Allosteric Inhibition and Catabolite Inactivation of Purified Biodegradative Threonine Dehydratase of *Salmonella typhimurium*†

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ABSTRACT: Biodegradative threonine dehydratase of *Salmonella typhimurium* LT2 has been purified by affinity chromatography on AMP-Sepharose. In the presence of AMP, an allosteric effector, the enzyme exhibits a molecular weight of about 140 000 and is composed of four subunits of 36 000 each. The purified protein showed an absorbance maximum at 413 nm characteristic of enzymes containing bound pyridoxal phosphate; chemical determination indicated 2 mol of pyridoxal phosphate per 140 000 g. The K_m value for pyridoxal phosphate for the apoenzyme was 5 μM. Titrations of sulfhydryl groups with and without sodium dodecyl sulfate showed three-SH groups per 36 000 g, one of which is "buried". The catalytic activity of the enzyme was stimulated by AMP as well as by CMP, GMP, UMP, and ADP. The K_m for L-threonine was decreased by 15-fold when assayed with 3 mM AMP relative to that without AMP; the ratio of V_{max} values with and without AMP was 6. The enzyme activity was reversibly inhibited by pyruvate in the presence of AMP and revealed a strictly noncompetitive inhibition with respect to L-threonine and a "mixed" kinetic relationship in terms of AMP. Inhibition by pyruvate was independent of enzyme concentration. Incubation of the enzyme under assay conditions with [^{14}C]pyruvate for 5 min showed no protein-bound radioactivity. In addition to allosteric regulation, the threonine dehydratase was also subjected to catabolite inactivation by several intermediary metabolites, including pyruvate. The rate of enzyme inactivation was decreased with increasing protein concentration; AMP was obligatorily required for the inactivation process. Gel-filtration data showed that enzyme inactivation by pyruvate led to dissociation of the native enzyme; the inactive dissociated form of the enzyme contained 1 mol of firmly bound pyruvate per 140 000 g. Dialysis of the dissociated form of the enzyme against AMP and dithiothreitol resulted in reassociation to yield active enzyme with pyruvate still attached to the protein. We conclude that, despite the overall similarity in the control of biodegradative threonine dehydratases from *Escherichia coli* and *S. typhimurium*, the molecular mechanisms for the allosteric inhibition and catabolite inactivation by pyruvate of the enzymes from these closely related organisms are distinct and presumably reflect the differences in the structural features and the association-dissociation behavior of these two proteins.

Considerable evidence has been accumulated on the structure and regulation of the biodegradative threonine dehydratase (EC 4.2.1.16) from *Escherichia coli* (Hirata et al., 1965; Phillips and Wood, 1965; Whanger et al., 1968; Shizuta et al., 1969, 1973; Gerlt et al., 1973; Feldman and Datta, 1975; Dunne and Wood, 1975; Shizuta and Hayaishi, 1976; Saeki et al., 1977). In the buffer containing AMP, the enzyme has a molecular weight of about 147 000 and is composed of four identical subunits of approximately 38 000 each; there are 4 mol of pyridoxal phosphate per 147 000 g, and the tetramer can bind 4 mol of AMP per mol of protein. The enzyme is inducible, requires cyclic 3′,5′-AMP for its synthesis, and shows catabolite repression by glucose; AMP is an allosteric effector for this enzyme. More recent experiments have uncovered that the *E. coli* enzyme is inhibited by the reaction products, α-ketobutyrate and pyruvate, in the *absence* of AMP (Shizuta et al., 1973) and exhibits catabolite inactivation by several intermediary metabolites (Feldman and Datta, 1975). In contrast to the above studies with the *E. coli* enzyme, very little information is available on the biodegradative threonine dehydratase from *Salmonella typhimurium*. In one published report, Luginbuhl et al. (1974) have shown that the enzyme is inducible and requires 3′,5′-cAMP for its synthesis; in crude extract, the enzyme shows a requirement for AMP for catalytic activity. In preliminary experiments on the control of threonine dehydratase from *S. typhimurium* by intermediary metabolites, we observed (Bhadra and Datta, 1976) that the enzyme was not only subjected to catabolite inactivation by pyruvate but was also reversibly inhibited by the keto acid during enzyme assay in the *presence* of AMP. Thus, the same enzyme isolated from two closely related organisms appeared to be somewhat different and prompted us to investigate the structure and regulation of this enzyme from *S. typhimurium*. We report here the purification of the enzyme using affinity chromatography on AMP-Sepharose column. Studies with the purified enzyme show that, although the enzyme isolated from *S. typhimurium* has a similar molecular size and subunit structure and is related antigenically to the enzyme from *E. coli*, significant differences exist with respect to the number of sulfhydryl groups, pyridoxal phosphate content, and AMP binding sites; further, the activity of the *S. typhimurium* enzyme is stimulated significantly by ADP. We also found that the mechanisms of allosteric inhibition and catabolite inactivation of these two enzymes are different, especially in terms of the role of AMP and the conformational states of the enzyme from *S. typhimurium*.

Experimental Section

Reagents. Allo-free L-threonine, L-isoleucine, and 5,5′-dithiobis(2-nitrobenzoate) were obtained from Calbiochem, and sodium pyruvate, dithiothreitol, sodium dodecyl sulfate, triethanolamine hydrochloride (99% purity), iodoacetate, D,L-penicillamine, pyridoxal 5′-phosphate, NAD$^+$, NADP$^+$, and ribose nucleoside mono-, di-, and triphosphates were obtained from Sigma. [U-^{14}C]Pyruvic acid (specific activity, 10 mCi/mmol) was purchased from Amersham/Searle. The reagents for polyacrylamide gel electrophoresis and recrystalline

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TABLE I: Purification of Biodegradative Threonine Dehydratase from *Salmonella typhimurium*.

Fraction and step	Units[a] per mL	Units[a] Total	Total protein[b] (mg)	Sp. act. (units/mg of protein)
(1) Sonic extract	84	13 400	9500	1.4
(2) Streptomycin treatment	94	13 300	9100	1.5
(3) Ammonium sulfate	210	10 500	5200	2.0
(4) AMP-Sepharose column eluate (conc)	2390	4 300	17	253
(5) Sephadex G-200 eluate (conc)	3150	4 100	7.8	526

[a] Micromoles of α-ketobutyrate per minute at 25 °C. [b] Estimated by the method of Lowry et al. (1951).

guanidine hydrochloride were from Eastman Organic Chemicals. Dimethyl suberimidate was a product of Pierce Chemical Co. AMP-Sepharose was bought from Pharmacia. The following reference proteins were obtained from Worthington: yeast alcohol dehydrogenase, alkaline phosphatase, β-galactosidase, and horseradish peroxidase. Purified immunoglobulin was bought from Schwartz-Mann. Low-molecular-weight marker proteins were supplied by BDH Biochemicals. All ingredients of bacterial growth media were purchased from Difco Laboratories. All other chemicals were of reagent grade.

Bacterial Strain and Culture Conditions. Salmonella typhimurium LT2 was obtained from Dr. H. J. Whitfield. Stock cultures were maintained on nutrient agar slabs. Large-scale cultures were grown anaerobically at 37 °C in the tryptone-yeast extract medium of Umbarger and Brown (1957) supplemented with pyridoxine (1 mg/L).

Enzyme Assay. Threonine dehydratase activity was measured colorimetrically (Friedemann and Haugen, 1943) as modified by Feldman and Datta (1975). Spectrophotometric assay for enzyme activity in the presence of pyruvate was performed by monitoring the increase in absorbance at 310 nm as described by Shizuta et al. (1973). In both procedures, the standard incubation mixture contained, in a final volume of 1 mL, the following components: 100 μmol of potassium phosphate buffer, pH 8.0, 50 μmol of L-threonine, 3 μmol of AMP, 10 μmol of L-isoleucine (to inhibit biosynthetic threonine dehydratase, if present), and a rate-limiting amount of enzyme. One unit of enzyme is defined as the amount required to produce 1 μmol of α-ketobutyrate/min at 25 °C. The concentration of α-ketobutyrate was determined from the molar absorbancies of 25.6 at 310 nm or of 4000 at 540 nm. In some experiments, the data were expressed as change in absorbance at 310 nm per minute. All values were within the linear range of the assays employed.

The molecular weight of the enzyme was determined on calibrated Sephadex G-200 columns as described by Siegel and Monty (1966). The locations of the marker enzymes were established as described previously (Feldberg and Datta, 1971).

Molecular Size and Number of Subunits. Estimation of the subunit molecular weight was made by $NaDodSO_4$[1]-polyacrylamide gel electrophoresis of reduced and alkylated protein by the method of Weber et al. (1972). To determine the number of subunits in the native enzyme, protein samples cross-linked with dimethyl suberimidate (Davies and Stark, 1970) were electrophoresed on 5% acrylamide gel slab in the $NaDodSO_4$-borate-acetate buffer system and stained with Coomassie blue according to the method of Fairbanks et al. (1971).

Sulfhydryl Titration. The number of sulfhydryl groups was obtained by titration with Nbs_2 as described by Ellman (1959) in 100 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA and 3 mM AMP.

Pyridoxal phosphate was analyzed by the fluorometric method of Adams (1969).

Preparation of Apoenzyme. Purified enzyme was incubated at 37 °C for 20 h in 100 mM potassium phosphate buffer (pH 8.0), containing 3 mM AMP, 2 mM DTT, 3 mM NaN_3, 0.2 mM phenylmethanesulfonyl fluoride, and 100 mM D,L-penicillamine, and dialyzed for 16 h at 4 °C against the same buffer but without penicillamine (two changes, 200 volumes each). Prior to assay, the apoenzyme was incubated for 30 min at 37 °C in the dark in the complete reaction mixture with pyridoxal phosphate but without threonine.

Immunological Techniques. Antibodies against purified threonine dehydratase of *E. coli* (a gift from David Merberg) were raised in rabbits by the general procedure outlined by Brown (1957), except that the route of injection was intradermal (I. J. Goldstein, personal communication). Ouchterlony double-diffusion technique, as described by Goldstein and So (1965), was employed to assess the immunological identity of the enzymes from *E. coli* and *S. typhimurium*.

Other Methods. Protein was estimated by the method of Lowry et al. (1951) using bovine serum albumin as the standard. Spectral data were obtained by the use of a Cary 15 recording spectrophotometer.

Results

Enzyme Purification. Large-scale tryptone-yeast extract medium (60–70 L/batch) was inoculated with a 1% inoculum of cells grown aerobically at 37 °C for 16–20 h. The culture was incubated anaerobically at 37 °C, and cells were harvested when the enzyme activity per milliliter of culture reached a maximum value, usually after a period of 5–7 h. The yield of cells was about 1 g/L of medium and contained about 200 units of enzyme per gram wet weight of cells. Throughout the purification procedure, the temperature was maintained between 0 and 4 °C.

Seventy grams of wet weight cells were suspended in 100 mL of 100 mM potassium phosphate buffer, pH 6.8, containing 5 mM AMP, 2 mM DTT, and 1 mM phenylmethanesulfonyl fluoride (buffer A), and the suspension was subjected to sonic oscillation for a total period of 10 min in 1-min pulses. The extract was centrifuged at 40 000*g* for 60 min, and the supernatant fluid was retained (fraction 1, Table I). A solution of streptomycin prepared in buffer A was added dropwise to fraction 1 over a period of 10 min with continuous stirring (to reach a final concentration of 3%), and the mixture was centrifuged at 40 000*g* for 60 min. The supernatant fluid was collected (fraction 2). To each 100 mL of fraction 2, 28 g of solid ammonium sulfate was added gradually with continuous stirring. After an additional stirring for 60 min, the mixture was centrifuged at 40 000*g* for 60 min and the pellet otained was dissolved in a minimum volume of buffer A. The solution was dialyzed for 16 h against 40 volumes of buffer A. The dialyzed material (fraction 3) was applied on a Sephadex G-25

[1] Abbreviations are: $NaDodSO_4$, sodium dodecyl sulfate; Nbs_2, 5,5′-dithiobis(2-nitrobenzoate); DTT, dithiothreitol; NAD, nicotinamide adenine dinucleotide; NADP, NAD phosphate; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride; EDTA, (ethylenedinitrilo)tetraacetic acid.

TABLE II: Estimation of Molecular Weight by Gel Filtration on Sephadex G-200.

Elution buffer[a] suppl	Initial enzyme concn units/mL	Initial enzyme concn μg/mL	App mol wt
	0.53	1	111 000
	6.4	12	113 000
(A) AMP (3 mM)	34	65	126 000
	170	323	126 000
	1700	3230	136 000
(B) AMP (3 mM + DTT (2 mM)	6.4	12	112 000
(C) AMP (5 mM)	325	618	144 000
(D) AMP (5 mM) + DTT (5 mM)	325	618	130 000

[a] 100 mM potassium phosphate, pH 8.0.

column (55 × 2.5 cm) equilibrated in 100 mM potassium phosphate buffer, pH 8.0, containing 1 mM DTT and 1 mM phenylmethanesulfonyl fluoride (buffer AC) to remove AMP from the solution; the column was eluted with the same buffer. The pooled material containing enzyme activity was applied on an AMP-Sepharose column (20 mL gel volume; 2 μmol of AMP bound/mL gel) equilibrated with buffer AC. The column was washed with 2 column volumes of buffer AC containing 1 mM each of NAD^+ and $NADP^+$ followed by a large excess of buffer AC (about 1000 mL) until the eluate had negligible absorbance at 280 nm. The enzyme was eluted with buffer AC containing 10 mM AMP. After a two-step concentration procedure by ultrafiltration and precipitation with solid ammonium sulfate to give 60% saturation (fraction 4), the enzyme was applied on a Sephadex G-200 column (76 × 1.6 cm) which was equilibrated with buffer A and eluted with the same buffer at a flow rate of 6 mL/h. Fractions containing enzyme activity were pooled and concentrated by ultrafiltration (fraction 5). The preparation had a specific activity of 526; the yield was 30%[2] (Table I).

The enzyme solution showed maximum stability (half-life 5–6 weeks) when stored at 4 or −20 °C in 100 mM potassium phosphate buffer, pH 6.8, containing 10 mM AMP plus 2 mM DTT at a protein concentration of 1 mg/mL or higher.

Polyacrylamide gel electrophoresis of the purified enzyme according to Brewer and Ashworth (1969) and stained with Coomassie blue revealed two major bands comprising greater than 80% of the total protein and three minor bands of lesser mobilities. When a duplicate gel was sliced in 5-mm sections, each slice homogenized in 100 mM potassium phosphate buffer, pH 8.0, containing 3 mM AMP and assayed for threonine dehydratase activity by the colorimetric method, all bands showed enzyme activity, indicating enzyme aggregation during gel electrophoresis.

Molecular Weight of the Enzyme. The molecular weight of the native enzyme was estimated by gel filtration on Sephadex G-200 as a function of protein concentration in several buffer solutions.

The results in Table II show that in 100 mM potassium phosphate buffer, pH 8.0, containing 3 mM AMP the apparent molecular weight of the enzyme varied from 111 000 at an initial protein concentration of 1 μg/mL to a value of 136 000 at 3230 μg/mL; at 618 μg/mL of protein and at a higher concentration of AMP, the molecular weight reached the same approximate limiting value as that seen with the highest protein concentration but at lower levels of AMP. Addition of DTT did not show any apparent effect at low protein concentration but decreased the molecular size somewhat at a high concentration of the enzyme. These data are indicative of a rapid equilibrium between more than one active form of the enzyme in solution. Similar observations have been made for the threonine dehydratase from *E. coli;* Hayaishi and his collaborators (Shizuta et al., 1969; 1973) have proposed a dimer–tetramer association–dissociation reaction, whereas, based on active enzyme centrifugation and other data, a monomer–tetramer interconversion with a minimum molecular weight of 70 000 for the active enzyme has been proposed by Wood and his colleagues (Gerlt et al., 1973; Menson, 1976).

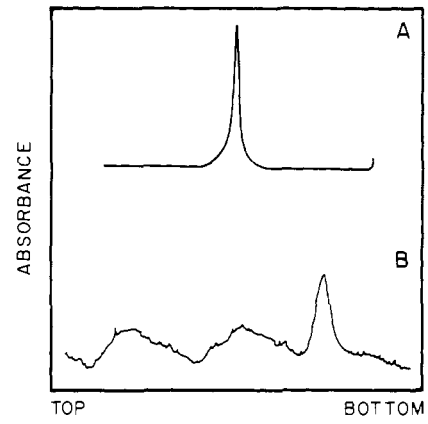

FIGURE 1: Densitometric scan of polyacrylamide gels. (A) Thirty micrograms of reduced and alkylated enzyme (see Experimental Section) was subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis and stained with Coomassie blue. The gel was scanned at 595 nm in a Gilford spectrophotometer equipped with a linear transport. (B) Purified enzyme was cross-linked with 2% dimethyl suberimidate and alkylated, and 30 μg of protein was subjected to $NaDodSO_4$ gel electrophoresis as described under Experimental Section. The gel was scanned using an Ortec densitometer Model 4310.

Molecular Size and the Number of Subunits. Heating at 100 °C of the purified enzyme with 1 to 2% $NaDodSO_4$ in 1% β-mercaptoethanol followed by $NaDodSO_4$–polyacrylamide gel electrophoresis revealed a major band and several minor bands of lesser mobilities, raising the possibility of incomplete denaturation and disaggregation of the enzyme subunits. In view of this, lyophilized enzyme was dissolved in hot 7 M guanidine hydrochloride, reduced with β-mercaptoethanol, and alkylated extensively with iodoacetate by method 2 of Weber et al. (1972). A single protein band was observed upon $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 1A); the molecular weight of this species was 36 000 ± 2000, as estimated from the calibration curve with several standard polypeptide chains.

To determine the number of subunits in the native enzyme, purified threonine dehydratase in buffer solution containing AMP was cross-linked with dimethyl suberimidate followed by reduction and alkylation as described above. When the cross-linked alkylated enzyme was subjected to gel electrophoresis in $NaDodSO_4$ and stained with Coomassie blue, three species of polypeptides were observed (Figure 1B) corresponding to molecular weights of 37 000, 72 000, and 142 000. All three bands were somewhat diffused due to extensive alkylation of the protein (cf. Weber et al., 1972). No distinct

[2] In recent experiments, sonic extracts prepared in 100 mM potassium phosphate buffer, pH 6.8, containing 2 mM DTT, 3 mM NaN_3, 0.2 mM phenylmethanesulfonyl fluoride, 1 mM NAD^+, and 0.2 mM $NADP^+$ were applied directly on AMP-Sepharose column; following washing with buffer AC, the enzyme was eluted with AMP and further purified by fractionating through a Bio-Gel P-200 column equilibrated in buffer A. Although the yield was somewhat less, the enzyme isolated by this procedure was judged pure by $NaDodSO_4$ gel electrophoresis.

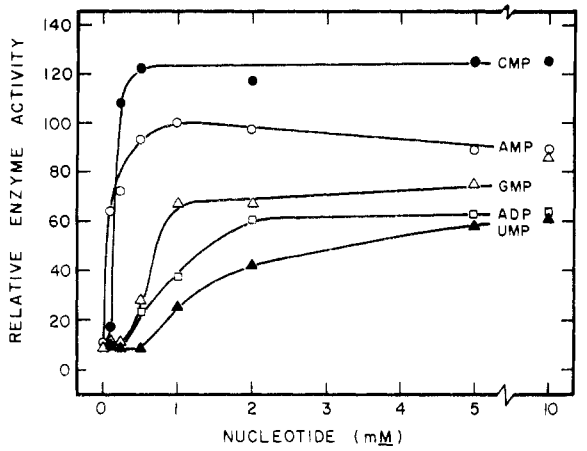

FIGURE 2: Stimulatory effects of various nucleotides. Stock enzyme was freed of AMP by dialysis for 16 h at 4 °C against 400 volumes of 100 mM potassium phosphate buffer, pH 7.0, containing 2 mM DTT followed by gel filtration on a Sephadex G-50 column (9 × 1 cm) in the same buffer. The assay mixture contained 50 mM L-threonine, 2 mM DTT, and 5 μg of protein in 100 mM potassium phosphate buffer, pH 8.0; the concentrations of nucleotides were as indicated. The enzyme assays were done spectrophotometrically. The data are expressed as relative enzyme activity to that seen with 1 mM AMP (1.41 μmol of α-ketobutyrate produced/min).

species in the molecular-weight range 100 000–110 000 was seen, despite several attempts to cross-link protein under a variety of experimental conditions.

The cumulative data seen thus far strongly suggest that in the presence of AMP the biodegradative threonine dehydratase of *S. typhimurium* has a molecular weight of about 140 000 g and consists of four subunits of 36 000 each. According to Saeki et al. (1977), the *E. coli* enzyme has a molecular weight of 147 000 and is composed of four identical subunits of 38 000 each.

Spectral Characteristics. The purified AMP-free enzyme showed an absorption maximum at 413 nm typical of enzymes containing pyridoxal phosphate as a hydrogen-bonded Schiff base. An $A_{413nm}^{1\%,1cm}$ value of 0.93 was calculated; this value is less than that found for the *E. coli* enzyme (Shizuta et al., 1969) and is consistent with the chemical analysis of pyridoxal phosphate shown below.

Pyridoxal Phosphate Content. The activity of the purified enzyme was independent of added pyridoxal phosphate. Assay of enzyme activity with increasing concentrations of hydroxylamine, penicillamine, and cysteine led to a loss of catalytic activity; the concentrations required for a 50% loss of enzyme activity were 0.26, 40, and 22 mM, respectively. Addition of pyridoxal phosphate to the inactive apoenzyme regenerated greater than 90% of the original enzyme activity; a K_m of 5 μM for pyridoxal phosphate was calculated from the data.

Chemical analysis of the pyridoxal phosphate content of the native enzyme at protein concentrations ranging from 0.04 to 0.16 nmol revealed an average value of 1.98 ± 0.27 mol of pyridoxal phosphate per 140 000 g. It is important to recall that the biodegradative threonine dehydratase from *E. coli* shows 4 mol of pyridoxal phosphate per mol of enzyme (Shizuta et al., 1969), whereas the *biosynthetic* enzyme from *S. typhimurium* has 2 mol per mol of protein (Burns and Zarlengo, 1968). Thus, with respect to the number of coenzyme molecules bound, the two enzymes from *S. typhimurium* have one-half the number of pyridoxal phosphate as compared to the *E. coli* threonine dehydratase.

Sulfhydryl Titration. Freshly purified enzyme dialyzed exhaustively against 100 mM potassium phosphate buffer, pH 7.0, containing 3 mM AMP was titrated with Nbs_2 according to the Ellman procedure. In the native enzyme, 8.4 mol of -SH/140 000 g was reacted with Nbs_2, whereas 11.6 mol of -SH was titrated in the presence of 0.15% $NaDodSO_4$, indicating that, on the average, each subunit of molecular weight 36 000 contains three -SH groups, one of which is buried in the protein interior.

Dependence of Catalytic Activity on Nucleotides. A large number of experiments with *E. coli* enzyme show an obligatory requirement of AMP for enzyme activity. Further, using a variety of AMP analogues as well as other natural nucleotides, the nature of the reactive groups for enzyme–ligand interactions has been clarified (Nakazawa et al., 1967; Rabinowitz et al., 1968). With the threonine dehydratase of *S. typhimurium* we have found that in 100 mM potassium phosphate buffer, pH 8.0 (at a protein concentration ranging from 4 to 90 μg/mL), the ratio of the V_{max} values (as determined from the Lineweaver–Burk plot) of the enzyme without AMP to that with 3 mM AMP was, on the average, 0.160; the K_m values for L-threonine with and without AMP were approximately 8 and 125 mM, respectively. Dunne et al. (1973) have observed a similar effect of AMP on the K_m for L-threonine for the *E. coli* enzyme; however, they have reported identical V_{max} values when assayed in the presence or absence of AMP after an appropriate correction for the instability of the AMP-free enzyme. It should be emphasized here that, unlike the situation with the *E. coli* threonine dehydratase (cf. Dunne et al., 1973), the AMP-free enzyme from *S. typhimurium* is stable in phosphate buffer containing DTT; the specific activity of the AMP-free enzyme, when assayed with saturating concentrations of AMP, was the same as that found with the enzyme stored in AMP.

The effect of increasing AMP concentration on enzyme activity presented in Figure 2 reveals that the nucleotide saturation kinetics is of the normal Michaelis–Menten type and at higher concentration (>2 mM) AMP was inhibitory. The concentration of AMP required for one-half the maximal velocity was 0.05 mM.

Figure 2 also depicts the stimulatory effects of various other nucleotides on threonine dehydratase of *S. typhimurium*. It is clear that GMP, CMP, UMP, as well as ADP are enzyme activators. The other nucleoside diphosphates and all four nucleoside triphosphates were inactive. At a concentration of 10 mM each, maximum stimulation of enzyme activity (as expressed by the ratio of activity with the nucleotide to that obtained without the nucleotide) was 5.5, 6.3, 8.0, and 15.0 for UMP, ADP, GMP, and CMP, respectively; the concentration of the individual nucleotide required for one-half the maximum velocity was 1.2, 0.8, 0.7, and 0.2 mM, respectively. In the context of the effects of various nucleotides, two points are of special interest: (1) that a high concentration of AMP (>2 mM) is inhibitory to the *S. typhimurium* threonine dehydratase and (2) that significant stimulation of enzyme activity is seen with ADP (see Figure 2). In terms of the ADP effect, the enzyme from *S. typhimurium* more closely resembles the ADP-activated threonine dehydratase of *Clostridium tetanomorphum* (Whiteley and Tahara, 1966; Nakazawa and Hayaishi, 1967; Vanquickenborne and Phillips, 1968); very little, if any, stimulation of enzyme activity by ADP has been seen with the enzyme from *E. coli* (Hirata et al., 1965; Nakazawa et al., 1967; Rabinowitz et al., 1968).

Allosteric Inhibition of Enzyme Activity. An assay of the dehydratase activity of *S. typhimurium* with an increasing concentration of pyruvate at varying levels of AMP showed pronounced inhibition at all levels of AMP, although the inhibitory effect of pyruvate was progressively diminished as the concentration of AMP was raised (Figure 3). To analyze the

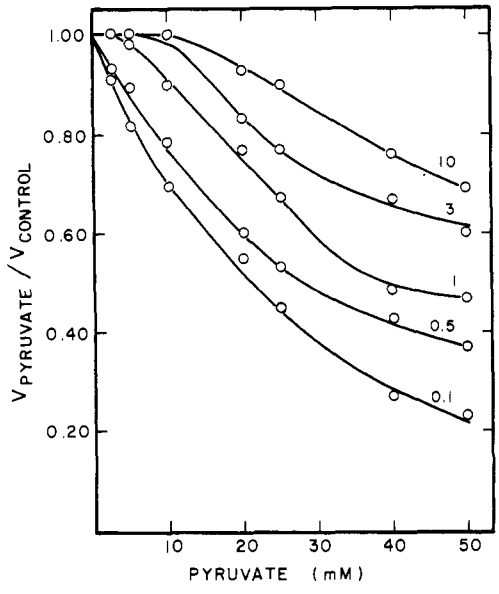

FIGURE 3: Inhibition of enzyme activity as a function of pyruvate concentration at different levels of AMP. Enzyme activity (7 μg of protein) was measured by the spectrophotometric procedure at 310 nm using a Gilford recording spectrophotometer. The figure next to each curve indicates the concentration of AMP present during the assay.

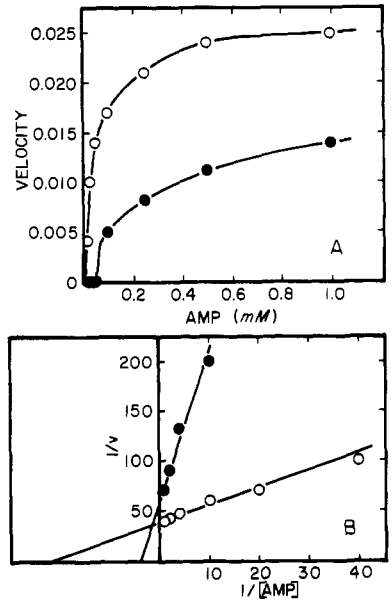

FIGURE 4: AMP saturation curves in the presence and absence of pyruvate. AMP-free enzyme was prepared as described in legend of Figure 2. Nine micrograms of protein was assayed by the spectrophotometric procedure: (○) no pyruvate; (●) 25 mM pyruvate. (A) Data expressed as Δ310 nm/min; (B) data expressed as reciprocal velocity vs. reciprocal AMP concentration.

kinetic relationship between AMP and pyruvate, enzyme activity was measured as a function of AMP concentration with and without the inhibitor. The results displayed in Figure 4A reveal that, whereas in the absence of pyruvate the AMP saturation curve was hyperbolic in nature, in the presence of pyruvate a sigmoid relationship was observed between AMP concentration vs. enzyme activity; at infinite AMP concentration (as seen from the Lineweaver–Burk plots of the results obtained at higher levels of AMP, see Figure 4B), pyruvate was still able to inhibit enzyme activity. These data strongly suggest that pyruvate and AMP occupy separate sites on the enzyme molecule. Further, since the kinetics of inhibition by pyruvate with varying concentration of L-threonine revealed a strictly noncompetitive relationship between the substrate and inhibitor, we conclude that the binding sites for pyruvate and AMP are distinct from that required for the binding of threonine. These findings are different from those seen with the *E. coli* enzyme; Shizuta et al. (1973) had concluded that AMP and α-ketobutyrate (or pyruvate) can occupy the same site on the enzyme molecule and that inhibition of enzyme activity in the absence of AMP results from binding of α-ketobutyrate to the AMP binding site.

The product inhibition by pyruvate of the *S. typhimurium* threonine dehydratase was freely reversible; enzyme preincubated with 40 mM pyruvate for 5 min in the presence or absence of AMP under the assay condition but without L-threonine was fully active when pyruvate was diluted out during the assay. Further, purified threonine dehydratase incubated with 50 mM [^{14}C]pyruvate (sp act. 1.25 mCi/mmol) for 5 min at 25 °C in the complete reaction mixture containing 3 mM AMP and 50 mM L-threonine and passed through a Sephadex G-50 column showed no radioactivity associated with the enzyme; when assayed with pyruvate, the enzyme eluted from the Sephadex G-50 column showed a similar extent of inhibition as compared to the native enzyme that has not been exposed to pyruvate. These results show that, during short-term incubation, the inhibition of enzyme activity by pyruvate did not lead to irreversible association of the inhibitor with the enzyme molecule. (For apparent covalent attachment of pyruvate during catabolite inactivation, see below.)

An important aspect of the allosteric effect of pyruvate on the *S. typhimurium* enzyme is that the extent of inhibition was not dependent on protein concentration within an 80-fold concentration range tested (1–80 μg/mL); in contrast, the inhibition by α-ketobutyrate of the *E. coli* enzyme was greatly enhanced at low protein concentration, and less inhibition was observed at a high concentration of the enzyme during the assay (Shizuta et al., 1973).

In addition to pyruvate, α-ketobutyrate, a product of the threonine deamination reaction, also inhibited the activity of *S. typhimurium* dehydratase. No inhibition was seen with the other keto acids, including oxaloacetate, α-ketoglutarate, and glyoxylate. These results are reminiscent of those reported for the *E. coli* enzyme (Shizuta et al., 1973), with the exception that significant inhibition of enzyme activity from *S. typhimurium* was observed in the *presence* of AMP. For example, during assays with 1 mM AMP, a 25 mM concentration of α-ketobutyrate inhibited enzyme activity by 50%; under identical conditions, no inhibition of the enzyme from *E. coli* was observed (Shizuta et al., 1973).

Ligand-Mediated Association–Dissociation Behavior of the Enzyme. Previous studies on the *E. coli* threonine dehydratase have shown that AMP influences the state of oligomerization of the dehydratase (Dunne and Wood, 1975; Shizuta and Hayaishi, 1976). The results presented below indicate that the enzyme from *S. typhimurium* can also exist in distinct molecular forms in the presence and absence of various ligands.

As mentioned earlier, the AMP-free enzyme from *S. typhimurium* was quite stable in the presence of dithiothreitol; however, when purified dehydratase was incubated at 37 °C in phosphate buffer in the absence of AMP *and* dithiothreitol, 50% of the initial enzyme activity was lost in about 20 min (Figure 5). A considerable *protection* against inactivation was afforded when the phosphate buffer was supplemented with either 50 mM pyruvate or 2.5 mM AMP. (When both pyruvate and AMP were added to the phosphate buffer, a more

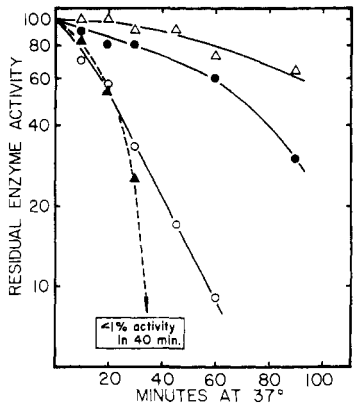

FIGURE 5: Heat inactivation profile of threonine dehydratase. Purified enzyme was dialyzed exhaustively at 4 °C against 100 mM potassium phosphate buffer, pH 8.0, containing 1 mM NaN_3 and 0.2 mM phenylmethanesulfonyl fluoride to remove dithiothreitol and AMP. The enzyme (300 μg/mL) was incubated in the phosphate buffer at 37 °C with the supplements as indicated, and aliquots (6 μg of protein) were withdrawn at various time periods and assayed for enzyme activity by the routine spectrophotometric method. Concentrations of various components during the incubation at 37 °C were as follows: (○) none; (●) 2.5 mM AMP; (△) 50 mM pyruvate; (▲) 2.5 mM AMP plus 50 mM pyruvate. The data are expressed as log of residual enzyme activity relative to zero time control.

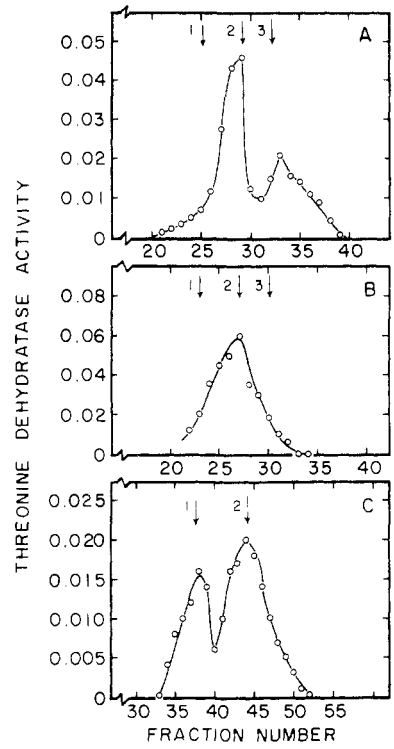

FIGURE 6: Gel-filtration profiles of threonine dehydratase in the presence or absence of AMP and pyruvate. For A and B, the AMP-free enzyme, prepared as described in the legend of Figure 2, was mixed with the reference proteins and passed through Sephadex G-200 columns at 4 °C equilibrated with 100 mM potassium phosphate buffer, pH 6.8, containing 3 mM NaN_3 and 0.2 mM phenylmethanesulfonyl fluoride with or without 50 mM pyruvate. The columns were eluted with the respective buffers at a flow rate of 3 mL/h, and 2.8-mL fractions were collected. One-hundred-microliter aliquots were assayed for enzyme activity following incubation with 3 mM AMP and 2 mM dithiothreitol (see Feldman and Datta, 1975). (A) 440 μg of enzyme was applied on a 76 × 1.6 cm column without pyruvate; enzyme activity assayed colorimetrically. (B) 460 μg of enzyme was applied on 68 × 1.6 cm column with 50 mM pyruvate; enzyme activity assayed spectrophotometrically. (C) Purified enzyme (1.15 mg/mL), partially inactivated by dialysis against 100 mM potassium phosphate buffer, pH 8.0, containing 3 mM AMP and 50 mM pyruvate at 4 °C for 40 h (60% inactivation), was mixed with the reference proteins and passed through a Sephadex G-200 column (85 × 1.6 cm) equilibrated in the same buffer. The flow rate was 8 mL/h and 1.8-mL fractions were collected. The profile of pyruvate-inactivated enzyme was determined by treatment of column fractions with 2 mM dithiothreitol for 60 min at 25 °C to reactivate the enzyme according to Feldman and Datta (1975). In a control experiment, an aliquot of enzyme treated without pyruvate but otherwise under identical conditions was passed through a separate calibrated Sephadex G-200 column equilibrated with 100 mM potassium phosphate buffer, pH 8.0, containing 3 mM AMP. The elution profile of this enzyme (not shown) revealed one peak corresponding to molecular weight of about 135 000. The reference proteins were (1) yeast alcohol dehydrogenase, molecular weight 142 000; (2) alkaline phosphatase, molecular weight 80 000; (3) horseradish peroxidase, molecular weight 40 000.

rapid inactivation was seen than that observed for the enzyme in buffer alone; this point will be discussed later under catabolite inactivation.) The lability of the AMP-free enzyme and the different extent of protection afforded by saturating concentrations of AMP and pyruvate (see Figure 5) suggest different "conformational" states of the protein when these ligands individually occupy their respective sites on the enzyme molecule. Gel-filtration data of the AMP-free enzyme in the presence and absence of pyruvate (Figure 6) strengthen this interpretation: whereas in the absence of AMP the enzyme showed two molecular species with apparent molecular weights of 78 000 and 36 000 (Figure 6A), only one form of threonine dehydratase, corresponding to the molecular weight of about 78 000 (Figure 6B), was seen when pyruvate was included in the gel-filtration buffer, indicating that the binding of pyruvate to the AMP-free enzyme stabilized the monomer–dimer association–dissociation equilibrium in favor of the dimeric form of the enzyme. It may be recalled that the apparent molecular weight of the enzyme in the presence of AMP (at a similar protein concentration) was approximately 140 000; as described below under catabolite inactivation, incubation of this form of the enzyme with pyruvate also resulted in dissociation to yield the enzyme dimer (see Figure 6C).

The above results clearly indicate that the *S. typhimurium* enzyme shows altered association–dissociation behavior when AMP and/or pyruvate are bound to their respective sites on the protein molecule. Since the inhibitory effect of pyruvate was progressively increased with decreasing concentrations of AMP (see Figure 3) and complete inhibition was observed when the concentration of AMP was less than that required for 50% stimulation of enzyme activity (i.e., <0.05 mM, cf. Figure 4A), it seems plausible, therefore, that the expression of catalytic activity is dependent on the ligand-mediated physical states of the enzyme. The following experiments depicted in Figure 7 provide some evidence in this regard. When the enzyme was preincubated for 5 min in the *absence* of AMP with either pyruvate or L-threonine plus pyruvate (curves 6 and 7, respectively), the steady-state rate of product formation was preceded by a lag of approximately 3 min; further, the rate of product formation was less than when the inhibitor was added simultaneously with AMP or to the enzyme preincubated with AMP (cf. curves 6 and 7 with 2, 4, and 8). Control experiments show that (1) preincubation of enzyme *with* AMP prior to the addition of either threonine (curve 3) or threonine plus pyruvate (curve 4) did not exhibit a lag period; (2) the steady-state rate of product formation was reached instantaneously when AMP was added to the enzyme preincubated with threonine for 5 min (curve 5); (3) the simultaneous addition of AMP, threonine, and pyruvate (curve 2) or the addition of pyruvate during deamination of threonine (curve 8) did not produce any lag. Two facts emerge from these data: (1) the transition from the AMP-free (i.e., less active dissociated) form to that of the AMP-bound active tetrameric state is rapid, and (2) pyruvate

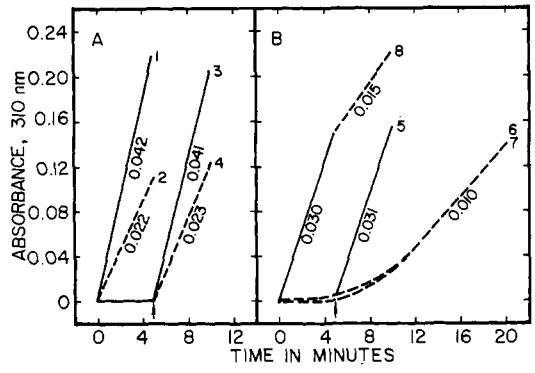

FIGURE 7: Effects of order of addition of various components on enzyme activity. A routine spectrophotometric assay at 25 °C was employed. An increase in absorbance at 310 nm, shown in the ordinate, has been corrected for the absorbance due to pyruvate when present in the assay mixture. The figure next to each curve refers to the rate of product formation as expressed by ΔA_{310nm} min^{-1}. Concentrations of various components, when present, were 3 mM AMP, 50 mM L-threonine, and 25 mM pyruvate. Concentrations of AMP-free enzyme, prepared as described in the legend of Figure 2, were 10 μg for A and 8 μg for B. The following assay conditions were used: *curve 1,* AMP plus threonine added at zero time; *curve 2,* AMP, threonine, and pyruvate added at zero time; *curves 3 and 4,* enzyme preincubated with AMP for 5 min followed by the addition of threonine and threonine plus pyruvate, respectively; *curve 5,* enzyme preincubated with threonine followed by the addition of AMP; *curves 6 and 7,* enzyme preincubated with either pyruvate or threonine plus pyruvate, respectively, followed by the addition of AMP plus threonine or AMP, respectively; *curve 8,* AMP plus threonine added at zero time followed by pyruvate at 5 min.

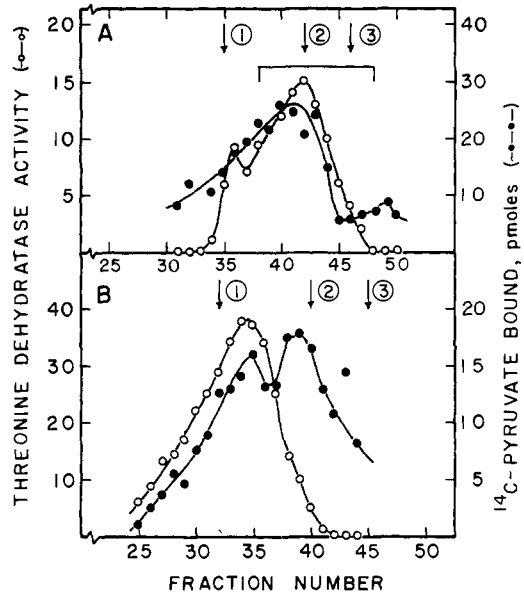

FIGURE 8: Binding of [^{14}C]pyruvate to threonine dehydratase. (A) Purified enzyme (575 μg) free of dithiothreitol was incubated at 4 °C in 100 mM potassium phosphate buffer, pH 8.0, containing 3 mM AMP and 50 mM [^{14}C]pyruvate (1.24 mCi/mmol) in a total volume of 1.5 mL and dialyzed at 4 °C for 24 h against the same buffer but with 50 mM nonradioactive pyruvate (five changes, 100 volumes each). The inactive enzyme (97% inactivation) was passed through a Sephadex G-200 column (76 × 1.6 cm) equilibrated in the above buffer. The column was eluted at a flow rate of about 7 mL/h and 1.7-mL fractions were collected. Threonine dehydratase activities were determined after treating aliquots with 2 mM dithiothreitol for 60 min at 25 °C. Radioactivity of aliquots was determined in 3a70B scintillation fluid (Research Product International) using a Packard Tri-Carb spectrometer. The profiles seen in B were obtained by pooling fractions 38–48 from A, concentrating by vacuum dialysis, dialyzing for 40 h at 4 °C against 100 mM potassium phosphate buffer, pH 8.0, containing 15 mM AMP and 2 mM dithiothreitol, and passing through a calibrated Sephadex G-200 column equilibrated with the same buffer. The numbered arrows at the top of each figure indicate the positions of the reference proteins (see the legend to Figure 6).

interferes with the rapid conversion from the AMP-free to the AMP-bound form of the enzyme. A more drastic decrease in the steady-state rate of product formation by the AMP-free enzyme *pretreated* with pyruvate [as compared to that seen when the enzyme was preincubated with AMP followed by the addition of pyruvate (curve 4)] may be explained by assuming that once pyruvate has stabilized the less active dimer conversion to the active form cannot be facilitated by the low concentration of AMP present during the assay. A more direct evidence in support of this notion is given below (cf. Figure 8B).

Catabolite Inactivation of Threonine Dehydratase. In 1975, Feldman and Datta reported a new mode of regulation of biodegradative threonine dehydratase of *E. coli,* designated as catabolite inactivation, in which the enzyme was rapidly *inactivated* by several intermediary metabolites, including pyruvate, in both intact cells and in purified enzyme preparations. The results documented below show that the biodegradative threonine dehydratase of *S. typhimurium* is also subjected to catabolite inactivation control.

When starved resting cells of *S. typhimurium* were incubated for various lengths of time with pyruvate in phosphate buffer containing AMP and the residual enzyme activity was measured after dilution to lower the concentration of pyruvate below that which is inhibitory, progressive inactivation of the enzyme was observed; similar inactivation kinetics was seen with purified enzyme preparations. In both cases, very little or no inactivation could be detected in the absence of pyruvate. As reported for the *E. coli* enzyme (Feldman and Datta, 1975), four other keto acids also inactivated the dehydratase from *S. typhimurium;* upon incubation of the enzyme (250 μg/mL in phosphate buffer containing 3 mM AMP) at 37 °C for 60 min with a 50 mM concentration of the metabolites, the extent of inactivation by α-ketobutyrate, oxaloacetate, phosphoenolpyruvate, and glyoxylate were 63, 72, 76, and 83%, respectively. α-Ketoglutarate was inactive.

A detailed analysis of the catabolite inactivation of the *S. typhimurium* enzyme by pyruvate showed that the rate of enzyme inactivation (as measured by the time required in minutes to obtain a 50% loss of enzyme activity, $t_{1/2}$, relative to the control sample not treated with pyruvate) decreased by 40-fold when the protein concentration was increased from 25 to 400 μg/mL, and a maximum inactivation rate was seen with 9 mM AMP. In fact, the requirement of AMP for catabolite inactivation by pyruvate was obligatory. As seen in Figure 5, pyruvate and AMP, individually, protected the AMP-free enzyme during incubation at 37 °C, whereas in the presence of pyruvate *plus* AMP the enzyme inactivated more rapidly than that seen in buffer alone, indicating a synergistic effect of these ligands.[3] A significant protection by either pyruvate or AMP of the AMP-free enzyme when contrasted with the time-dependent loss in enzyme activity in the presence of AMP plus pyruvate clearly indicates that the binding of pyruvate per se in the *absence* of AMP does not lead to catabolite inactivation.

Mechanism of Catabolite Inactivation. From several lines

[3] An important aspect of the data is that the inactivation profile of the AMP-free enzyme in buffer alone was identical to that seen with the enzyme in the presence of AMP plus pyruvate up to about 20 min; however, the inactivation process was accelerated more rapidly after 20 min of incubation when the enzyme was exposed to AMP plus pyruvate. This result suggests that the rapid loss of enzyme activity in the presence of these ligands is due to catabolite inactivation which is a time-dependent process.

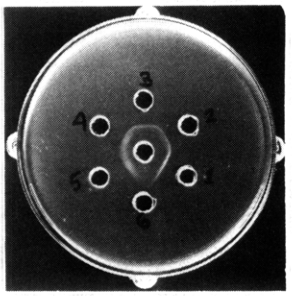

FIGURE 9: Immunological identity of threonine dehydratases from *E. coli* and *S. typhimurium*. The center well contained antibodies (rabbit) directed against purified enzyme from *E. coli*. *Wells 1 and 3:* 20 μg of purified enzyme of *E. coli*. *Wells 2 and 4:* 30 μg of purified enzyme from *S. typhimurium*. *Well 5:* 30 μg of pyruvate-treated inactive enzyme from *S. typhimurium*. *Well 6:* buffer.

of evidence, Feldman and Datta (1975) proposed a mechanism for the catabolite inactivation by pyruvate of the *E. coli* threonine dehydratase: "covalent" attachment of pyruvate and dissociation of the oligomer to yield inactive enzyme. The data obtained with the *S. typhimurium* dehydratase show that inactivation by pyruvate also led to a dissociation of the enzyme. The molecular size of the dissociated form, as determined by gel filtration on calibrated Sephadex G-200 (cf. Figure 6C), was found to be about 67 000 as compared to the native enzyme which has a molecular weight of about 135 000. Further, enzyme inactivated by [^{14}C]pyruvate followed by gel filtration on Sephadex G-200 showed protein-bound radioactivity (Figure 8A). The results depicted in Figure 8B show that the pyruvate-dissociated form of the enzyme may be reassociated in the presence of high concentrations of AMP and dithiothreitol to the native oligomeric form with pyruvate still attached to the protein; upon reassociation, the gel-filtration profiles of both enzyme activity and radioactivity shifted to the higher molecular weight region. (The peak of radioactivity appearing on the leading edge of Figure 8B, corresponding to the dissociated inactive form of the enzyme, is presumably due to an incomplete oligomerization of the protein during dialysis against AMP and dithiothreitol.)

To determine the number of pyruvate molecules tightly bound to the dissociated form, purified enzyme was inactivated by incubating at 37 °C for 4 h with 50 mM [^{14}C]pyruvate and passed through a Sephadex G-200 column to separate the 70 000 molecular weight species from the native undissociated form, as described in the legend for Figure 8. When the pooled fraction corresponding to the dissociated form of the enzyme was analyzed for radioactivity, approximately 1 mol of pyruvate was found to be attached per mol of enzyme; as reported earlier (Feldman and Datta, 1975), the same stoichiometry was also seen with enzyme from *E. coli*.

Discussion

The results presented here show that the molecular weight and subunit structure of the biodegradative threonine dehydratase from *S. typhimurium* are similar to the enzyme isolated from *E. coli*. Nevertheless, the differences in the pyridoxal phosphate content, the number of free sulfhydryl groups, and the existence of ADP binding sites on the *S. typhimurium* dehydratase strongly suggest that these proteins are to some extent different in their chemical composition. In view of this, it was surprising to find that antibodies directed toward the purified *E. coli* enzyme showed a cross-reaction with the enzyme from *S. typhimurium*, and, as shown in Figure 9, the line of identity (without a spur) indicates that they are immunologically identical. We interpret these results to mean that, despite subtle structural differences, the gross three-dimensional configurations of these two proteins are quite similar and that the physical domains of pyridoxal phosphate and ADP binding sites or the cysteinyl residues do not contribute significantly toward antigenicity. It would be interesting to compare the amino acid sequence of the dehydratases; considering the genetic relatedness of these two organisms, a high degree of sequence homology is not unexpected between these two proteins.

In the context of metabolic regulation, both enzymes are inhibited by the reaction products, α-ketobutyrate and pyruvate; they are also subjected to catabolite inactivation by several intermediary metabolites. However, significant differences exist as to the role of AMP in the product inhibition as well as in the catabolite inactivation by pyruvate. The following observations with the *S. typhimurium* dehydratase are relevant in this regard: (a) The *inhibition of enzyme activity* by pyruvate was seen at all concentrations of AMP and was independent of protein concentration; a strictly noncompetitive nature of the inhibition kinetics with respect to L-threonine and the fact that at infinite concentration of AMP pyruvate was still able to inhibit enzyme activity suggest that AMP and pyruvate binding sites are different from each other as well as separate from the binding site for threonine. (b) Product inhibition by pyruvate was freely reversible, and during short-term incubation the inhibitor did not bind "covalently" to the enzyme. (c) The less active AMP-free enzyme appears to be present in a monomer–dimer association–dissociation equilibrium as compared to the fully active AMP-bound form which exists as a tetramer; binding of pyruvate to the AMP-free enzyme interfered with the rapid transition from the AMP-free form to the AMP-bound form. (d) When AMP-free enzyme was preincubated with pyruvate, inhibition by pyruvate was greater than when the inhibitor was added simultaneously with AMP; further, pyruvate stabilized the AMP-free form by perturbing the monomer–dimer equilibrium in favor of the dimeric state of the enzyme. These findings suggest altered physical states of the protein when AMP and pyruvate are bound on the enzyme molecule. (e) *Catabolite inactivation* by pyruvate showed an obligatory requirement for AMP and was optimal at a high concentration of AMP.

The cumulative data summarized above clearly indicate that, despite the overall similarity in the structure and regulation of threonine dehydratases from *E. coli* and *S. typhimurium*, the molecular mechanisms involved in the product inhibition and catabolite inactivation are distinct and presumably reflect the subtle differences in the structural features and the association–dissociation behavior of these two proteins in the presence of various ligands. This is a clear example of the biochemical diversity in allosteric regulation of the same enzyme isolated from two bacterial species. It is reasonable to assume that the distinct mechanism of enzyme–modifier interactions in each case is peculiarly suited to the physiology and metabolism of these two organisms in question.

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Isotope-Trapping Experiments with Rabbit Liver Fructose Bisphosphatase†

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ABSTRACT: Isotope-trapping experiments with metal-free rabbit liver fructose 1,6-bisphosphatase have shown that enzyme-bound D-fructose 1,6-bisphosphate completely dissociates prior to enzyme turnover initiated by Mn^{2+} as the catalytic metal. The exchange rate of the binary enzyme-D-fructose 1,6-bisphosphate complex with the substrate pool is, therefore, more rapid than its conversion to products, suggesting that structural Mn^{2+} is necessary for productive substrate binding. Rapid-quench isotope-trapping experiments confirm the requirement for structural Mn^{2+} ions for productive binding to occur. These experiments also show that an ordered formation of the enzyme-$Mn^{2+}{}_s$-D-fructose 1,6-bisphosphate ternary complex which features metal-ion addition prior to substrate constitutes a catalytically competent pathway in the mechanism of fructose 1,6-bisphosphatase and that all four subunits are active in a single turnover event.

Fructose 1,6-bisphosphatase (EC 3.1.3.11, D-fructose 1,6-bisphosphate 1-phosphohydrolase, FBPase[1]) catalyzes the hydrolysis of D-fructose 1,6-bisphosphate (fru-1,6-P_2) to D-fructose 6-phosphate (fru-6-P) and orthophosphate (P_i) in the presence of a required divalent metal cation cofactor. Neutral rabbit liver FBPase[2] has been shown to bind four Mn^{2+} ions per mole of enzyme tetramer. An additional second set of four Mn^{2+} ions is also bound to the enzyme in the presence of the substrate analogue ($\alpha + \beta$) methyl D-fructofuranoside 1,6-diphosphate (Libby et al., 1975). For purposes of discussion, the first set of Mn^{2+} ions has been designated structural Mn^{2+} ($Mn^{2+}{}_s$) and the second set as catalytic Mn^{2+} ($Mn^{2+}{}_c$). The existence of this second set of binding sites in the presence of

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[1] The abbreviations FBPase, fru-1,6-P_2, fru-6-P and P_i are used throughout this paper to symbolize fructose 1,6-bisphosphatase, D-fructose 1,6-bisphosphate, D-fructose 6-phosphate, and ortho phosphate, respectively; other abbreviations used are: NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced NADP, EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

[2] Neutral enzyme exhibits optimal activity at pH 7.5 in the presence of known activators, in contrast to the proteolytically modified alkaline form of the enzyme, which exhibits maximal activity at alkaline pH.

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