

BBA 33237

**Cd<sup>2+</sup> activation of L-threonine dehydrogenase from *Escherichia coli* K-12**

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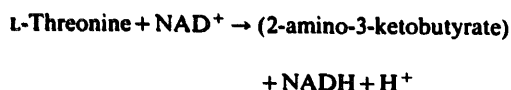
(Received 9 August 1988)

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

Homogeneous preparations of L-threonine dehydrogenase (L-threonine:NAD<sup>+</sup> 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<sup>2+</sup> stimulates dehydrogenase activity approx. 10-fold; this activation is concentration-dependent and is saturable with an activation  $K_d = 0.9 \mu\text{M}$ . Full activation by Cd<sup>2+</sup> is obtained in the absence of added thiols. The pH-activity profile of the Cd<sup>2+</sup>-activated enzyme conforms to a theoretical curve for one-proton ionization with a  $\text{p}K_a = 7.85$ . Mn<sup>2+</sup>, the only other activating metal ion, competes with Cd<sup>2+</sup> for the same binding site.  $K_m$  values for L-threonine and NAD<sup>+</sup> as well as the  $V_{\text{max}}$  for 'demetallized', Cd<sup>2+</sup>-activated, and Mn<sup>2+</sup>-activated threonine dehydrogenase were determined and compared.

**Introduction**

Threonine dehydrogenase (L-threonine:NAD<sup>+</sup> 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



where the apparent product, i.e., 2-amino-3-ketobutyrate, either rapidly decarboxylates with liberation of aminoacetone or is cleaved by a CoA-de-

pendent 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 ( $M_r \approx 80\,000$ ), 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<sup>2+</sup>. 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<sup>2+</sup> under standard assay conditions.

Among numerous other metal ions tested, we found that Cd<sup>2+</sup> 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  $\text{Cd}^{2+}$ -activated enzyme. Activation by  $\text{Cd}^{2+}$ , in contrast to that by  $\text{Mn}^{2+}$ , 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  $\text{Cd}^{2+}$ -stimulated threonine dehydrogenase under conditions where activation by  $\text{Mn}^{2+}$  is not possible.

## Materials and Methods

### Chemicals

$\text{NAD}^+$  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).  $^{54}\text{MnCl}_2$  in 0.5 M HCl ( $> 40$  Ci/g) and a  $0.1 \mu\text{Ci } ^{54}\text{Mn}^{2+}$  reference source were products of New England Nuclear (Boston, MA).  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 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  $\mu\text{g}$  of the dehydrogenase for 30 min at  $37^\circ\text{C}$  in 200  $\mu\text{mol}$  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  $\mu\text{mol}$  of

L-threonine plus 5  $\mu\text{mol}$  of  $\text{NAD}^+$ . One unit of enzyme activity is defined as that amount which catalyzed the formation of 1.0  $\mu\text{mol}$  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 $\text{Cd}^{2+}$ and $\text{Mn}^{2+}$ 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  $80^\circ\text{C}$ ) as a primary standard. For  $\text{Mn}^{2+}$ , 3 drops of 5% (w/v) potassium sodium tartrate followed by 2-3 ml of  $\text{NH}_3/\text{NH}_4^+$  buffer (pH 10.0) were added to a solution containing approx. 0.5 mmol of  $\text{Mn}^{2+}$ . This solution was then heated to  $70^\circ\text{C}$  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  $\text{Cd}^{2+}$  solutions, except that ascorbic acid and potassium sodium tartrate were not added.

### Binding of $\text{Cd}^{2+}$ and $\text{Mn}^{2+}$ by threonine dehydrogenase

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

### Results

#### Activation of *E. coli* threonine dehydrogenase activity by $\text{Cd}^{2+}$

$\text{Cd}^{2+}$  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  $\text{Cd}^{2+}$  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

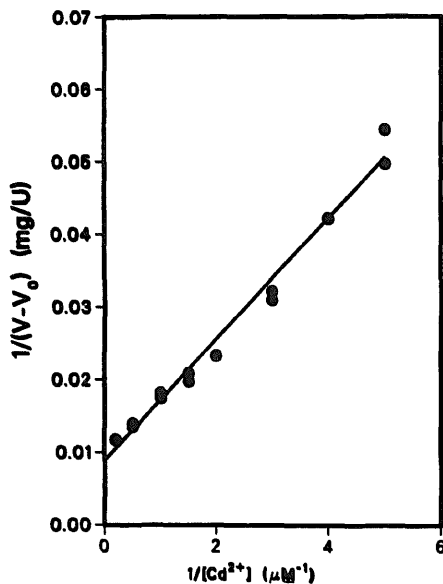


Fig. 1.  $\text{Cd}^{2+}$ -dependent stimulation of *E. coli* threonine dehydrogenase activity. 'Demetallized', thiol-free enzyme (0.5  $\mu\text{g}$ ) was incubated for 30 min at 37°C in 0.4 M Tris-HCl buffer (pH 8.4) with the concentrations of  $\text{Cd}^{2+}$  shown before the assay was initiated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

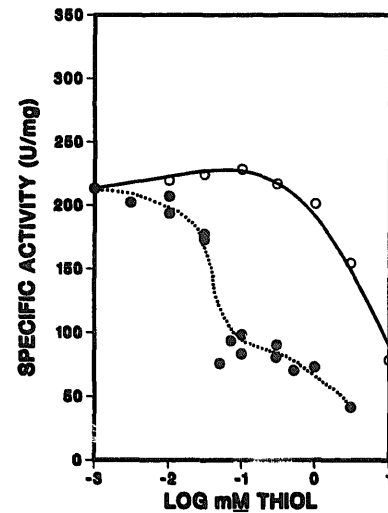


Fig. 2. Inhibition of the  $\text{Cd}^{2+}$ -activating effect on threonine dehydrogenase by 2-mercaptoethanol or dithiothreitol. Thiol-free, 'demetallized' enzyme (0.5  $\mu\text{g}$ ) was first incubated for 30 min at 37°C in 0.4 M Tris-HCl buffer (pH 8.4) containing 30  $\mu\text{M}$   $\text{Cd}^{2+}$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

instance was 12.5 units/mg. As can be seen, activation by  $\text{Cd}^{2+}$  is saturable with a metal ion-independent level of activity of 112 units/mg and an activation  $K_d = 0.9 \mu\text{M}$ , which value is 10-fold lower than that obtained with  $\text{Mn}^{2+}$  [7]. Subsequent results showed that greater than 95% of full activation is obtained by incubating the enzyme with  $\text{Cd}^{2+}$  for only 5 min at 37°C.

#### Effect of thiols on threonine dehydrogenase activation by $\text{Cd}^{2+}$

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

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

#### *pH dependence of $\text{Cd}^{2+}$ -activated dehydrogenase activity*

The pH-activity profile of  $\text{Cd}^{2+}$ -activated threonine dehydrogenase was determined and compared directly with that for the  $\text{Mn}^{2+}$ -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  $\text{Cd}^{2+}$ - and the  $\text{Mn}^{2+}$ -activated enzyme are very similar. In both cases, the data conform closely to a theoretic-

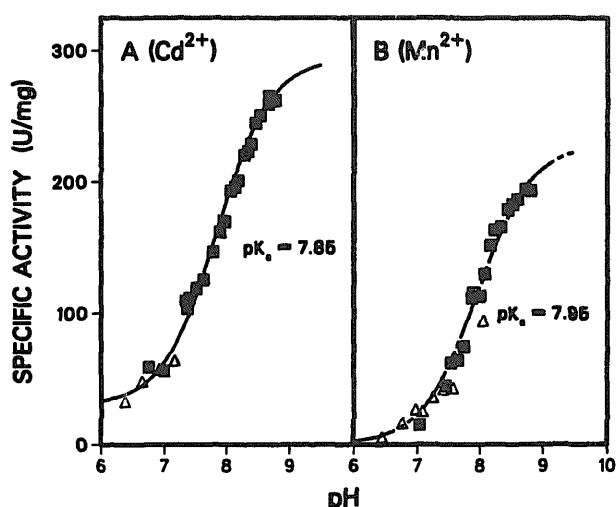


Fig. 3. pH-Activity profile of metal ion-activated threonine dehydrogenase. Thiol-free, 'demetallized' enzyme ( $0.5 \mu\text{g}$ ) was first incubated for 30 min at  $37^\circ\text{C}$  in  $0.8 \text{ M}$  buffer containing either (A) just  $70 \mu\text{M}$   $\text{Cd}^{2+}$  or (B)  $250 \mu\text{M}$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

cal curve for one proton ionization, based on the Michaelis pH-function equation of:

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

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

#### *Competition between $\text{Cd}^{2+}$ and $\text{Mn}^{2+}$ for binding to threonine dehydrogenase*

Since both ions stimulate dehydrogenase activity, the ability of  $\text{Cd}^{2+}$  to displace  $\text{Mn}^{2+}$  from the enzyme was examined. Two samples of threonine dehydrogenase were incubated with  $^{54}\text{Mn}^{2+}$ ; one sample served as a control to measure total binding of  $^{54}\text{Mn}^{2+}$ , whereas  $30 \text{ nmol}$  of  $\text{Cd}^{2+}$  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.,  $0.9 \mu\text{M}$  and  $9 \mu\text{M}$  for  $\text{Cd}^{2+}$  and  $\text{Mn}^{2+}$ , respectively. If competition for the same binding site occurred, an approx. 50% displacement of  $\text{Mn}^{2+}$  by  $\text{Cd}^{2+}$  at a  $\text{Mn}^{2+}/\text{Cd}^{2+}$  ratio of 7:1 would be expected. The amount of  $^{54}\text{Mn}^{2+}$  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  $\text{Mn}^{2+}$  were not maintained throughout this procedure, the true stoichiometry of  $\text{Mn}^{2+}$  binding to the dehydrogenase was not observed in these studies. The results are presented in Fig. 4. Whereas  $1.11 \text{ mol}$  of  $\text{Mn}^{2+}/\text{mol}$  of enzyme tetramer were bound in the control incubation mixture (a value consistent with results obtained previously by this method), only  $0.42 \text{ mol}$  (a 62% decrease) was bound by the enzyme sample exposed to  $\text{Cd}^{2+}$ . Total dehydrogenase activity eluted from the incubation mixture containing  $\text{Cd}^{2+}$  was slightly lower than for the control, a factor attributable to the inhibition of  $\text{Cd}^{2+}$ -activation by 2-mercaptoethanol (see Fig. 2). A thiol concentra-

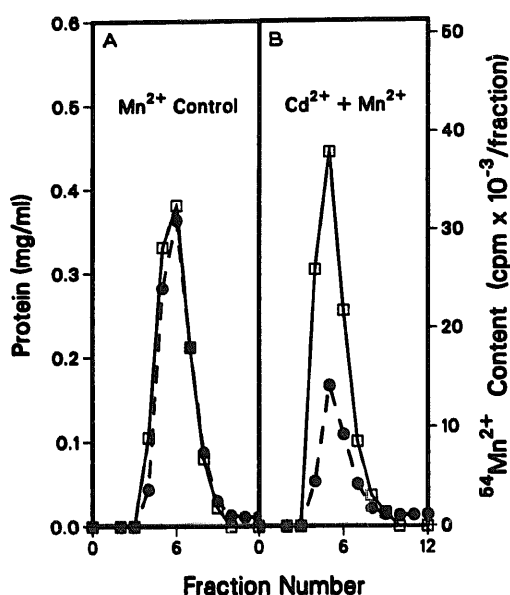


Fig. 4. Competitive metal ion binding by threonine dehydrogenase. (A) Thiol-free, 'demetallized' enzyme (1.25 mg) was incubated for 3 h at 4°C in 50 mM Tris-HCl buffer (pH 8.4) containing 1 mM 2-mercaptoethanol plus 200 nmol  $^{54}\text{Mn}^{2+}$  ( $71.6 \cdot 10^3$  dpm/nmol). (B) A second sample (1.25 mg) of enzyme was treated identically except that the incubation mixture also contained 30 nmol  $\text{Cd}^{2+}$ . 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 ( $\square$ — $\square$ ), and radioactivity ( $\bullet$ — $\bullet$ ).

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

#### Kinetic parameters for $\text{Cd}^{2+}$ -activated threonine dehydrogenase

Values obtained previously for the  $\text{Mn}^{2+}$ -activated enzyme [7] were complicated by the finding that 2-mercaptoethanol, although required for maximal  $\text{Mn}^{2+}$  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  $\text{NAD}^+$  were obtained.

Several attempts were made to examine the steady-state kinetics of  $\text{Mn}^{2+}$ -activated threonine dehydrogenase activity in a thiol-free environ-

ment. For example, the enzyme was first activated by 250  $\mu\text{M}$   $\text{Mn}^{2+}$  in the presence of 5 mM 2-mercaptoethanol; free  $\text{Mn}^{2+}$  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  $\text{Mn}^{2+}$  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  $\text{Cd}^{2+}$ -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  $\text{Mn}^{2+}$ -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  $\text{NAD}^+$  are evident with the thiol-dependent  $\text{Mn}^{2+}$ -activated form of the enzyme, whereas the same values for the  $\text{Cd}^{2+}$ -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.  $\text{Mn}^{2+}$ -activated dehydrogenase was prepared by incubating in 0.4 M Tris-HCl buffer (pH 8.4) containing 250  $\mu\text{M}$   $\text{Mn}^{2+}$  plus 5 mM 2-mercaptoethanol at 37°C for 30 min; substrates were then added and assays carried out.  $\text{Cd}^{2+}$ -activated enzyme was incubated and assayed in the same manner but in buffer containing only 70  $\mu\text{M}$   $\text{Cd}^{2+}$ .

Enzyme preparation	$V_{\max}$ (units/mg)	$K_m$ (L-threonine)	$K_m$ ( $\text{NAD}^+$ ) (mM)
'Demetallized'	28.3	1.1	0.11
$\text{Mn}^{2+}$ -activated	685	211	0.60
$\text{Cd}^{2+}$ -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  $0.002 \text{ min}^{-1}$ . 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  $250 \mu\text{M}$   $Mn^{2+}$  and with the

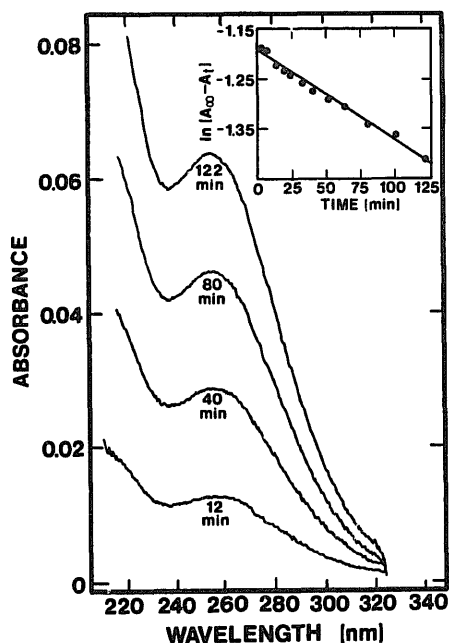


Fig. 5. Formation of  $Mn^{3+}$  from  $Mn^{2+}$  under normal assay conditions.  $1 \text{ mM}$   $Mn^{2+}$  was added to  $0.4 \text{ M}$  Tris-HCl buffer (pH 8.4) at  $37^\circ\text{C}$ . Aliquots ( $50 \mu\text{l}$ ) were withdrawn at the times indicated, diluted 20-fold in  $0.1 \text{ M}$  sodium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

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.

The effect of  $Mn^{3+}$  on enzyme activity was also examined. Manganese(III) acetate was dissolved in  $0.4 \text{ M}$  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  $259.5 \text{ nm}$ . When threonine dehydrogenase was incubated with  $250 \mu\text{M}$   $Mn^{3+}$ , no stimulation or inhibition of activity was observed. Addition, however, of  $5 \text{ mM}$  2-mercaptoethanol to reaction mixtures containing  $250 \mu\text{M}$   $Mn^{3+}$  caused a 10-fold activation of dehydrogenase activity implying that  $Mn^{3+}$  was reduced by the thiol to  $Mn^{2+}$ . If  $250 \mu\text{M}$   $Mn^{3+}$  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  $\text{Cd}^{2+}$  competes with  $\text{Mn}^{2+}$  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  $\text{Cd}^{2+}$ -activated enzyme has a 28% higher limiting activity rate at  $\text{pH} > 12$ . In addition, whereas the metal ion-free and the  $\text{Mn}^{2+}$ -activated enzyme at  $\text{pH} 6$  have 2 (unpublished data) and 0 units of activity/mg, respectively,  $\text{Cd}^{2+}$ -activated threonine dehydrogenase retains a significant fraction of its maximal activity at this pH indicating that  $\text{Cd}^{2+}$  (unlike  $\text{Mn}^{2+}$ ) 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  $\text{p}K_a \cong 7.9 (\pm 0.05 \text{ units})$ .

There is a sizable difference in the concentration of these two metal ions required to activate threonine dehydrogenase; the activation  $K_d$  for  $\text{Cd}^{2+}$  is about 10-times lower than that for  $\text{Mn}^{2+}$ . The  $V_{\text{max}}/K_{\text{m}(\text{threonine})}$  value for the  $\text{Cd}^{2+}$ -activated enzyme is also 9-times greater than that for  $\text{Mn}^{2+}$ -activated threonine dehydrogenase. Since the micromolar concentrations of  $\text{Cd}^{2+}$  required for full enzyme activation is toxic to *E. coli* cells, it does not seem likely that  $\text{Cd}^{2+}$  plays a meaningful role in regulating threonine dehydrogenase activity in vivo. Rather, the 2.8-fold higher  $V_{\text{max}}$  value for the  $\text{Mn}^{2+}$ -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  $\text{Cd}^{2+}$  occurs in the absence of exogenous thiols, whereas activation by  $\text{Mn}^{2+}$  is only seen in the presence of such compounds. Two possibilities were considered to explain the thiol-dependence of  $\text{Mn}^{2+}$ -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 pub-

lished). 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  $\text{Mn}^{2+}$  or  $\text{Cd}^{2+}$ . This is not seen with threonine dehydrogenase from *E. coli*. While the  $\text{Cd}^{2+}$ -activated enzyme maintains full activity for several hours in the absence of 2-mercaptoethanol, activation by  $\text{Mn}^{2+}$  is both incomplete and unstable in the absence of an added thiol. The second possibility considered, therefore, was that 2-mercaptoethanol keeps  $\text{Mn}^{2+}$  (which is an activator) from being oxidized to  $\text{Mn}^{3+}$  (which is not). It is known that while  $\text{Mn}^{2+}$  is the most stable ionic species,  $\text{Mn}^{3+}$  can readily form in the presence of oxygen [13]. The results shown in Fig. 5 indicate that  $\text{Mn}^{2+}$  is indeed oxidized to  $\text{Mn}^{3+}$  under the assay conditions used in our studies. Ezra and co-workers [14] reported that  $\text{Mn}^{3+}$  is reduced to  $\text{Mn}^{2+}$  by 2-mercaptoethanol. We observed the same; full activation of threonine dehydrogenase by  $\text{Mn}^{3+}$  in the presence of 2-mercaptoethanol as well as disappearance of the ultraviolet band for the  $\text{Mn}^{3+}$ -pyrophosphate complex following the addition of 2-mercaptoethanol to a solution containing  $\text{Mn}^{3+}$  demonstrated the reduction of  $\text{Mn}^{3+} \rightarrow \text{Mn}^{2+}$  in our system. When, however, threonine dehydrogenase is incubated directly with  $\text{Mn}^{3+}$ , no significant effect on either the metal ion-free or the  $\text{Cd}^{2+}$ -activated enzyme was seen. Although no precise conclusions regarding the basis for the thiol dependence of enzyme activation by  $\text{Mn}^{2+}$  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  $\text{Mn}^{2+}$  than in the presence of  $\text{Cd}^{2+}$ , possibly due to the formation of  $\text{Mn}^{3+}$  on the protein surface after  $\text{Mn}^{2+}$  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  $\text{Mn}^{2+}$ -activation, of course, complicates such efforts. Knowing on the basis of these studies that

the thiol-independent activation of the enzyme by  $\text{Cd}^{2+}$  is very similar to thiol-dependent activation by  $\text{Mn}^{2+}$ , we are now able to use the  $\text{Cd}^{2+}$ -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.

### Acknowledgement

The authors are indebted to Dr. G.W. Jourdian for allowing them to make extensive use of the Tracor Analytic Gamma-Trac 1191 counter in his research laboratories.

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