L-Threonine Dehydrogenase from *Escherichia coli* K-12: Thiol-Dependent Activation by Mn^{2+} †

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ABSTRACT: Addition of 1 mM Mn^{2+} to all solutions in the final chromatographic step used to purify L-threonine dehydrogenase (L-threonine:NAD^+ oxidoreductase, EC 1.1.1.103) from extracts of *Escherichia coli* K-12 routinely provides 30–40 mg of pure enzyme per 100 g wet weight of cells with specific activity = 20–30 units/mg. Enzyme dialyzed exhaustively against buffers containing Chelex-100 resin has a specific activity = 8 units/mg and contains 0.003 or 0.02 mol of Mn^{2+}/mol of enzyme as determined by radiolabeling studies with $^{54}Mn^{2+}$ or by atomic absorption spectroscopy, respectively. Dehydrogenase activity is completely abolished by low concentrations of either Hg^{2+} or Ag^+; of a large spectrum of other metal ions tested, only Mn^{2+} and Cd^{2+} have an activating effect. Activation of threonine dehydrogenase by Mn^{2+} is thiol-dependent and is saturable with an activation K_d = 9.0 μM and a V_{max} = 105 units/mg. Stoichiometry of Mn^{2+} binding was found to be 0.86 mol of Mn^{2+}/mol of enzyme subunit with a dissociation constant (K_d) = 8.5 μM. Mn^{2+} appears to interact directly with threonine dehydrogenase; gel filtration studies with the dehydrogenase plus $^{54}Mn^{2+}$ in the presence of either NAD^+, NADH, L-threonine, or combinations thereof show that only Mn^{2+} coelutes with the enzyme whereas all other ligands elute in the salt front and the stoichiometry of the dehydrogenase–Mn^{2+} interaction is not affected in any instance. A theoretical curve fit to data for the pH–activity profile of Mn^{2+}-saturated enzyme has a pK_a = 7.95 for one proton ionization. The data establish L-threonine dehydrogenase of *E. coli* to be a metal ion activated enzyme.

The reaction catalyzed by threonine dehydrogenase (EC 1.1.1.103) has recently been shown to initiate the primary route for threonine utilization in both eukaryotes (Dale, 1978) and prokaryotes (Boylan & Dekker, 1983; Komatsubara et al., 1978). This is the only pathway for L-threonine utilization that is detected in chicken liver (Aoyama & Motokawa, 1981), and it accounts for 87% of the L-threonine degraded in the liver of normally fed rats (Bird & Nunn, 1983).

Although other investigators have reported that cell-free extracts of numerous microorganisms and mammalian tissues catalyze the NAD^+-dependent[1] oxidation of threonine, our laboratories were the first to succeed in obtaining threonine dehydrogenase in pure form (Boylan & Dekker, 1981). This was accomplished by fractionating extracts of a mutant strain of *Escherichia coli* K-12 (designated *E. coli* SBD76), which was obtained by forcing wild-type cells to grown on L-threonine as the sole carbon source. Of the three known enzymes that initiate threonine catabolism (i.e., an aldolase, a dehydratase, and threonine dehydrogenase), only the level of the latter enzyme is highly elevated in this mutant. In first studies to establish some of its general properties, we found that the pure dehydrogenase is a tetramer (M_r 140 000) of apparently identical subunits and that activity was stimulated 2–3-fold by added Mn^{2+} (Boylan & Dekker, 1981). In this paper, we report isolating the enzyme by a modified procedure that takes advantage of the interaction of Mn^{2+} with the dehydrogenase; significantly higher yields of the pure enzyme are obtained. In addition, results are described that quantitatively establish the Mn^{2+}–threonine dehydrogenase interaction and provide some insight on the role of Mn^{2+} in catalysis.

Materials and Methods

Our mutant *E. coli* strain SBD76 was isolated and grown in large batches as previously described (Boylan & Dekker, 1981, 1983). Likewise, Blue Dextran–Sepharose 4B was synthesized as outlined before (Boylan & Dekker, 1981) with the following modifications. After the gel was reacted with Blue Dextran, removed by filtration, and washed with 1 M KCl, it was suspended and stirred for 24 h at 4 °C in 100 mL of 0.15 M Tris-HCl buffer (pH 8.6) containing 4 mg/mL bovine serum albumin (Ramadoss et al., 1983). Thereafter, the gel was washed with 2 L of 1 M KCl to remove noncovalently bound albumin and finally resuspended in 10 mM Tris-HCl buffer (pH 7.5) for storage. On the basis of the absorbance of the wash fluid at 280 nm, it was estimated that 0.6 g of Blue Dextran was covalently bound to the Sepharose.

Protein Determinations. The level of protein in cell extracts and in partially purified enzyme fractions was measured by the method of Lowry et al. (1951) with crystalline bovine serine albumin as the standard. The concentration of pure threonine dehydrogenase preparations was determined more precisely by dry weight measurements (Kupke & Dorrier, 1978). Five milligrams of TDH was lyophilized and dried under vacuum to minimum weight. Multiple samples were then carefully weighed, the mass was recorded every 30 s for 10 min, and these values were extrapolated to zero time. These samples were then dissolved, and the absorbance spectrum from 320 to 240 nm was recorded on a Cary 219 spectrophotometer against a blank containing only buffer. An ex-

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[1] Abbreviations: Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced form of NAD^+; SDS, sodium dodecyl sulfate; TDH, L-threonine dehydrogenase; Tris, tris(hydroxymethyl)aminomethane.

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tinction coefficient of 1.106 ± 0.005/mg/mL was determined for pure threonine dehydrogenase at 280 nm. To confirm this value, 200 μg of each sample was hydrolyzed in vacuo in 6 N HCl for 40 h at 110 °C. Total residue weight was then determined on the basis of amino acid analyses with a Beckman amino acid analyzer (Model 120C). Protein concentration based on total residue weight agreed within 5% of that determined by the dry weight method.

Assays for L-*Threonine Dehydrogenase Activity.* In Assay I, the amount of aminoacetone formed from L-threonine was determined colorimetrically by a modification (Gibson et al., 1958) of the method of Mauzerall and Granick (1956). TDH activity in crude extracts or in partially purified fractions was determined by this method. Assay II, in which NADH formation was monitored at 340 nm, was used in studies with samples of the pure dehydrogenase. Measurements were made with the use of a Cary 219 spectrophotometer equipped with a thermostated multicuvette changer. Mixtures (0.98 mL) containing 200 μmol of Tris-HCl buffer (pH 8.4), 125 μmol of L-threonine, and 5 μmol of NAD^+ were first incubated for 5 min at 37 °C. The reaction was then initiated by adding enzyme (20 μL). One unit of enzyme activity is defined as that amount which catalyzed the formation of 1.0 μmol of NADH/min at 37 °C. Specific activity is expressed as units per milligram of protein. For Mn^{2+}-dependent kinetic studies, Assay II was modified as follows. Mixtures (0.5 mL) containing 200 μmol of Tris-HCl buffer (pH 8.4), 2.5 μmol of 2-mercaptoethanol, plus designated amounts of enzyme and Mn^{2+} were first incubated for 30 min at 37 °C. The reaction was then initiated by adding a mixture (0.5 mL) containing 125 μmol of L-threonine plus 5 μmol of NAD^+. All solutions were maintained at 37 °C; this was designated Assay IIm.

Removal of Contaminating Metal Ions. All metal ion free studies were done in polyethylene and polystyrene plasticware that had been thoroughly rinsed with ethanol followed by glass double-distilled water (Thiers, 1957). The buffer solution used in these studies [50 mM Tris-HCl (pH 8.4) containing 5 mM 2-mercaptoethanol] was freed of contaminating metal ions by passage through a 30-mL syringe filled with Chelex-100. All other solutions were treated batchwise with Chelex-100. Dialysis tubing was boiled twice in 50 mM EDTA, then boiled several times in glass double-distilled water, and finally rinsed with glass double-distilled water. Cuvettes used for spectrophotometric measurements were soaked for 2 h in 95% ethanol/1 N HCl (1:1) and then rinsed sequentially with 50 mM EDTA, water, and ethanol. All glassware used in the standardization of Mn^{2+} solutions was soaked in a hot nitric acid/sulfuric acid (3:1) mixture and then rinsed with glass double-distilled water and oven-dried.

Binding Studies with Mn^{2+}. Quantitative measurement of the amount of Mn^{2+} bound by TDH was carried out by the gel filtration method of Hummel and Dreyer (1962). To obtain reproducible results, several precautions were taken. All solutions were made and stored in metal ion free polyethylene bottles. All glass and metal components were coated with Poly-Kote (1% dichlorodimethylsilane in benzene), and only Teflon-coated plastic tubing and connectors were used. The column (33 × 0.6 cm) of Sephadex G-25 (fine) had a zero dead volume; the Sephadex was previously dialyzed against Chelex-100 resin. The temperature of the column was maintained at 11 ± 1 °C, and the column was equilibrated with 50 mM Tris-HCl buffer (pH 8.4) containing 5 mM 2-mercaptoethanol and 5–40 μM $^{54}Mn^{2+}$ (sp act. = 800–3500 cpm/nmol). Stock solutions of $MnCl_2 \cdot 4H_2O$ were standardized by titration against EDTA (Flaschka & Amin, 1953). At each Mn^{2+} concentration, three TDH samples (0.32–0.77 mg) were preequilibrated with a Mn^{2+} concentration identical with that of the column; each was then applied to and subsequently eluted from the column. One-milliliter fractions were collected and assayed for TDH activity (Assay II), protein content (A_{280}), and level of radioactivity (using a Tracor Analytic Gamma-Trac 1191 counter with a window of 735–935 keV and counting efficiency of 13.9%). The difference between base-line radioactivity and that of each fraction in the trough was determined; the amount of Mn^{2+} bound was calculated from the relationship:

$$\text{nmol of } Mn^{2+} \text{ bound} = \sum_i (\Delta\text{cpm}_i)(\text{mL}_i)/\text{sp act.} \quad (1)$$

where Δcpm_i is the difference between the radioactivity of fraction i and base-line radioactivity, mL_i is the volume of fraction i, and sp act. is the specific activity of the $^{54}Mn^{2+}$ solution. Data were plotted according to Scatchard (1949).

Other Materials. Blue Dextran was a product of Pharmacia, Inc. Chelex-100 (200–400 mesh) was obtained from Bio-Rad Laboratories, cyanogen bromide from Pierce Chemical Co., and manganese-54 from New England Nuclear. DEAE-Sephadex A-50-120, Sepharose 4B, and Sephadex G-25 were purchased from Sigma Chemical Co. All other compounds were of the highest quality commercially available.

RESULTS

Modified Procedure for Preparing Homogeneous Threonine Dehydrogenase. All operations were carried out at 4 °C unless otherwise stated, and all buffers contained 1 mM 2-mercaptoethanol plus 0.02% sodium azide.

For each preparation, 100 g (wet weight) of *E. coli* SBD76 cell paste was used. The first three fractionation steps (homogenization, controlled heat denaturation, and DEAE-Sephadex chromatography) were carried out as previously described (Boylan & Dekker, 1981). The last and most effective step in purifying TDH is its absorption to and elution from a Blue Dextran–Sepharose column. However, as initially developed and applied, this step was somewhat inconsistent in yield and reproducibility. A recent report indicated that the binding of several enzymes to a variety of immobilized triazine dyes, including Cibacron Blue F3GA (the triazine dye in Blue Dextran), could be improved by adding divalent metal ions to the loading and eluting buffers (Hughes et al., 1982). As indicated earlier, we had found that *E. coli* TDH activity was stimulated 2–3-fold by added Mn^{2+}; the effect, therefore, of metal ions on the final Blue Dextran–Sepharose 4B affinity chromatography step was tested. Metal ions were added to the buffers only during this step. In first studies, 2 mM Mn^{2+} (the activating metal ion) and 2 mM Zn^{2+} (a nonactivating metal ion) were separately examined. Both metal ions greatly enhanced the binding of TDH to the affinity resin, but pure TDH was specifically eluted by NAD^+ only from the Mn^{2+}-treated column. 2,2′-Dipyridyl had to be added in order to elute TDH activity from the Zn^{2+}-equilibrated column, and enzyme preparations so obtained contained more than 20 contaminating proteins as determined by SDS–polyacrylamide gel electrophoresis.

The apparent improved binding of TDH to the affinity column in the presence of Mn^{2+} was tested by running three separate Blue Dextran–Sepharose 4B columns in parallel; no Mn^{2+} was added to column A whereas 10 μM and 1 mM Mn^{2+} were added to all eluting buffers used on columns B and C, respectively. As can be seen in Figure 1, the binding of TDH activity improved dramatically with increasing concentrations of Mn^{2+}; 1 mM Mn^{2+} allowed for greater than 99% binding of applied dehydrogenase activity. After columns B

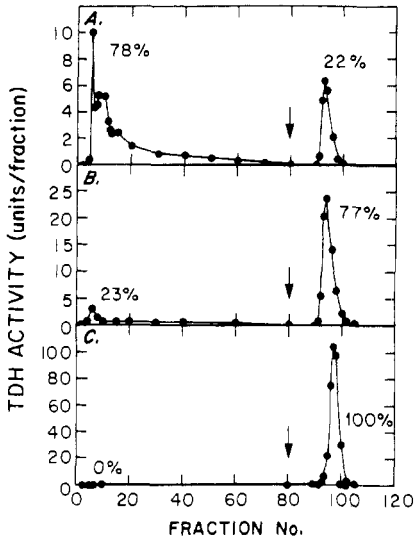

FIGURE 1: Effect of Mn^{2+} on Blue Dextran–Sepharose affinity chromatography of threonine dehydrogenase from *E. coli* K-12. TDH activity, eluted from a DEAE-Sephadex column, was applied to and eluted from three individual Blue Dextran–Sepharose columns (10-mL bed volume) with 10 mM Tris-HCl buffer (pH 7.5) containing 0 (A), 10 μM (B), and 1 mM (C) Mn^{2+} in all buffers and enzyme samples. After the columns were washed with 500 mL of the appropriate buffer, a 100-mL pulse of 5 mM NAD^+ in buffer was applied (as indicated by the arrow) to elute the TDH. Percent recovery shown in each frame is based on a total of 3.7 mg of TDH eluted from column C.

and C were washed to removed contaminating proteins, TDH activity was specifically eluted by NAD^+ in the presence of Mn^{2+}.

For routine purification purposes, therefore, 1 mM Mn^{2+} was added to the enzyme preparation as obtained after DEAE-Sephadex column chromatography (i.e., step 3), and the solution was applied to a Blue Dextran–Sepharose column (5 × 2.5 cm) that had been equilibrated with 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM Mn^{2+}. The column was then washed with 500 mL of the 1 mM Mn^{2+}–buffer solution, which was followed by a 100-mL pulse of 5 mM NAD^+ in the same buffer to elute the enzyme. Fractions (1 mL) were collected in tubes containing 0.14 mL of 0.4 M Tris-HCl buffer (pH 8.4). TDH activity is eluted as a single sharp peak. Mn^{2+} and NAD^+ were removed by exhaustive dialysis against 50 mM Tris-HCl buffer (pH 7.4) containing 3.5 g/L Chelex-100 resin. The pH was then returned to 8.4 by dialysis against 50 mM Tris-HCl buffer (pH 8.4) containing 5 mM 2-mercaptoethanol. Enzyme so prepared is stable for about 2 months when stored at 4 °C. A final yield of 30–40 mg of pure TDH/100 g wet weight of cells with specific activity $\simeq$ 20 has consistently been obtained in 17 different enzyme preparations.

Quantitative Determination of the Mn^{2+} Content of "Demetalized" TDH. To ascertain whether all the Mn^{2+} had been removed from the dehydrogenase by the foregoing procedure and whether TDH might have some Mn^{2+}-independent activity, the Mn^{2+} content of the "demetallized" enzyme was measured by radiolabeled studies with $^{54}Mn^{2+}$ and also by atomic absorption spectroscopy. For this purpose, TDH (5 mg) was incubated with 100 nmol of $^{54}Mn^{2+}$ (5300 cpm/nmol) for 3 h at 4 °C and subsequently applied to and eluted from a column of Sephadex G-25. A single peak of TDH activity was eluted containing 3.5 mg of protein and 0.1 mol of Mn^{2+}/mol of TDH. This sample of enzyme was then dialyzed for 60 h against several changes of 50 mM Tris-HCl buffer (pH 8.4) containing 3.5 g/L Chelex-100. Radioactivity measurements indicated the Mn^{2+} content of the dehydrogenase had now been reduced to 0.003 mol of Mn^{2+}/mol of enzyme. This same enzyme sample was also analyzed by atomic absorption spectroscopy (a Perkin-Elmer Model 5000 atomic absorption instrument equipped with a HGA 500 graphite furnace was used); the Mn^{2+} content was found to be 0.02 mol of Mn^{2+}/mol. Such "fully demetalized" enzyme retained a level of activity = 8 units/mg. Studies with numerous different enzyme samples have established that freshly prepared, "metal ion free" TDH has a basal level of activity $\simeq$ 15–20 units/mg.

Effect of Different Metal Ions on TDH Activity. For this study, pure TDH was dialyzed extensively against buffer containing 10 mM EDTA followed by exhaustive dialysis to remove the EDTA. It should be noted that the thiol-dependent activation of TDH activity by Mn^{2+} (see studies described later) was not fully realized when these experiments were done; hence, the TDH preparation used here contained 1 mM 2-mercaptoethanol while no thiol was added to the assay solutions.

Enzyme activity was measured by Assay II after homogeneous TDH (1 μg) was incubated for 5 min at 37 °C in buffer containing either 64 μM or 3.2 mM of the metal ions indicated (all as their chloride salts). Only Mn^{2+} and Cd^{2+} had an activating effect; under the experimental conditions used here, these two metal ions stimulated TDH activity about 4-fold and 6-fold, respectively. Activity was completely abolished by 64 μM Hg^{2+} or Ag^{2+}, and 20–50% inhibition was observed in the presence of 3.2 mM Cu^{2+}, Ni^{2+}, or Be^{2+}. In contrast, the following metal ions had no effect on TDH activity: Li^+, Na^+, K^+, Rb^+, Cs^+, Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Co^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+}, Al^{3+} [as $K_2Al_2(SO_4)\cdot 24H_2O$], Sn^{2+}, V^{4+} (as $VOSO_4$), and Mo^{6+} [as $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$].

Thiol Requirement and Optimal Mn^{2+} Concentration for TDH Activation. In the early stages of our studies with pure TDH, we observed that stimulation of activity by added Mn^{2+} decreased when enzyme preparations were stored at 4 °C for 2–3 weeks. Although the pure enzyme was routinely stored in buffers containing 1 mM 2-mercaptoethanol, the loss of TDH activation by Mn^{2+} due to oxidation of exogenous thiol seemed likely. To examine this possibility, 2-mercaptoethanol was removed from a sample of TDH by exhaustive dialysis. The enzyme was then incubated with 100 μM Mn^{2+} and increasing concentrations of 2-mercaptoethanol. The data obtained are shown in Figure 2; similar results were seen with all reduced thiols tested. Optimal activation of TDH activity by Mn^{2+} was seen in the presence of 0.5 mM dithiothreitol or L-cysteine and with 5 mM concentrations of either 2-mercaptoethanol, α-thioglycerol, reduced glutathione, or mercaptoacetic acid. Activation of TDH activity by Mn^{2+}, therefore, is dependent on the presence of a reduced thiol in all enzyme stock solutions and assay buffers.

With such results in hand, the time dependence of TDH activation by Mn^{2+} was readily established. For this purpose, TDH was incubated with a given concentration (e.g., 50 or 100 μM) of Mn^{2+} plus 2-mercaptoethanol for 0–60 min and subsequently assayed for enzyme activity. TDH activity consistently increased with time of incubation to a maximum value after 20–30 min; Mn^{2+}-saturated enzyme remained fully active up to 6 h.

The effect of increasing Mn^{2+} concentrations on the activity of "demetalized" enzyme was also determined. The "demetalized" TDH used in these studies (prepared as described earlier) had a basal level of activity = 16 units/mg. It was incubated with increasing concentrations of Mn^{2+}, and dehydrogenase activity was subsequently measured by Assay IIm.

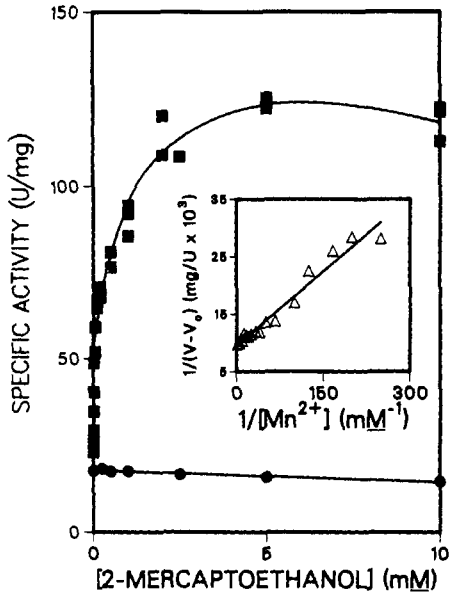

FIGURE 2: Thiol-dependent stimulation of *E. coli* threonine dehydrogenase activity by Mn^{2+}. A sample of "demetalized" TDH (0.8 μg) was subjected to a prior incubation at 37 °C for 30 min in 200 μmol of Tris-HCl buffer (pH 8.4) containing no Mn^{2+} (●) or 100 mM Mn^{2+} (■) and varying concentrations of 2-mercaptoethanol. The reaction was initiated by adding 125 μmol of L-threonine plus 5 μmol of NAD^+. (Inset) Mn^{2+}-dependent stimulation of threonine dehydrogenase activity. A sample of "demetalized" TDH (0.8 μg) was subjected to a prior incubation at 37 °C for 30 min with 0.4 M Tris-HCl buffer (pH 8.4), 5 mM 2-mercaptoethanol, and varying Mn^{2+} concentrations (0.5-mL total volume). The reaction was initiated by addition of 125 μmol of L-threonine plus 5 μmol of NAD^+ (Assay IIm).

The data, presented as a double-reciprocal plot in Figure 2 (inset), show that activation of TDH by Mn^{2+} is saturable with an activation K_d = 9.0 μM and a $V_{Mn^{2+}}$ = 105 units/mg. [The Mn^{2+}-dependent velocity ($V_{Mn^{2+}}$) was obtained by subtracting the Mn^{2+}-independent velocity (V_0 = 16 units/mg) from the overall reaction rate at each Mn^{2+} concentration.]

Effect of pH on Activity of "Demetalized" and Mn^{2+}-Saturated TDH. The pH of the reaction mixture had a different effect on the activity of the "demetalized" and the Mn^{2+}-saturated enzyme. To obtain a pH–activity profile for the "demetalized" enzyme, and so as to best be able to compare these data with previously published results (Boylan & Dekker, 1981), a sample of TDH was dialyzed extensively against thiol-free buffers containing Chelex-100 resin, and the dehydrogenase-catalyzed reaction was initiated by adding the enzyme to a cuvette containing all assay components already warmed to 37 °C. We found that prior incubation of TDH above pH 8.8 resulted in a severe loss of activity. Over the pH range of 6.0–11.5, activity increased steadily from pH 6.0 to an optimum at pH 10.8, followed by a precipitous decline thereafter. These data are identical with those reported in our first paper (Boylan & Dekker, 1981) except that the pH optimum found here (i.e., 10.8) is 0.5 pH unit higher.

Unlike the "demetalized" enzyme, Mn^{2+}-saturated (thiol-dependent) TDH has a pH–activity profile that fits a theoretical curve for the Michaelis pH–function for one-proton ionization, i.e.

$$V = V_{max}/(1 + [H^+]/K_a) \quad (2)$$

where V_{max} is the maximum obtainable catalytic rate, $[H^+]$ is the hydrogen ion concentration, and K_a is the ionization constant for a group on the enzyme. The one-proton ionization curve we obtained has a pK_a = 7.95, a V = 229 units/mg at pH >12, and a V = 0 unit/mg at pH <6. Such data suggest that the observed increase in TDH activity caused by Mn^{2+} may be due to ionization of a single residue on the enzyme.

Kinetic Parameters for "Demetalized" and Mn^{2+}-Saturated TDH. For studies with the "demetalized" enzyme, Mn^{2+} and 2-mercaptoethanol were removed by exhaustive dialysis against buffer containing Chelex resin; TDH activity was followed by Assay II at 37 °C. Secondary plots of the data (slopes and intercepts vs. $1/[NAD^+]$) were used to obtain actual values. K_m values determined for "demetalized" TDH (i.e., 0.11 mM for NAD^+ and 1.1 mM for L-threonine) were quite similar to those we published earlier (i.e., 0.19 mM for NAD^+ and 1.43 mM for L-threonine) for TDH having an unknown Mn^{2+} content (Boylan & Dekker, 1981). On the other hand, a V_{max} = 28.3 units/mg was obtained in these studies, which is only half the value published previously (i.e., 57 units/mg); this difference is most likely due to residual amounts of Mn^{2+} bound to the enzyme in our first studies.

For kinetic studies with metal-saturated enzyme (250 μM Mn^{2+}), TDH activity was measured by Assay IIm. As expected, secondary plots of the data showed a 24-fold increase in V_{max} (i.e., 685 units/mg) for Mn^{2+}-saturated TDH. K_m values of 0.60 and 221 mM were found for NAD^+ and L-threonine, respectively. The higher K_m (5.5-fold) for NAD^+ is significant but still within a reasonable physiological range, whereas the K_m for L-threonine is unusually large. To examine what effect varying concentrations of 2-mercaptoethanol might have on the "demetalized" enzyme, a series of assays were performed with a saturating concentration of NAD^+ (5 mM), 2–10 mM L-threonine, and 2, 4, 6, and 8 mM 2-mercaptoethanol. The reaction was initiated by adding TDH. A Lineweaver–Burke plot of $1/V$ vs. $1/$[L-threonine] gave straight lines that intersected to the left of the y axis, indicating mixed noncompetitive inhibition by 2-mercaptoethanol. A linear secondary plot of apparent K_m vs. 2-mercaptoethanol concentration was obtained from which an apparent K_m of 2.5 mM L-threonine was predicted for assays done in the presence of 5 mM 2-mercaptoethanol. This is a 2.3-fold increase over the actual K_m determined for L-threonine in the absence of 2-mercaptoethanol. If it is assumed that added 2-mercaptoethanol has a similar effect on the K_m value for L-threonine in assays using Mn^{2+}-saturated TDH, the K_m under these conditions may actually be less than 100 mM.

Evidence for Interaction of Mn^{2+} with TDH. Mn^{2+} and Mg^{2+} are known to form complexes with substrates in nucleotide-dependent enzyme-catalyzed reactions. To determine whether Mn^{2+} interacts with TDH or forms a complex with one of the substrates or products of this reaction, experiments were performed with radioactive ^{54}Mn. Samples of "demetalized" TDH were subjected to a prior incubation for 3 h at 4 °C with $^{54}Mn^{2+}$. The mixtures were then applied to and eluted from columns (50 × 1 cm) of Sephadex G-25 to separate the Mn^{2+}–TDH complex from the free Mn^{2+}.[2] Protein, dehydrogenase activity, and radioactivity all coeluted from the column as one symmetrical peak (Figure 3); Mn^{2+} and TDH were found to interact with a stoichiometry of

[2] This column chromatographic procedure should not be confused with the Hummel–Dreyer method described under Materials and Methods. These initial studies to demonstrate a possible interaction of Mn^{2+} with TDH were performed by applying samples of enzyme that had been mixed with large excesses of Mn^{2+} to columns of Sephadex G-25 that had been equilibrated only with 50 mM Tris-HCl buffer (pH 8.4); the sole intent here was to separate free Mn^{2+} from Mn^{2+} that was bound to the enzyme (see Figure 3, Table I). Unlike the Hummel–Dreyer binding method, saturating levels of Mn^{2+} were not maintained throughout this procedure; hence, the true stoichiometry of Mn^{2+} binding to TDH under saturating conditions was never observed.

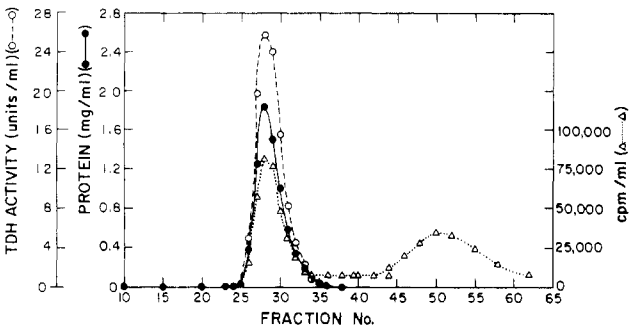

FIGURE 3: Chromatography of *E. coli* threonine dehydrogenase on Sephadex G-25 with radioactive Mn^{2+}; coelution of protein, enzymatic activity, and radioactivity. "Demetalized" TDH (1.7 mg) was incubated for 3 h in 50 mM Tris-HCl buffer (pH 8.4) containing 5 mM 2-mercaptoethanol and 1 μmol of $^{54}Mn^{2+}$. The sample was then applied to and eluted from a column (50 × 1 cm) of Sephadex G-25 at 10 mL/h. Protein (●) was determined by A_{280}, dehydrogenase activity (○) by Assay II, and radioactivity (△) on a Tracor Analytic Gamma-Trac 1191 set at 735–935 keV.

Table I: Binding of Mn^{2+} to TDH As Determined by Column Chromatography on Sephadex G-25[a]

sample	TDH (mg)	Mn^{2+} added (nmol)	sp act. of Mn^{2+} (cpm/nmol)	additions	Mn^{2+} bound (mol of Mn^{2+}/mol of TDH)
1	0	500	3400	buffer only	0
2	1.94	100	18000	none	1.0
3	1.94	500	3600	none	1.7
4	1.94	1000	1800	none	1.4
5	1.94	500	3530	10 μmol of NAD^+	1.5
6	1.94	500	3560	10 μmol of NADH	1.4
7	1.94	500	3600	75 μmol of L-threonine	1.2

[a] Samples of "demetalized" TDH (1.94 mg) were subjected to a prior incubation at 4 °C for 3 h in 50 mM Tris-HCl buffer (pH 8.4) containing 5 mM 2-mercaptoethanol, varying concentrations of radioactive $^{54}Mn^{2+}$, and the additions indicated. The samples were then applied to and eluted from columns (50 × 1 cm) of Sephadex G-25 with buffers containing only 50 mM Tris-HCl buffer (pH 8.4) and 5 mM 2-mercaptoethanol.

1.0–1.7 mol of Mn^{2+}/mol of TDH (Table I). To test the effect that L-threonine, NAD^+, or NADH might have on the interaction of Mn^{2+} with TDH, three samples of the enzyme were subjected to a prior incubation in the same manner with radiolabeled Mn^{2+} and either 10 μmol of NAD^+, 10 μmol of NADH, or 75 μmol of L-threonine. The elution of NAD^+ was detected by its absorbance at 280 nm, of NADH by the ratio of its absorbance at 340 and 260 nm, of protein by A_{280}, and of L-threonine by reaction with ninhydrin (Rosen, 1957). [To distinguish NAD^+ from protein, the ultraviolet spectrum (320–240 nm) was taken for samples containing material that absorbed at 280 nm; the spectra showed that NAD^+ and protein were well separated.] In all cases, only Mn^{2+} coeluted with TDH; all other ligands eluted from the column with excess Mn^{2+} in the salt front. There was no evidence for the formation of a tight ternary complex between TDH–Mn^{2+} and any of these three ligands, and the stoichiometry of the TDH–Mn^{2+} interaction was not significantly affected in any instance (Table I). It seems quite clear, therefore, that Mn^{2+} interacts directly with TDH, although the possibility of enzyme-bound Mn^{2+} interacting with the substrate(s) or product(s) at the enzyme surface during catalysis has not been eliminated.

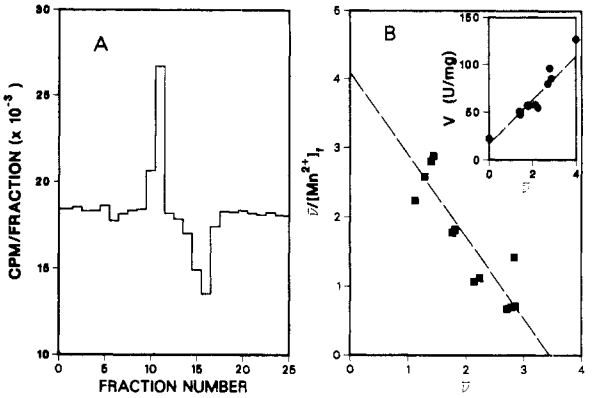

FIGURE 4: Quantitative determination of Mn^{2+} binding to *E. coli* threonine dehydrogenase by gel filtration. Samples of "demetalized" TDH (0.32–0.77 mg in 1.0-mL total volume) containing the same Mn^{2+} concentration as the equilibrating buffer were applied to and eluted from a column (33 × 0.6 cm) of Sephadex G-25 at 0.75 mL/h. One-milliliter fractions were collected and assayed for protein, enzymatic activity, and radioactivity. The Mn^{2+} concentration ranged from 5 to 40 μM. See Materials and Methods for details. (A) A typical TDH elution profile in the presence of 40 μM Mn^{2+}. (B) Bound Mn^{2+} was calculated by eq 1, and the data were plotted according to Scatchard. (Inset) Data points include all samples eluted; the specific activity of "demetalized" enzyme is plotted as $\bar{\nu} = 0$ and that of Mn^{2+}-saturated enzyme as $\bar{\nu} = 4.0$ mol of Mn^{2+}/mol of TDH.

Quantitative Determination of Mn^{2+} Binding to TDH. The binding of Mn^{2+} to TDH was quantified by the method of Hummel and Dreyer (1962). For these studies, 12.6 mg of metal ion free TDH was prepared as described earlier. TDH samples were applied to and eluted from the $^{54}Mn^{2+}$-saturated column as described under Materials and Methods. A typical elution profile can be seen in Figure 4A. Results from a series of experiments using columns saturated with 5–40 μM Mn^{2+} were plotted according to Scatchard (Figure 4B). The stoichiometry of binding was found to be 3.45 mol of Mn^{2+}/mol of TDH (i.e., 0.86 mol of Mn^{2+}/subunit) with a dissociation constant (K_d) equal to 8.5 μM; this value correlates well with the activation K_d of 9.0 μM determined previously. It was also found that the specific activity of TDH increased linearly with increasing Mn^{2+} content (Figure 4B, inset).

Discussion

Threonine dehydrogenase from both chicken (Aoyama & Motokawa, 1981) and goat (Ray & Ray, 1985) liver is a single polypeptide of $M_r \simeq 88\,000$ whereas the enzyme from *E. coli* is a tetramer (M_r 140 000) of apparently identical subunits. No information has been presented regarding the effect of various cations on the activity of TDH from goat liver, but the chicken liver enzyme has been found to be inhibited ~50% by 1.0 mM Mn^{2+}. *E. coli* TDH activity, on the other hand, is stimulated by added Mn^{2+}.

All data obtained indicate that TDH from *E. coli* is a metal ion activated enzyme (rather than a metalloenzyme). For example, it is necessary to add Mn^{2+} to the isolated enzyme for maximal activity. *E. coli* TDH also has a basal level of activity ($V_0 \simeq 20$ units/mg) that is not Mn^{2+} dependent. A $K_d = 8.5$ μM for Mn^{2+} was obtained by gel filtration studies, which value is typical of metal ion activated enzymes (Malmström & Rosenberg, 1959). Furthermore, the stimulation of *E. coli* TDH activity by Mn^{2+} is saturable; half-maximal stimulation is seen at 9.0 μM Mn^{2+}, implying that stimulation of activity is the direct result of Mn^{2+} binding.

Williams recently proposed that Mn^{2+} may be involved in both catalysis and regulation of some Mn^{2+}-activated enzymes (Williams, 1982). After observing the activation of phos-

phoenolpyruvate carboxykinase by Mn^{2+}, Schramm proposed that the following set of criteria should be considered in ascertaining whether this metal ion might have a role in the regulation of enzymatic activity (Schramm, 1982): (1) The kinetic constant(s) for the interaction of Mn^{2+} must approximate the intracellular free Mn^{2+} concentration at physiological substrate concentrations. (2) The binding site(s) for Mn^{2+} must discriminate strongly against Mg^{2+}. (3) The amount of intracellular, exchangeable Mn^{2+} must approximate or exceed the molarity of target enzymes required for observed metabolic flux. (4) The intracellular free Mn^{2+} must change in response to altered physiological or hormonal states.

Only one group has resported on the intracellular concentration of Mn^{2+} in *E. coli*; Silver et al. found that such cells have a specific active-transport system for Mn^{2+} (Silver & Kralovic, 1969; Silver et al., 1970) and can develop an intracellular concentration of 15 μM Mn^{2+} of which 85% is exchangeable with the medium (Silver & Jasper, 1977). The binding and activation constants reported here for TDH and Mn^{2+} are in this concentration range. Second, we have found that Mg^{2+} itself has no effect on TDH activity and that TDH activation by 100 μM Mn^{2+} is not inhibited by a 500-fold excess of Mg^{2+}, indicating that TDH selectively interacts with Mn^{2+} in the presence of excess Mg^{2+}. Third, on the basis of the level of TDH activity found in crude extracts, *E. coli* SBD76 cells contain approximately 100 mg of TDH/100 g (wet weight) of cells. Using a M_r of 140000 (Boylan & Dekker, 1981) and the estimate that the water content of *E. coli* cells is 70% (Ingraham et al., 1983), we calculate the intracellular concentration of TDH in our mutant cells to be approximately 40 μM. Initial studies in our laboratory with extracts of wild-type *E. coli* K-12 indicate that they contain about 2 μM TDH, which concentration is well below the reported level of Mn^{2+} in the cell. Hence, the interaction between Mn^{2+} and *E. coli* TDH conforms to Schramm's first three criteria. No data are yet available for *E. coli* cells relative to the fourth criterion.

Activation of *E. coli* TDH by Mn^{2+} is thiol-dependent; maximal levels of activity for many enzymes require the addition of both a metal ion and a reduced thiol (Dixon & Webb, 1979). However, in most cases the relationship between the metal ion activation effect and the role of added thiols in catalysis is not well understood. The thiol dependence of TDH activation by Mn^{2+} could relate to the oxidation state of the sulfhydryl groups of the enzyme. Such seems to be true for phosphoenolpyruvate carboxykinase from rat liver cytosol where Brinkworth et al. (1981) observed a Mn^{2+}-sensitive and a Mn^{2+}-insensitive form of the enzyme. Incubation of the homogeneous carboxykinase with either oxidized glutathione or Mn^{2+} alone resulted in a rapid loss of its sensitivity to stimulation by Mn^{2+} when stored. Even though some carboxykinase activity was retained in this state, sensitivity to activation was recovered by incubating the enzyme with Mn^{2+} in the presence of dithiothreitol. On the other hand, the thiol dependence of TDH activation by Mn^{2+} may also relate to the oxidation state of manganese. On the basis of EPR studies of the Mn^{2+} content of *Staphylococcus aureus* cells, Ezra and co-workers concluded that manganese exists as Mn^{2+} under anaerobic conditions and as Mn^{3+} when oxygen is present. They also found that the addition of 2-mercaptoethanol to an oxygenated suspension of *S. aureus* cells resulted in the reduction of Mn^{3+} to Mn^{2+} (Ezra et al., 1984). Similar results have been reported by Archibald and Fridovich (1982) in studies with lactic acid bacteria.

It seems quite unlikely that Mn^{2+} would oxidize any sulfhydryl group of *E. coli* TDH since that would convert Mn^{2+} to Mn^0. If, however, the oxidative behavior of Mn^{2+} seen in *S. aureus* also takes place in *E. coli* cell suspensions and/or in TDH assay mixtures, Mn^{3+} could very well be present in the absence of 2-mercaptoethanol, and a reducing thiol may be necessary to keep the manganese in the divalent state. Otherwise, Mn^{3+} could react with free sulfhydryl groups of TDH, forming Mn^{2+} and oxidized, inactive enzyme.

At present, only a simple model of the Mn^{2+} interaction with *E. coli* TDH can be postulated. The improved binding of TDH to Blue Dextran–Sepharose and its increased activity in the presence of added metal ion suggest that a conformational change to a more active enzyme occurs in the presence of Mn^{2+}. Possibly, added Mn^{2+} may perturb an equilibrium between an inactive (or less active) and an active form of TDH favoring the active conformation. The low level of Mn^{2+}-independent activity represents that small percentage of enzyme that resides in the active conformation in the absence of added metal ion. Such a view allows for the possibility of TDH activity being regulated in vivo by fluctuations in the Mn^{2+} concentration. More specifically, the pH and thiol dependence of TDH activation by Mn^{2+} raises the question of whether a sulfhydryl group (or groups) on the enzyme might be involved either in binding directly to Mn^{2+} or in the formation of a Mn^{2+}-binding domain on the enzyme; this was the focus of subsequent investigations, which will be reported separately.

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Registry No. NAD, 53-84-9; TDH, 9067-99-6; $HO(CH_2)_2SH$, 60-24-2; Mn(2+), 16397-91-4; L-threonine, 72-19-5.

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Selective Carboxymethylation of Cysteine-174 of the $\beta_2\beta_2$ and $\beta_1\beta_1$ Human Liver Alcohol Dehydrogenase Isoenzymes by Iodoacetate†

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ABSTRACT: The $\beta_1\beta_1$ and $\beta_2\beta_2$ human liver alcohol dehydrogenase isoenzymes differ by only one residue at the coenzyme-binding site; Arg-47 in β_1 is replaced by His in the β_2 subunit. Since Arg-47 is thought to facilitate the carboxymethylation of Cys-46 in horse liver alcohol dehydrogenase by binding halo acids in a Michaelis–Menten complex prior to inactivation, the specificity and kinetics of modification of the two human liver $\beta\beta$ isoenzymes with iodoacetate were compared. Both of the $\beta\beta$ isoenzymes were inactivated by treatment with iodo[^{14}C]acetate, and one Cys per subunit was carboxymethylated. Cys-174, which is a ligand to the active-site zinc atom in horse liver alcohol dehydrogenase, was selectively carboxymethylated in each of the human $\beta\beta$ isoenzymes; less than 15% of the iodo[^{14}C]acetate incorporated into the enzyme appeared in Cys-46. Therefore, the three-dimensional structure of the basic amino acids in the anion-binding site of the human $\beta\beta$ isoenzymes appears to be different from that of horse liver alcohol dehydrogenase. The kinetics of alkylation are consistent with the formation of a Michaelis–Menten complex before inactivation of the isoenzymes. The average K_i values for iodoacetate were 10 and 16 mM for $\beta_1\beta_1$ and $\beta_2\beta_2$, respectively, and maximal rate constants for inactivation were 0.22 and 0.17 min^{-1}, respectively. From these data, it can be concluded that there is a relatively minor effect of the substitution of His for Arg at position 47 on the kinetics of inactivation.

A variety of active-site-directed chemical modifying reagents have been used to investigate the structure and mechanism of horse liver alcohol dehydrogenase. The halo acids iodoacetate and 3-bromopropionate have been shown to selectively alkylate the Cys-46 and Cys-174 residues, respectively, both of which are active-site zinc ligands (Li & Vallee, 1964; Harris, 1964; Chadha & Plapp, 1984). Enzyme alkylated with iodoacetate retains less than 5% of the original catalytic activity (Reynolds & McKinley-McKee, 1970). The kinetics of inactivation by these reagents indicate that a high-affinity, reversible enzyme–inhibitor Michaelis–Menten complex is formed prior to covalent modification (Reynolds & McKinley–McKee, 1969; Chadha & Plapp, 1984). NAD^+ and NADH competitively inhibit inactivation by iodoacetate. On the basis of X-ray crystallographic studies of the Cys-46 carboxymethylated enzyme (Zeppezauer et al., 1975) and chemical modification studies of Arg residues (Lange et al., 1975), it has been suggested that iodoacetate binds to Arg-47 prior to alkylation of Cys-46 to form the reversible Michaelis–Menten complex. The alkylation reaction of Cys-46 in horse liver alcohol dehydrogenase by iodoacetate has been used to investigate the specificity and affinity of anions, such as NAD(H) analogues, $Pt(CN)^{4-}$, phosphate, and halides, for the anion-binding site (Reynolds & McKinley-McKee, 1969; Reynolds et al., 1970; Zeppezauer et al., 1975; Dahl & McKinley-McKee, 1980).

Human liver alcohol dehydrogenase exists in multiple molecular forms, and a genetic model accounts for this multiplicity (Smith et al., 1971; Bosron & Li, 1981; Vallee & Bazzone, 1983). The model assumes that there are five separate gene loci with polymorphism at two loci. The two alloenzymes that are produced at ADH_2, $\beta_1\beta_1$ and $\beta_2\beta_2$, exhibit different pH optima for ethanol oxidation (10.5 and 8.5, respectively), V_{max} values for ethanol oxidation at pH 7.5 (9.2 and 400 min^{-1}), and K_m values for NAD^+ (7.4 and 180 μM). Since the β_1 subunit predominates in Caucasians while the β_2 subunit occurs most frequently in Orientals (Yin et al., 1984a), it was suggested that such polymorphism of alcohol dehydrogenase isoenzymes may contribute to differences in the alcohol elimination rate between these racial groups (Stamatoyannopoulos et al., 1975).

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