay. After the enzyme was approx. 25, 50 and 75% inactivated, aliquots containing 2 mg of protein were removed, a 10-fold excess of 2-mercaptoethanol was added, and all samples were applied to and eluted from columns (50×1 cm) of Sephadex G-25. Dehydrogenase activity, protein (A_{280}), and radioactivity (^{14}CMCys content, except in the control) eluted as a single peak in each case. (A) Threonine dehydrogenase-containing fractions from each column were pooled and assayed as indicated. (B) Aliquots (0.7–0.9 mg of enzyme) were first removed and incubated for 3 h at 4° C with 200 nmol of $^{54}Mn^{2+}$ (98900 dpm/nmol) in 50 mM Tris-HCl buffer (pH 8.4) containing 5 mM 2-mercaptoethanol. These samples were then again applied to and eluted from columns of Sephadex G-25 and the pooled fractions assayed as before. (C) Activation by Mn^{2+} was determined for the control and the partially inactivated enzyme samples. The maximal Mn^{2+}-dependent activity was then plotted against the ^{14}CMCys content.

enzyme subunit (Fig. 1C), while the activation K_d (determined as described in Ref. 12) for Mn^{2+} remained essentially constant at 7.4 ± 1.3 μM for all enzyme samples.

Kinetics of threonine dehydrogenase modification with iodoacetate

The kinetics of enzyme modification and inactivation by iodoacetate were examined under pseudo-first-order reaction conditions to confirm the presence of a sulfhydryl group which was uniquely reactive toward this reagent. The results obtained are shown in Fig. 2. A slow but measurable rate of activity loss (k_0) was seen with the control; the iodoacetate-dependent pseudo-first-order inactivation rate constant (k'_{obs}) was obtained by subtracting k_0 from the observed rate constant (k_{obs}) at each concentration of iodoacetate. A plot of k'_{obs} vs. [iodoacetate] did not intersect the origin, indicating that the alkylation of threonine dehydrogenase by iodoacetate does not follow simple second-order kinetics. To explain such a deviation, Kitz and Wilson [25] proposed a two-step inactivation reaction in which the formation of a reversible enzyme-inhibitor complex (E · I) of the enzyme (E) with the inhibitor (I) is followed by irreversible alkylation to form modified enzyme (E′) [25,26].

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E \cdot I \xrightarrow{k_3} E' \qquad (3)$$

This mechanism can be described by the following rate equation:

$$k'_{obs} = \frac{k_3}{1 + K_i/[I]} \qquad (4)$$

where $K_i = (k_2 + k_3)/k_1$ and k'_{obs} is the observed pseudo-first-order rate constant for the inactivation reaction. A plot of $1/k'_{obs}$ vs. $1/[I]$ will have an intercept $= 1/k_3$ and a slope $= K_i/k_3$. From the slope and intercept of the line in the inset of Fig. 2, a $K_i = 6.65$ mM was determined for enzyme-inhibitor complex formation, indicating a weak interaction of iodoacetate with threonine dehydrogenase prior to the irreversible alkylation reaction. This weak interaction explains why such a large excess of iodoacetate (100–1000-fold over total enzyme thiol content) is required to inactivate threonine dehydrogenase. The rate constant for the second step, k_3, was 0.151 min^{-1}, yielding a second-order rate constant (k_3/K_i) of 0.023 $mM^{-1} \cdot min^{-1}$ for this two-step inactivation reaction.

Protection of E. coli threonine dehydrogenase against inactivation by iodoacetate

The effect of NAD^+, NADH and L-threonine on inactivation by iodoacetate was examined. Approx. 15% protection was seen in the presence of 5 mM NAD^+ (about 50-times its K_m value), whereas L-threonine, at a

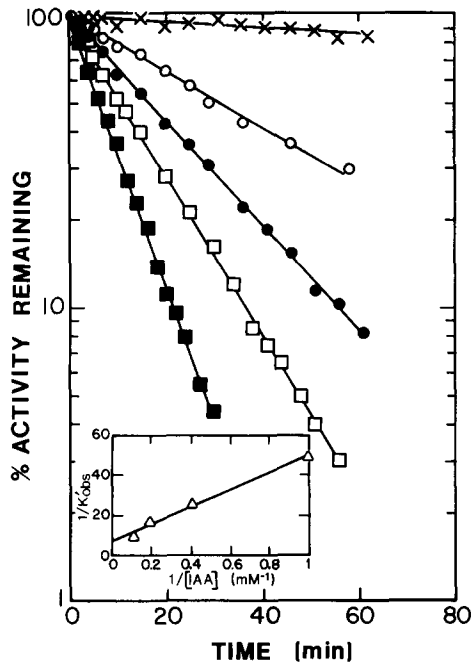

Fig. 2. Kinetics of inactivation of 'Mn^{2+}-free' threonine dehydrogenase by iodoacetate. Solutions (1.8 ml) containing 350 μmol of Tris-HCl buffer (pH 8.4) and the following final concentrations of iodoacetate were incubated at 37 °C for 5 min: 0 (×), 1.0 mM (○), 2.5 mM (●), 5.0 mM (□), 10 mM (■). A sample of enzyme (0.69 mg/ml) was also incubated separately for 5 min at 37 °C; 0.2-ml aliquots were then removed, added to the tubes containing the iodoacetate, and mixed. Aliquots (0.1 ml) of the reaction mixtures were removed at specified times, diluted 10-fold, and assayed for dehydrogenase activity. (Inset) Secondary plot of the data [25,26].

concentration equivalent to 5-times its K_m (5 mM), provided about 30% protection (Fig. 3A, lower curve). NADH was the most effective compound tested; 40% protection was observed at a concentration of 0.5 mM (Fig. 3B, lower curve). The K_m for NADH in the reverse direction of the reaction (NADH + 2-amino-3-oxobutyrate → NAD^+ + L-threonine) has not been determined because the amino-oxo acid is very unstable and unavailable. As an alternative, the inhibitory effect of NADH against NAD^+ in the forward reaction was examined. Inhibition by NADH was found to be competitive with NAD^+ with a $K_i = 41$ μM. Hence, the protective effect of NADH was evident at 10–12-times its K_i, whereas little protection was seen with NAD^+ at concentrations 50-times its K_m value.

The combined effect of NADH plus L-threonine was nearly additive; 60 to 70% protection was seen in the presence of saturating levels of both (Fig. 3A and B, upper curves). The kinetics of inactivation of threonine dehydrogenase by iodoacetate were then determined in the presence of 0.5 mM NADH. The experimental conditions were identical to those used in the earlier inactivation kinetic studies, except that 0.5 mM NADH was included in the reaction mixture. As before, the plot of k'_{obs} vs. [iodoacetate] failed to intersect the origin. When the data were plotted according to equation 4, a K_i of 27.5 mM, an inactivation rate constant (k_3) of 0.164 min^{-1}, and a second-order rate constant of 0.006 $mM^{-1} \cdot min^{-1}$ were obtained. Similar studies done in the presence of 20 mM L-threonine gave a K_i of 18.7 mM, a k_3 of 0.132 min^{-1}, and a second-order rate constant of 0.007 $mM^{-1} \cdot min^{-1}$.

Inactivation of Cd^{2+}-saturated threonine dehydrogenase by iodoacetate

As already noted, threonine dehydrogenase activation by Mn^{2+} is thiol-dependent. Complete binding of Mn^{2+} to threonine dehydrogenase is also seen only in the presence of an exogenous thiol [12]. It was not possible, therefore, to measure the kinetics of inactivation by iodoacetate for the Mn^{2+}-saturated enzyme. Cd^{2+}, however, also activates *E. coli* threonine dehydrogenase and most of the properties of the enzyme activated by these two metal ions are very similar [13]. The one significant difference is that Cd^{2+}-activation of threonine dehydrogenase is not thiol-dependent. Therefore, the kinetics of inactivation of metal ion-saturated threonine dehydrogenase by iodoacetate were

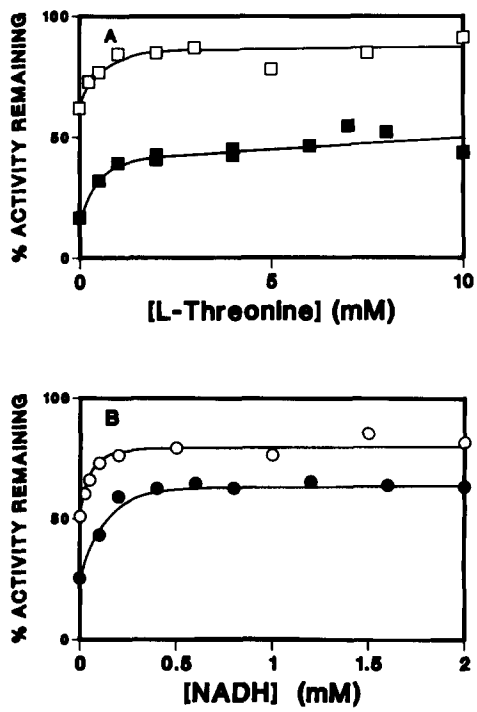

Fig. 3. Protective effects of threonine and NADH against inactivation of threonine dehydrogenase by iodoacetate. A concentration of iodoacetate was used that caused ≈ 80% inactivation in the control. The degree of protection observed was then indicated by the difference betwen the activity remaining in the protected sample and the control. (A) Enzyme (2.8 μg) was incubated with L-threonine (■) or L-threonine plus 1.0 mM NADH (□) and 5 mM iodoacetate in 0.2 M Tris-HCl buffer (pH 8.4) at 37 °C for 20 min (total vol. = 0.2 ml). Aliquots (0.1 ml) were then removed, diluted 10-fold, and assayed for dehydrogenase activity. (B) Enzyme was incubated with NADH (●) or NADH plus 20 mM L-threonine (○) as indicated for (A) above.

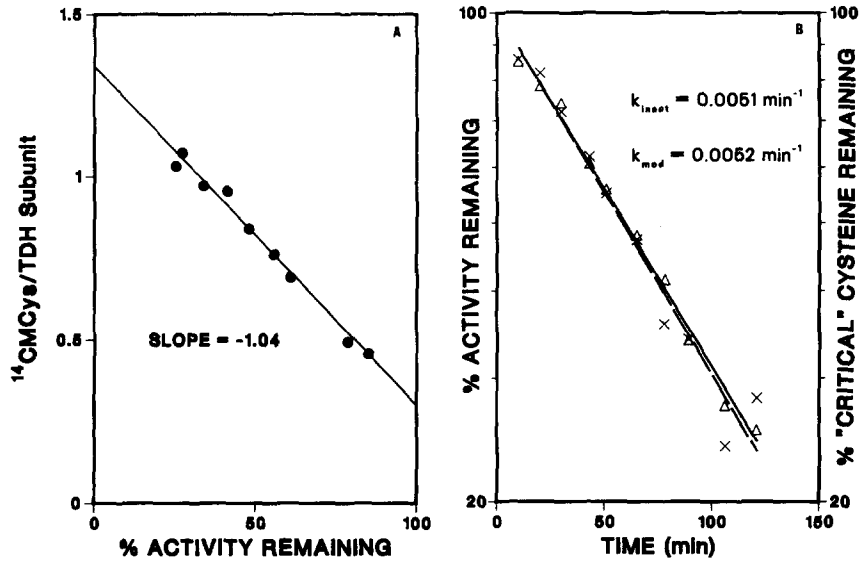

Fig. 4. Comparative rates of carboxymethylation and inactivation of threonine dehydrogenase by iodoacetate. (A) 'Mn^{2+}-free' enzyme (8.0 mg) in 0.2 M Tris-HCl buffer (pH 8.4) was incubated for 15 min before a final concentration of 1.0 mM iodo[1-^{14}C]acetate (5570 dpm/nmol) was added (total reaction vol. = 20 ml). Six aliquots (2 ml) were withdrawn at fixed times, the reaction quenched by adding a 10-fold excess of 2-mercaptoethanol, and the mixtures applied to and eluted from a column (50×1 cm) of Sephadex G-25. The protein-containing fractions were pooled and the levels of dehydrogenase activity, protein, and radioactivity determined. Three aliquots (2 ml), removed at 20, 51 and 106 min, were dialyzed exhaustively against water, then lyophilized, and finally hydrolyzed in vacuo in constant boiling 6 M HCl for 45 min at 150 °C. Amino acid analyses showed that > 95% of the radioactivity incorporated was due to CMCys formation. Total CMCys formation is plotted against the dehydrogenase activity remaining. (B) Rate of loss of activity (△) and of the 'critical' cysteine residue (×). The 'critical' cysteine was determined by the relationship: % 'Critical' Cysteine Remaining = [1 − (^{14}CMCys incorporated − 0.30)]×100, where the value of 0.30 is taken from the *y*-intercept (100% activity) in Fig. 4A.

examined following enzyme activation by 10 μM Cd^{2+} (a concentration ten times the activation K_d for Cd^{2+}). Enzyme inactivation occurred much more rapidly in the presence of Cd^{2+} than in its absence. When the concentration of iodoacetate was varied from 0.5 mM to as high as 20 mM, an inactivation rate constant (k_3) of 5.3 min^{-1} and a K_i of 51.9 mM were obtained, yielding a second-order rate constant of 0.102 $mM^{-1} \cdot min^{-1}$.

Reaction of iodo[1-^{14}C]acetate with 'Mn^{2+}-free' threonine dehydrogenase: comparison of the rate of modification with the rate of inactivation

In Fig. 1, the modification of a single equivalent of cysteine per threonine dehydrogenase subunit was shown to correlate well not only with the loss of Mn^{2+}-dependent and Mn^{2+}-independent enzymatic activity, but also with the loss of the Mn^{2+} binding capacity of the enzyme. To confirm these findings, the rates of thiol modification and of enzyme inactivation by iodo[1-^{14}C]acetate were examined and compared using the 'Mn^{2+}-free' enzyme.

A plot of the equivalents of ^{14}CMCys formed per subunit against the percent of enzymatic activity remaining gave a line whose slope indicated that complete inactivation of threonine dehydrogenase correlated with the modification of 1.04 equivalents of cysteine per enzyme subunit (Fig. 4A). Total incorporation of radioactivity extrapolates to 1.34 ^{14}CMCys/subunit at 0% enzymatic activity and to 0.30 ^{14}CMCys/subunit when the dehydrogenase is fully (100%) active. It appears that 0.30 of an equivalent of ^{14}CMCys/subunit was formed very rapidly and had no detectable effect on threonine dehydrogenase activity. Such non-specific labeling was attributed to a small amount of denatured enzyme present in this preparation. The rates of inactivation and modification of the 'critical' equivalent of cysteine (i.e., that involved in inactivation) are compared in Fig. 4B. As can be seen, the two rates are identical within experimental error, further supporting the conclusion that carboxymethylation of a single equivalent of cysteine per threonine dehydrogenase subunit results in enzyme inactivation.

Discussion

The observed sensitivity of *E. coli* threonine dehydrogenase to inactivation by thiol-specific reagents and the dependence of enzyme activation by Mn^{2+} on the presence of added thiols [12] prompted this study. A total of six cystine/2 residues per enzyme subunit was found by two different methods; this value is in complete agreement with other independently obtained data [11,27]. Titration under denaturing conditions with several different thiol-specific reagents demonstrated the presence of four free sulfhydryl groups per enzyme subunit, and selective labeling with iodo[1-^{14}C]acetate

as well as application of the specific 5-nitrothiosulfobenzoate assay showed the presence of one disulfide bond in each subunit.

The effect of thiol modification on enzyme activity was then examined. Two bulky reagents, Nbs_2 and *p*-mercuribenzoate, both inactivated the enzyme at low concentrations; in fact, the reaction with stoichiometric quantities of *p*-mercuribenzoate was so rapid that the kinetics of inactivation could not be examined under pseudo-first order conditions. However, no evidence was found for the presence of a thiol uniquely reactive toward either Nbs_2 or *p*-mercuribenzoate. The small uncharged reagent, methyl methanethiosulfonate, also inactivated the enzyme but a large excess of this compound was required.

In contrast, we found that iodoacetic acid selectively modifies a single sulfhydryl group per subunit of *E. coli* threonine dehydrogenase. The formation of one equivalent of carboxymethylcysteine per subunit results in complete loss of the Mn^{2+}-independent level of dehydrogenase activity as well as of the ability of the enzyme to bind and hence be stimulated by Mn^{2+}. This selective modification by iodoacetate causes complete loss of function; the enzyme is either completely active or completely inactive – thus, the activation K_d remains constant while the Mn^{2+}-dependent activity decreases linearly with the extent of carboxymethylation. The identical rates of sulfhydryl group modification and of enzyme inactivation shown in Fig. 4B further indicate that the loss of dehydrogenase activity is caused by modification of a unique cysteine residue in each dehydrogenase subunit rather than partial modification of several cysteine residues.

The two-step reaction mechanism for the inactivation of threonine dehydrogenase by iodoacetate is typical of affinity labeling reactions; the affinity label initially binds to the enzyme and subsequently reacts with the susceptible residue. Similar results were obtained in the modification of horse liver alcohol dehydrogenase [28] and the $\beta_1\beta_1$ and $\beta_2\beta_2$ isozymes of human liver alcohol dehydrogenase [29]. In all three of these cases, a single sulfhydryl group per subunit was modified by iodoacetate via a two-step reaction mechanism; Cys-46 was modified in the horse liver enzyme, while Cys-174 was modified in both isozymes of human liver alcohol dehydrogenase. Interestingly, the kinetic constants for *E. coli* threonine dehydrogenase (not activated by Cd^{2+}) and the two human isozymes of alcohol dehydrogenase are quite similar: k_3 values of 0.22, 0.17, and 0.15 min^{-1} and K_i values of 10, 16 and 7 mM were found for the human $\beta_1\beta_1$ isozyme, the human $\beta_2\beta_2$ isozyme, and threonine dehydrogenase, respectively. A similar K_i of 6 mM has been reported for the horse liver enzyme [29].

Substrates, however, show differing protective results with the human liver alcohol dehydrogenase isozymes and threonine dehydrogenase. Saturating concentrations of NADH gave complete protection to both isozymes of human alcohol dehydrogenase and NADH was shown to competitively inhibit inactivation by iodoacetate [29]. With *E. coli* threonine dehydrogenase, on the other hand, only partial protection against inactivation was obtained with saturating concentrations of NADH. Most likely, both threonine and NADH exert their protective effect by interfering with the initial binding of iodoacetate to threonine dehydrogenase; however, the failure of either compound to provide complete protection at saturating concentrations rules against direct competition between either of these two compounds and the modifier. Rather, the observed protection might be due to stabilization of the enzyme in a conformation that is less susceptible to attack by iodoacetate.

The different effects seen with iodoacetic acid and iodoacetamide also provide some clues regarding the inactivation caused by iodoacetate. Whereas iodoacetate rapidly and specifically inactivates threonine dehydrogenase, similar concentrations of iodoacetamide have very little effect. Initial formation of the enzyme-inhibitor complex, therefore, may depend on an ionic interaction between the carboxyl group of iodoacetate and a positively charged residue near the site of alkylation. Also, the failure of high concentrations of NAD^+ to provide significant protection against inactivation may be due to charge–charge repulsion between this compound and a positive charge near the susceptible thiol. Similar results were obtained with horse liver alcohol dehydrogenase. This enzyme is inactivated much more rapidly by iodoacetate than by iodoacetamide [28], and inactivation by iodoacetamide followed a simple bimolecular reaction with no evidence for initial inhibitor-enzyme complex formation prior to alkylation. Reversible formation of an enzyme-inhibitor complex between iodoacetate and the horse liver enzyme is attributed to ionic attraction between the carboxyl group of iodoacetate and the guanidinium group of Arg-47, which is adjacent to Cys-46, the site of alkylation.

Establishing that Cd^{2+}-activation of threonine dehydrogenase was not thiol dependent [13] proved to be useful in studying the kinetics of iodoacetate inactivation of the metal ion-activated enzyme. The K_i value was found to be significantly higher for the Cd^{2+}-activated than for the Cd^{2+}-free enzyme; the same was true for the kinetics of inactivation of the 'Mn^{2+}-free' dehydrogenase in the presence (as opposed to the absence) of either NADH or L-threonine. However, whereas the k_3 value for Mn^{2+}/Cd^{2+}-free threonine dehydrogenase is virtually the same as in the presence of either NADH or L-threonine, this rate constant for the second step in the inactivation reaction is increased > 30-fold in the presence of Cd^{2+} indicating that the reactivity (i.e., nucleophilicity) of the thiol group toward iodoacetate is markedly enhanced by Cd^{2+}. Our demonstration that carboxymethylation of a single equivalent

of cysteine results in concomitant loss of the enzyme's ability to bind Mn^{2+} and of stimulation of its activity by Mn^{2+} points to a role for the modified thiol as a ligand in the coordination sphere of the metal ion (Mn^{2+} or Cd^{2+}). The finding, however, that Cd^{2+} increases the rate of inactivation instead of protecting the susceptible thiol from alkylation rules against a direct interaction between the thiol and Cd^{2+} (or Mn^{2+}). Carboxymethylation of that thiol may cause a conformational change in the enzyme which interferes with Cd^{2+} (or Mn^{2+}) binding; the attached carboxymethyl group may block access to the metal ion binding site as well.

The finding of a uniquely reactive sulfhydryl group in threonine dehydrogenase is interesting in light of our earlier observation that the pH-activity profile of the Cd^{2+}- and the Mn^{2+}-activated enzyme fits a curve for deprotonation of a single group on the enzyme with a $pK_a \cong 7.9$. This ionization, which is seen in the presence of Cd^{2+} and Mn^{2+} (but not in their absence), may directly involve the thiol group of a cysteine side-chain or, alternatively, another group adjacent to it which makes it more reactive toward iodoacetate.

On the basis of our current results, it seems most likely that thiol modification of *E. coli* threonine dehydrogenase by iodoacetate has primarily a structural rather than a catalytic effect. The failure of saturating concentrations of either NADH or threonine to provide complete protection of enzyme activity appears to rule against the presence of a catalytic thiol in threonine dehydrogenase such as has been reported with pig muscle glyceraldehyde-3-phosphate dehydrogenase. Furthermore, it would be expected that the enzyme would be more susceptible to inactivation by a low molar excess of methyl methanethiosulfonate if a thiol were directly involved in catalysis.

The results in hand are most consistent with a model in which a positive charge in or near the microenvironment of the susceptible thiol forms an initial enzyme-inhibitor complex with iodoacetate prior to alkylation; this alkylation results in a change in enzyme conformation and/or active site leading to a loss of activity as well as metal ion binding capability.

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