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Cd^{2+} activation of L-threonine dehydrogenase from *Escherichia coli* K-12

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Key words: Threonine metabolism; L-Threonine dehydrogenase; Cadmium ion activation; Cadmium; (*E. coli* K-12)

Homogeneous preparations of L-threonine dehydrogenase (L-threonine:NAD^+ oxidoreductase, EC 1.1.1.103) from *Escherichia coli* K-12, after having been dialyzed against buffers containing Chelex-100 resin, have a basal level of activity of 10–20 units/mg. Added Cd^{2+} stimulates dehydrogenase activity approx. 10-fold; this activation is concentration-dependent and is saturable with an activation $K_d = 0.9\ \mu M$. Full activation by Cd^{2+} is obtained in the absence of added thiols. The pH-activity profile of the Cd^{2+}-activated enzyme conforms to a theoretical curve for one-proton ionization with a $pK_a = 7.85$. Mn^{2+}, the only other activating metal ion, competes with Cd^{2+} for the same binding site. K_m values for L-threonine and NAD^+ as well as the V_{max} for 'demetallized', Cd^{2+}-activated, and Mn^{2+}-activated threonine dehydrogenase were determined and compared.

Introduction

Threonine dehydrogenase (L-threonine:NAD^+ oxidoreductase, EC 1.1.1.103) initiates the primary route for threonine utilization in both eukaryotes [1] and prokaryotes [2,3]. The reaction catalyzed is

L-Threonine + NAD^+ → (2-amino-3-ketobutyrate)

$$+ NADH + H^+$$

where the apparent product, i.e., 2-amino-3-ketobutyrate, either rapidly decarboxylates with liberation of aminoacetone or is cleaved by a CoA-dependent ligase with formation of acetyl CoA plus glycine.

So far, threonine dehydrogenase has been isolated in pure form from extracts of chicken liver [4], goat liver [5], and *Escherichia coli* [6]. Initial studies indicate that this dehydrogenase from eukaryotic sources is markedly different from the *E. coli* enzyme. Goat and chicken liver threonine dehydrogenase are both single polypeptide chains ($M_r \cong 80\,000$), whereas the enzyme from *E. coli* K-12 is a tetramer ($M_r = 148\,800$) of apparently identical subunits. The chicken liver dehydrogenase is not sensitive to thiol-specific reagents but is inhibited approx. 50% by 1.0 mM Mn^{2+}. In contrast, threonine dehydrogenase from *E. coli* is readily inhibited by various thiol-reacting reagents, and the low basal level of activity that it manifests in the absence of metal ions is stimulated approx. 10-fold by 1.0 mM Mn^{2+} under standard assay conditions.

Among numerous other metal ions tested, we found that Cd^{2+} also stimulates *E. coli* threonine

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Abbreviations: SDS, sodium dodecyl sulfate; Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

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dehydrogenase activity [7]. We report here the kinetics, pH dependence and other properties of the Cd^{2+}-activated enzyme. Activation by Cd^{2+}, in contrast to that by Mn^{2+}, is not thiol-dependent; hence the general role of thiols in catalysis as well as their possible specific interaction with the metal ion can be examined with Cd^{2+}-stimulated threonine dehydrogenase under conditions where activation by Mn^{2+} is not possible.

Materials and Methods

Chemicals

NAD^+ was purchased from U.S. Biochemicals Corp. (Cleveland, OH). DEAE-Sephadex (A50–120) and Sepharose 4B were obtained from Sigma Chemical Co. (St. Louis, MO), Chelex-100 (200–400 mesh) from Bio-Rad Laboratories (Richmond, CA), and Eriochrome Black T and methyl red from Aldrich Chemical Co. (Milwaukee, WI). $^{54}MnCl_2$ in 0.5 M HCl (> 40 Ci/g) and a 0.1 μCi $^{54}Mn^{2+}$ reference source were products of New England Nuclear (Boston, MA). $MnCl_2 \cdot 4H_2O$, crystal, was Baker-analyzed reagent grade from J.T. Baker Chemical Co. (Phillipsburg, NJ), and manganese(III) acetate from Alfa Products (Danvers, MA). All other chemicals were of the highest quality commercially available.

Enzymes and enzyme assays

Homogeneous preparations of threonine dehydrogenase were obtained from extracts of a mutant strain of *E. coli* K-12 by procedures described previously [7]. 'Demetallized' enzyme was prepared by dialyzing the sample for 48 h against several changes of 50 mM Tris-HCl buffer (pH 7.4) containing 3.5 g/l of Chelex-100 resin followed by dialysis against 50 mM Tris-HCl buffer (pH 8.4). The purity of enzyme preparations was routinely confirmed by SDS-polyacrylamide gel electrophoresis [8].

Threonine dehydrogenase activity was measured by following the formation of NADH at 340 nm [7]. Specific activity of the enzyme was determined by first incubating 0.5–1.0 μg of the dehydrogenase for 30 min at 37 °C in 200 μmol of Tris-HCl buffer (pH 8.4) containing designated concentrations of metal ions and/or a thiol and then initiating the reaction by adding 125 μmol of L-threonine plus 5 μmol of NAD^+. One unit of enzyme activity is defined as that amount which catalyzed the formation of 1.0 μmol of NADH per min. A molar absorption coefficient of $6.22 \cdot 10^3$ was used for NADH [9]. The protein concentration of threonine dehydrogenase samples was determined from the absorbance at 280 nm using the previously reported absorption coefficient of 1.106 for a 1 mg/ml solution [7]. Spectral measurements were made with a Cary 219 recording spectrophotometer equipped with thermostatted cuvette holders and a timed cell programmer.

Removal of contaminating metal ions

All plasticware, glassware, dialysis tubing, and reagent solutions were carefully treated as described before [7].

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

This was done by the methods of Flaschka and Schwarzenbach [10,11]. Stock solutions of either metal ion were titrated directly, using EDTA (disodium salt, previously dried for 2 h at 80 °C) as a primary standard. For Mn^{2+}, 3 drops of 5% (w/v) potassium sodium tartrate followed by 2–3 ml of NH_3/NH_4^+ buffer (pH 10.0) were added to a solution containing approx. 0.5 mmol of Mn^{2+}. This solution was then heated to 70 °C after which 20–30 mg of ascorbic acid, 4 drops of methyl red solution (20 mg in 100 ml of 60%, v/v, ethanol), and 2 drops of Eriochrome Black T solution (200 mg in 15 ml of triethanolamine plus 5 ml of abs. ethanol) were added sequentially and the mixture was titrated to a red/green endpoint with 0.01 M EDTA. The same procedure was followed to standardize Cd^{2+} solutions, except that ascorbic acid and potassium sodium tartrate were not added.

Binding of Cd^{2+} and Mn^{2+} by threonine dehydrogenase

Co-elution of the enzyme and $^{54}Mn^{2+}$ from a gel-filtration column was used to examine possible competitive binding of Mn^{2+} and Cd^{2+}. Samples of 'demetallized' dehydrogenase (1–2 mg) were incubated for 3 h at 4 °C with 200 nmol of $^{54}Mn^{2+}$ ($71.6 \cdot 10^3$ dpm/nmol) in 50 mM Tris-HCl buffer (pH 8.4) containing 1 mM 2-mercaptoethanol with and without added Cd^{2+} (30 nmol). The mixture

was then applied to and eluted from a column (50 × 1 cm) of Sephadex G-25 (fine) at a flow rate of 10–12 ml/h. Fractions (1 ml) were assayed for protein, dehydrogenase activity, and radioactivity.

Measurement of radioactivity

The level of radioactivity in aqueous solutions containing $^{54}Mn^{2+}$ was measured directly by 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 $^{54}Mn^{2+}$.

Results

Activation of E. coli threonine dehydrogenase activity by Cd^{2+}

Cd^{2+} activation of dehydrogenase activity was found to be concentration-dependent. For these studies, 'demetallized' dehydrogenase was first dialyzed to remove exogenous thiols. It was then incubated for 30 min with different concentrations of Cd^{2+} and the level of activity subsequently determined. The data are shown in Fig. 1. The metal ion-independent level of activity (V_0) in this instance was 12.5 units/mg. As can be seen, activation by Cd^{2+} is saturable with a metal ion-dependent level of activity of 112 units/mg and an activation $K_d = 0.9$ μM, which value is 10-fold lower than that obtained with Mn^{2+} [7]. Subsequent results showed that greater than 95% of full activation is obtained by incubating the enzyme with Cd^{2+} for only 5 min at 37 °C.

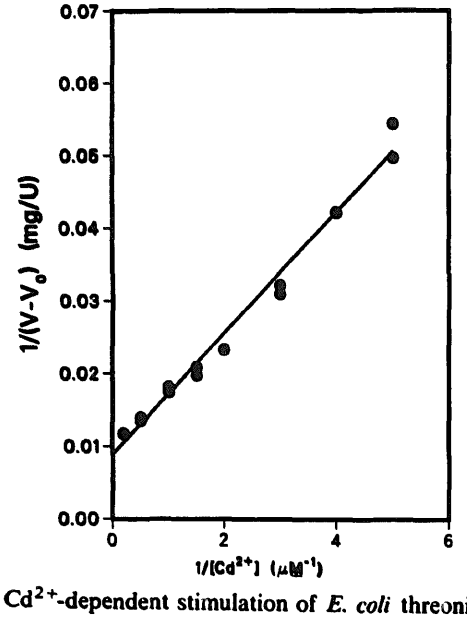

Fig. 1. Cd^{2+}-dependent stimulation of *E. coli* threonine dehydrogenase activity. 'Demetallized', thiol-free enzyme (0.5 μg) was incubated for 30 min at 37 °C in 0.4 M Tris-HCl buffer (pH 8.4) with the concentrations of Cd^{2+} shown before the assay was initiated by adding 125 μmol of L-threonine plus 5 μmol of NAD^+.

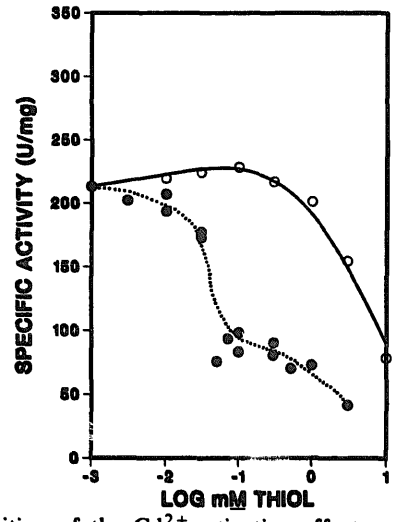

Fig. 2. Inhibition of the Cd^{2+}-activating effect on threonine dehydrogenase by 2-mercaptoethanol or dithiothreitol. Thiol-free, 'demetallized' enzyme (0.5 μg) was first incubated for 30 min at 37 °C in 0.4 M Tris-HCl buffer (pH 8.4) containing 30 μM Cd^{2+} and varying concentrations of either 2-mercaptoethanol (○) or dithiothreitol (●). The assay was then initiated as in Fig. 1.

Effect of thiols on threonine dehydrogenase activation by Cd^{2+}

The effect of various thiols on Cd^{2+} activation was examined. In first studies, samples of thiol-free 'demetallized' enzyme were incubated with 30 μM Cd^{2+} and 0–10 mM 2-mercaptoethanol. In contrast to activation by Mn^{2+} [7], full activation by Cd^{2+} is seen in the absence of any added thiol. High concentrations of 2-mercaptoethanol, in fact, inhibit activation by Cd^{2+}. Since it was thought this result might be due to chelation of Cd^{2+} by 2-mercaptoethanol, the known stronger chelation of Cd^{2+} by a vicinal dithiol was tested. For this purpose, samples of thiol-free, 'demetallized' dehydrogenase were incubated with 30 μM Cd^{2+} plus increasing concentrations of dithiothreitol. Whereas the activating effect of Cd^{2+} decreased slowly with increasing concentrations of 2-

mercaptoethanol (Fig. 2, upper curve), a sharp decline in the activation of threonine dehydrogenase by Cd^{2+} occurred at 50 μM dithiothreitol (Fig. 2, lower curve); this dithiothreitol concentration is just in excess of the Cd^{2+}. Furthermore, throughout the concentration range studied, dithiothreitol was a more effective inhibitor of Cd^{2+} activation than was 2-mercaptoethanol. Such data suggest that metal-ion chelation is the basis for the inhibition of dehydrogenase activation by Cd^{2+} in the presence of added thiols.

pH dependence of Cd^{2+}-activated dehydrogenase activity

The pH-activity profile of Cd^{2+}-activated threonine dehydrogenase was determined and compared directly with that for the Mn^{2+}-activated enzyme. For this purpose, 'demetallized' dehydrogenase was incubated with the respective metal ion in buffers over the pH range of 5.8–8.8 (incubation of the enzyme at pH values above 8.8 causes extensive loss of activity). As can be seen in Fig. 3, the pH-activity profiles of the Cd^{2+}- and the Mn^{2+}-activated enzyme are very similar. In both cases, the data conform closely to a theoretical curve for one proton ionization, based on the Michaelis pH-function equation of:

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

where V_{max} is the maximum velocity for the enzyme and K_a is the ionization constant for the proton on the enzyme. For Cd^{2+}-activated threonine dehydrogenase, the theoretical curve has a $pK_a = 7.85$, $V = 295$ units/mg at pH > 12, and $V_0 = 20$ units/mg at pH < 6. The fitted parameters for the Mn^{2+}-activated enzyme were reported earlier as $pK_a = 7.95$, $V = 229$ units/mg at pH > 12, and $V_0 = 0$ unit/mg at pH < 6 [7].

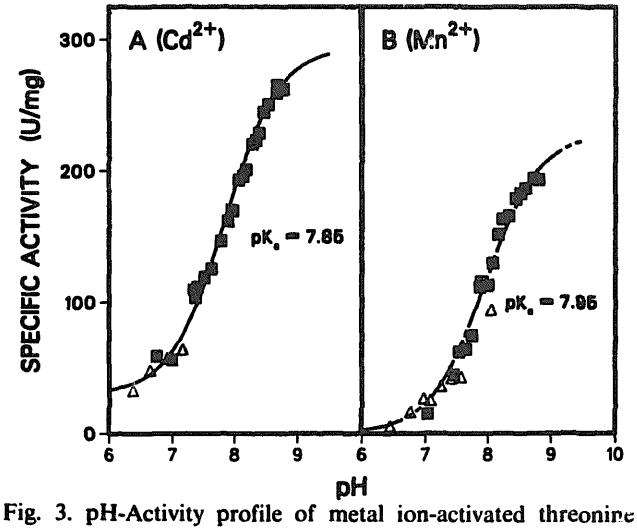

Fig. 3. pH-Activity profile of metal ion-activated threonine dehydrogenase. Thiol-free, 'demetallized' enzyme (0.5 μg) was first incubated for 30 min at 37 °C in 0.8 M buffer containing either (A) just 70 μM Cd^{2+} or (B) 250 μM Mn^{2+} plus 5 mM 2-mercaptoethanol. The assay was then initiated as in Fig. 1. Buffers used were Bistris-HCl (△) and Tris-HCl (■). The solid lines are theoretical curves based on the Michaelis pH function for ionization of a single residue.

Competition between Cd^{2+} and Mn^{2+} for binding to threonine dehydrogenase

Since both ions stimulate dehydrogenase activity, the ability of Cd^{2+} to displace Mn^{2+} from the enzyme was examined. Two samples of threonine dehydrogenase were incubated with $^{54}Mn^{2+}$; one sample served as a control to measure total binding of $^{54}Mn^{2+}$, whereas 30 nmol of Cd^{2+} were added to the second incubation mixture. The concentrations of the two metal ions used were based on their activation K_d values, i.e., 0.9 μM and 9 μM for Cd^{2+} and Mn^{2+}, respectively. If competition for the same binding site occurred, an approx. 50% displacement of Mn^{2+} by Cd^{2+} at a Mn^{2+}/Cd^{2+} ratio of 7 : 1 would be expected. The amount of $^{54}Mn^{2+}$ bound in each case was measured by eluting the enzyme-metal ion complex from a column of Sephadex G-25 and determining the co-eluted levels of enzyme activity, protein, and radioactivity. Since saturating concentrations of Mn^{2+} were not maintained throughout this procedure, the true stoichiometry of Mn^{2+} binding to the dehydrogenase was not observed in these studies. The results are presented in Fig. 4. Whereas 1.11 mol of Mn^{2+}/mol of enzyme tetramer were bound in the control incubation mixture (a value consistent with results obtained previously by this method), only 0.42 mol (a 62% decrease) was bound by the enzyme sample exposed to Cd^{2+}. Total dehydrogenase activity eluted from the incubation mixture containing Cd^{2+} was slightly lower than for the control, a factor attributable to the inhibition of Cd^{2+}-activation by 2-mercaptoethanol (see Fig. 2). A thiol concentra-

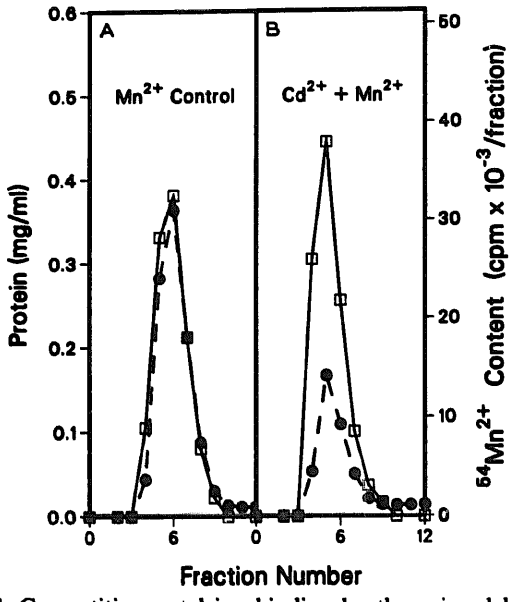

Fig. 4. Competitive metal ion binding by threonine dehydrogenase. (A) Thiol-free, 'demetallized' enzyme (1.25 mg) was incubated for 3 h at 4°C in 50 mM Tris-HCl buffer (pH 8.4) containing 1 mM 2-mercaptoethanol plus 200 nmol $^{54}Mn^{2+}$ ($71.6 \cdot 10^3$ dpm/nmol). (B) A second sample (1.25 mg) of enzyme was treated identically except that the incubation mixture also contained 30 nmol Cd^{2+}. Subsequently, each mixture was separately applied to and eluted from a column of Sephadex G-25 and the collected fractions (1 ml) were assayed for dehydrogenase activity, protein (□———□), and radioactivity (●- - - - -●).

tion of 1 mM was deliberately chosen, since this level allowed for 70–80% of maximal enzyme activation by Cd^{2+} as well as by Mn^{2+}. When such a binding experiment was repeated using a 2-fold excess of Cd^{2+} over $^{54}Mn^{2+}$ in the incubation mixture, more than 90% of the Mn^{2+} was displaced from the dehydrogenase.

Kinetic parameters for Cd^{2+}-activated threonine dehydrogenase

Values obtained previously for the Mn^{2+}-activated enzyme [7] were complicated by the finding that 2-mercaptoethanol, although required for maximal Mn^{2+} stimulation, inhibits the dehydrogenase-catalyzed reaction in a mixed noncompetitive manner. Hence, an unusually high K_m value for threonine and a somewhat higher value for NAD^+ were obtained.

Several attempts were made to examine the steady-state kinetics of Mn^{2+}-activated threonine dehydrogenase activity in a thiol-free environment. For example, the enzyme was first activated by 250 μM Mn^{2+} in the presence of 5 mM 2-mercaptoethanol; free Mn^{2+} and thiol were then rapidly removed by the column centrifugation technique of Penefsky [15]. Dehydrogenase so prepared was activated only 2–3-fold over metal ion-free enzyme, and this level of activity returned to that observed with the metal-free enzyme within 10 min of elution. Attempts were also made to activate the dehydrogenase with Mn^{2+} in the absence of 2-mercaptoethanol under anaerobic conditions, but it was found that even the metal ion-independent activity of the enzyme was lost during the repeated evacuation and flushing with nitrogen required for this method.

Such difficulties led us to determine the kinetic parameters of the Cd^{2+}-activated enzyme, which form of activation is not thiol-dependent. The values obtained are shown in Table I and compared with those for the 'demetallized' and the Mn^{2+}-activated enzyme. As can be seen, there is a large increase in V_{max} for both metal ion-activated forms of threonine dehydrogenase. The high K_m values for threonine and NAD^+ are evident with the thiol-dependent Mn^{2+}-activated form of the enzyme, whereas the same values for the Cd^{2+}-activated enzyme are within usual physiological ranges.

TABLE I

KINETIC PARAMETERS OF *E. COLI* THREONINE DEHYDROGENASE

'Demetallized' enzyme was obtained by dialysis against Chelex-100 resin as described in the text. Mn^{2+}-activated dehydrogenase was prepared by incubating in 0.4 M Tris-HCl buffer (pH 8.4) containing 250 μM Mn^{2+} plus 5 mM 2-mercaptoethanol at 37°C for 30 min; substrates were then added and assays carried out. Cd^{2+}-activated enzyme was incubated and assayed in the same manner but in buffer containing only 70 μM Cd^{2+}.

Enzyme preparation	V_{max} (units/mg)	K_m (L-threonine)	K_m (NAD^+) (mM)
'Demetallized'	28.3	1.1	0.11
Mn^{2+}-activated	685	211	0.60
Cd^{2+}-activated	243	8.2	0.18

Mn^{2+}/Mn^{3+} interconversion and its effect on threonine dehydrogenase activity

In considering possible reasons for the thiol-independent and the thiol-dependent stimulation of threonine dehydrogenase activity by Cd^{2+} and Mn^{2+}, respectively, we examined whether Mn^{2+} might undergo oxidation to Mn^{3+}. For this purpose, Mn^{2+} was incubated in the usual assay buffer and the formation of Mn^{3+} followed by measuring the appearance of an absorption band maximum at 259.5 nm which is reported to be characteristic of the Mn^{3+}-pyrophosphate complex [16]. As can be seen in Fig. 5, the oxidation of Mn^{2+} to Mn^{3+} proceeded exponentially to 20% conversion over a period of 2 h giving a first-order rate constant of 0.002 min^{-1}. The addition of 5 mM 2-mercaptoethanol to this mixture resulted in the disappearance of the Mn^{3+} · pyrophosphate spectral band. Since the normal assay mixture usually contained 4 nM enzyme as well as an initial concentration of 250 μM Mn^{2+} and with the first-order rate constant for Mn^{2+} oxidation now available, we determined that a Mn^{3+} concentration in excess of enzyme would be present after 5 min of the incubation period that preceded initiating the assay reaction by adding L-threonine plus NAD^+. Careful measurement of enzyme activation by Mn^{2+} in the absence of 2-mercaptoethanol showed that dehydrogenase activity was stimulated up to a maximum of approx. 5-fold within the first 5 min but thereafter (within the next 30 min) declined to the level seen with the metal ion-free enzyme.

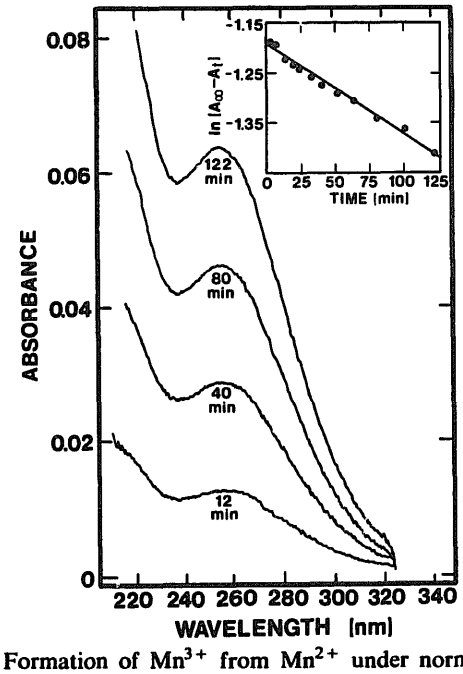

Fig. 5. Formation of Mn^{3+} from Mn^{2+} under normal assay conditions. 1 mM Mn^{2+} was added to 0.4 M Tris-HCl buffer (pH 8.4) at 37°C. Aliquots (50 μl) were withdrawn at the times indicated, diluted 20-fold in 0.1 M sodium pyrophosphate buffer (pH 6.5) and the ultraviolet spectrum then measured. (Inset) Determination of the first-order rate constant for the conversion of $Mn^{2+} \rightarrow Mn^{3+}$. A_∞ is the absorbance for 1 mM Mn^{3+} following dilution in the pyrophosphate buffer, based on an absorption coefficient of 6200 $M^{-1} \cdot cm^{-1}$ at 259.5 nm. A_t is the absorbance at 259.5 nm at time t.

The effect of Mn^{3+} on enzyme activity was also examined. Manganese(III) acetate was dissolved in 0.4 M Tris-HCl buffer (pH 8.4) and the solution immediately filtered to remove a precipitate which was assumed to be MnO_2. The concentration of Mn^{3+} was determined from the absorbance at 259.5 nm. When threonine dehydrogenase was incubated with 250 μM Mn^{3+}, no stimulation or inhibition of activity was observed. Addition, however, of 5 mM 2-mercaptoethanol to reaction mixtures containing 250 μM Mn^{3+} caused a 10-fold activation of dehydrogenase activity implying that Mn^{3+} was reduced by the thiol to Mn^{2+}. If 250 μM Mn^{3+} was added to enzyme fully activated by Cd^{2+}, no significant change occurred.

Discussion

Threonine dehydrogenase of *E. coli* is a metal ion-activated enzyme. If this dehydrogenase is dialyzed exhaustively against buffers containing Chelex-100 resin, it retains a low, metal ion-independent level of activity equal to about 10–20 units/mg. Of all metal ions tested, only Mn^{2+} and Cd^{2+} activate threonine dehydrogenase activity [6,7] and Mn^{2+} has been shown [7] to meet a number of criteria proposed by Schramm [12] in considering whether a metal ion might have a role in the regulation of enzymatic activity.

The properties of Cd^{2+}-activated threonine dehydrogenase, described in this paper, show that the Cd^{2+}- and Mn^{2+}-activated enzymes are similar in some respects but quite different in others. Full activation by either metal ion requires a prior incubation with the enzyme for about 5 min before the substrate and cosubstrate are added to initiate the reaction. Likewise, both metal ion-

activated forms of the enzyme manifest saturable, steady-state kinetics. Furthermore, the results of the column chromatographic study presented in Fig. 4 provide strong evidence that Cd^{2+} competes with Mn^{2+} for the same metal-ion binding site on this enzyme.

As is evident in Fig. 3, the pH-activity profiles for the two metal-ion activated forms of threonine dehydrogenase show some different characteristics. The fitted theoretical curve for the Cd^{2+}-activated enzyme has a 28% higher limiting activity rate at pH > 12. In addition, whereas the metal ion-free and the Mn^{2+}-activated enzyme at pH 6 have 2 (unpublished data) and 0 units of activity/mg, respectively, Cd^{2+}-activated threonine dehydrogenase retains a significant fraction of its maximal activity at this pH indicating that Cd^{2+} (unlike Mn^{2+}) is able to activate and possibly stabilize the enzyme at low pH values. Despite such differences, the most striking feature of Fig. 3 is that both plots fit a theoretical curve for one proton ionization with $pK_a \cong 7.9$ (± 0.05 units).

There is a sizable difference in the concentration of these two metal ions required to activate threonine dehydrogenase; the activation K_d for Cd^{2+} is about 10-times lower than that for Mn^{2+}. The $V_{max}/K_{m(threonine)}$ value for the Cd^{2+}-activated enzyme is also 9-times greater than that for Mn^{2+}-activated threonine dehydrogenase. Since the micromolar concentrations of Cd^{2+} required for full enzyme activation is toxic to *E. coli* cells, it does not seem likely that Cd^{2+} plays a meaningful role in regulating threonine dehydrogenase activity in vivo. Rather, the 2.8-fold higher V_{max} value for the Mn^{2+}-activated enzyme suggests that this metal ion is indeed the physiological activator.

The most interesting difference between these two activated forms of threonine dehydrogenase is that activation by Cd^{2+} occurs in the absence of exogenous thiols, whereas activation by Mn^{2+} is only seen in the presence of such compounds. Two possibilities were considered to explain the thiol-dependence of Mn^{2+}-activation. First, 2-mercaptoethanol might protect against the oxidation of an essential sulfhydryl group (or groups) in the enzyme that is (are) involved in the activation process. Some data have been obtained that indicate a relationship exists between thiol modification and enzyme activation (results to be published). If, however, a thiol like 2-mercaptoethanol only serves to protect a sulfhydryl group (or groups) in the enzyme from oxidation, one would expect that fully reduced enzyme from which the exogenous thiol had been rapidly removed would show full activation when subsequently incubated with either Mn^{2+} or Cd^{2+}. This is not seen with threonine dehydrogenase from *E. coli*. While the Cd^{2+}-activated enzyme maintains full activity for several hours in the absence of 2-mercaptoethanol, activation by Mn^{2+} is both incomplete and unstable in the absence of an added thiol. The second possibility considered, therefore, was that 2-mercaptoethanol keeps Mn^{2+} (which is an activator) from being oxidized to Mn^{3+} (which is not). It is known that while Mn^{2+} is the most stable ionic species, Mn^{3+} can readily form in the presence of oxygen [13]. The results shown in Fig. 5 indicate that Mn^{2+} is indeed oxidized to Mn^{3+} under the assay conditions used in our studies. Ezra and co-workers [14] reported that Mn^{3+} is reduced to Mn^{2+} by 2-mercaptoethanol. We observed the same; full activation of threonine dehydrogenase by Mn^{3+} in the presence of 2-mercaptoethanol as well as disappearance of the ultraviolet band for the Mn^{3+}-pyrophosphate complex following the addition of 2-mercaptoethanol to a solution containing Mn^{3+} demonstrated the reduction of $Mn^{3+} \rightarrow Mn^{2+}$ in our system. When, however, threonine dehydrogenase is incubated directly with Mn^{3+}, no significant effect on either the metal ion-free or the Cd^{2+}-activated enzyme was seen. Although no precise conclusions regarding the basis for the thiol dependence of enzyme activation by Mn^{2+} can be drawn from these data, this thiol requirement may imply a greater tendency for a sulfhydryl group (or groups) in the enzyme to be oxidized in the presence of Mn^{2+} than in the presence of Cd^{2+}, possibly due to the formation of Mn^{3+} on the protein surface after Mn^{2+} is bound by the enzyme.

Chemical modification studies with the metal ion-free and the metal-ion activated enzyme should give useful information concerning the possible involvement of protein sulfhydryl groups in the activation of threonine dehydrogenase by metal ions. The need to add an exogenous thiol for Mn^{2+}-activation, of course, complicates such efforts. Knowing on the basis of these studies that

the thiol-independent activation of the enzyme by Cd^{2+} is very similar to thiol-dependent activation by Mn^{2+}, we are now able to use the Cd^{2+}-activated dehydrogenase to examine the kinetics of sulfhydryl group modification as well as to study appropriate structure/function interrelationships of metal ion-free and metal ion-activated threonine dehydrogenase.

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