JOURNAL OF BACTERIOLOGY, Nov. 1969, p. 878-889
Vol. 100, No. 2
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Properties of Threonine Deaminase From a Bacterium Able to Use Threonine as Sole Source of Carbon

T. G. LESSIE AND H. R. WHITELEY

Department of Microbiology, University of Massachusetts, Amherst, Massachusetts 01002, and the Department of Microbiology, University of Washington, Seattle, Washington 98105

Received for publication 2 September 1969

A threonine deaminase susceptible to inhibition by isoleucine was purified over 3,000-fold from extracts of *Pseudomonas multivorans*, a bacterium able to use threonine or α-ketobutyrate as sole source of carbon and energy. The enzyme was characterized with respect to molecular weight, dissociation to subunits, and apparent affinities for threonine, isoleucine, and several other ligands. Certain features of the enzyme including its reversible dissociation to subunits, its high constitutive activity, its marked stability, and high apparent orders of binding for threonine and isoleucine were unusual compared to those of isoleucine-inhibitable enzymes from other bacteria. These findings suggested some relationship between properties of the enzyme and the ability of *P. multivorans* to use threonine as sole carbon source. However, mutant studies ruled out a direct role of the enzyme in threonine catabolism and indicated that another enzyme, threonine dehydrogenase, is essential for growth on threonine.

Two types of threonine deaminases (L-threonine hydrolyase, deaminating, EC 4.2.1.16) have been reported from microorganisms. The enzymes of one group participate in isoleucine biosynthesis and are subject to inhibition by isoleucine (1–4, 15, 21, 22). Those of another group, including an enzyme produced by *Escherichia coli* during anaerobic growth (11, 21, 24) and the enzyme from *Clostridium tetanomorphum* (9, 18, 23, 25), are activated by adenosine monophosphate (AMP) and adenosine diphosphate (ADP), respectively, and presumably play an important role in anaerobic threonine catabolism (20, 24).

The threonine deaminase from *Pseudomonas multivorans* was examined in an attempt to clarify the relationship between the "biosynthetic" and "catabolic" enzymes. This organism was selected because it can utilize threonine as sole source of carbon, nitrogen, and energy. Hence, if threonine deaminase functioned in the catabolism of threonine, it seemed possible that a new threonine deaminase of the "catabolic" type might be formed, or that a preexisting threonine deaminase of the "biosynthetic" type might be modified to permit it to function under conditions which would be inhibitory to an isoleucine-sensitive enzyme. This seemed reasonable since the organism could, under certain conditions, use α-ketobutyrate, the product of threonine deaminase action, as sole carbon and energy source. However, purification and characterization of the *P. multivorans* threonine deaminase indicated that the organism possessed only one threonine deaminase. This enzyme was strongly inhibited by isoleucine, but it differed in several respects from the isoleucine-inhibitable threonine deaminases from other bacteria. This study reports some kinetic and physical properties of the enzyme, as well as mutant studies which indicate that threonine deaminase is not directly involved in the catabolism of threonine by *P. multivorans*.

MATERIALS AND METHODS

Cultures. *P. multivorans* strain 249 (ATCC 17616), obtained from R. Y. Stanier, was used as the source of enzyme. All mutant strains were derived from this organism as described below.

Growth of bacteria. Cultures of *P. multivorans* were grown in media containing inorganic nutrients (12) supplemented with 2 g of L-threonine or 4 g of sodium citrate per liter. Bacteria were grown at 37 C in 70-liter batches under conditions of vigorous aeration in a 130-liter fermentor (New Brunswick Scientific Co., New Brunswick, N.J.). For mutant selection and determination of enzyme activities under different conditions of growth, the bacteria were grown in Erlenmeyer flasks filled to 20% of their nominal

capacity. Cultures were incubated with shaking at the specified temperatures.

Mutant selection. Mutants impaired in biosynthetic or catabolic functions were obtained by a modification of the well-known penicillin selection technique in which D-cycloserine was substituted for penicillin. Use of cycloserine was necessary since penicillin was ineffective with *P. multivorans*. D-Cycloserine inhibits cell wall biosynthesis (19) and effectively promotes formation of osmotically sensitive bacteria in growing cultures of *P. multivorans*. We have also used cycloserine to select mutant strains of other pseudomonads, and we suggest that this agent might aid in selection of mutant strains of other penicillin-resistant bacteria. Details of the selection procedure were as follows: 5 ml portions of fully grown cultures of strain 249 were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (100 μg/ml) at 35 C with shaking for 20 min. Bacteria were centrifuged, and washed, and were then suspended in 50 ml of citrate medium (supplemented with 20 μg of isoleucine or leucine per ml where required for growth of the desired mutants). The cultures were incubated until they attained a density equivalent to about 150 μg of protein per ml. Portions (25 ml) were then centrifuged, and the bacteria were washed and suspended in 50 ml of either (a) citrate medium for selection of isoleucine or leucine auxotrophs or (b) threonine medium for selection of bacteria unable to utilize threonine as sole carbon source. After the bacterial mass had doubled, D-cycloserine was added to a concentration of 100 μg/ml, and the cells were incubated until they again doubled in mass. Portions (30 ml) of the culture were centrifuged, and the pellets were washed twice with distilled water to promote lysis of the bacteria. This step was necessary since the high osmolarity of the medium otherwise prevented lysis. The surviving bacteria were centrifuged and resuspended in 50 ml of citrate medium (supplemented with leucine or isoleucine where appropriate), and then were grown to full density. The bacteria were then plated on solid media containing either (a) citrate and growth-limiting amounts of the required amino acids (i.e., 1 μg of leucine or isoleucine/ml for selection of the auxotrophic strains) or (b) limiting amounts of yeast extract (0.01%) for selection of mutants unable to utilize threonine as a carbon source. The cultures were diluted and plated to give approximately 200 colonies per plate. The plates were incubated until the majority of the colonies were relatively large, and then small colonies were tested for their ability to grow either (a) without isoleucine or leucine or (b) with threonine as carbon source. After one cycle of cycloserine treatment, the desired mutants comprised about 0.1% of the bacterial population.

Assays. Threonine deaminase activity was measured by following the production of α-ketobutyrate at 30 C in assay mixtures containing: 2×10^{-1} M tris (hydroxymethyl) amino methane (Tris) hydrochloride buffer (*p*H 8.5); 4×10^{-2} M mercaptoethanol; and 1.85×10^{-4} M pyridoxal phosphate, enzyme preparation, and 10^{-1} M L-threonine (adjusted to *p*H 8.5) in a final volume of 0.5 ml. After 20 min of incubation, 0.5 ml of 15% trichloroacetic acid was added to stop the reaction, and 0.2 ml of this mixture was mixed with 2 ml of 0.02% 2,4-dinitrophenylhydrazine in 0.4 N HCl. After 20 min, 2 ml of 2 N NaOH was added and α-ketobutyrate was determined as the dinitrophenylhydrazone by measuring the absorbance at 450 nm. An absorbancy of 1.0 was equivalent to 2 μmoles of α-ketobutyrate in the assay mixture.

Assays of samples from chromatographic columns and sucrose gradients were performed in a total volume of 0.1 ml. In these experiments, the reaction was stopped by addition of 1 ml of the above 2,4-dinitrophenylhydrazine reagent; 1 ml of 2 N NaOH was added after 20 min, and absorbancy at 450 nm was determined.

For assays of serine deaminase activity, L-serine was substituted for L-threonine in the above procedures. One unit of threonine deaminase or serine deaminase activity was defined as the amount of enzyme required to produce 1 μmole of α-ketobutyrate or pyruvate per min under the above assay conditions. Specific activity was defined as units per milligram of protein. Protein was determined according to Lowry et al. (13).

Threonine dehydrogenase activity was measured by following NAD reduction spectrophotometrically at 340 nm in assay mixtures containing 0.1 M Tris buffer (*p*H 8.5), 0.1 M L-threonine (*p*H 8.5), 5×10^{-4} M nicotinamide adenine dinucleotide (NAD), and enzyme in a final volume of 1 ml. Conversion of threonine to aminoacetone was determined in the same assay mixtures after incubation for 20 min. The reactions were stopped by addition of an equal volume of 15% trichloroacetic acid, and aminoacetone was measured by the procedure used by Gibson et al. (6) for determination of aminoketones. Activities are expressed as micromoles of product formed per minute per milligram of protein.

For determination of the specific activities of enzymes in crude extracts of bacteria grown under different conditions, the cultures were incubated until they attained a density equivalent to about 150 μg of protein per ml. Portions (30 ml) were centrifuged, and the cells were washed with 0.05 M Tris-hydrochloride buffer (*p*H 7.5) and suspended in 5 ml of the same buffer. The bacteria were disrupted by treatment for 2 min with a Branson probe sonifier (20 kc) operated at 70% full power. The preparations were centrifuged for 10 min at 12,000 × *g*, and the supernatant fractions, containing 1 to 2 mg of protein per ml, were used as a source of enzyme.

Estimation of molecular weight and sedimentation constants. Gel filtration through calibrated columns of Sephadex G-150 and G-200 were performed as described earlier (26). Sucrose gradients (5 to 20%) containing 0.05 M buffer, and other additions as specified, were used as described by Martin and Ames (16). Protein assays on fractions from gradients were performed with a scaled-down version of the Lowry method, using a total reaction mixture of 1.0 ml. Absorbance at 750 nm was determined in a spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.).

Materials. Hydroxylapatite was obtained from Bio-Rad Laboratories, Richmond, Calif. Amino acids

and nucleotides were obtained from Calbiochem, Los Angeles, Calif.

RESULTS

Preparation of crude extracts. Frozen bacteria from 35 liters of culture were thawed and suspended in 10^{-2} M potassium phosphate buffer (*p*H 7.0) containing 10^{-3} M L-isoleucine (IL buffer). Portions (50 ml) of the suspension were disrupted in a Raytheon 10 kc sonic oscillator for 20 min; the treated materials were combined and centrifuged, and the supernatant fractions (crude extract) were retained. All centrifugations were carried out at 4 C for 20 min at 12,000 $\times$ *g*.

Protamine sulfate fractionation. An equal volume of 1% (w/v) protamine sulfate (*p*H 7.0) was added gradually to the crude extract; the mixture was stirred for 30 min at 0 C and centrifuged. The activity of the resulting supernatant fraction was higher than that of the crude extract (Table 1). This increase was obtained consistently and may represent removal of an inhibitory substance. It should be noted that when crude extracts were prepared from less-concentrated cell suspensions (1 mg of protein/ml), the specific activity was slightly higher (0.4), and the total activity of the preparations was greater or equal to that recovered after treatment with protamine sulfate.

Ammonium sulfate fractionation. Solid ammonium sulfate was added slowly with stirring at 0 C to the protamine supernatant fraction to give a final saturation of 35%. In this and subsequent ammonium sulfate fractionation steps, the suspensions were stirred for 30 min at 0 C prior to centrifugation. Ammonium sulfate was added in successive steps to the supernatant fractions obtained by centrifugation to give 40 and 50% saturation, respectively. The precipitates obtained by centrifuging the 0 to 35%, the 35 to 40% and the 40 to 50% saturated preparations were dissolved in IL buffer and stored at 0 C.

Heat step. The fraction precipitated by 35 to 40% saturation with ammonium sulfate was heated at 55 C for 10 min and centrifuged to remove denatured protein.

Chromatography on hydroxylapatite. The supernatant fraction was dialyzed overnight against 1 liter of IL buffer at 4 C and adsorbed to a 70-ml column (1.6 by 35 cm) of hydroxylapatite equilibrated with IL buffer. The column was eluted at 4 C with a linear gradient of 0.01 to 0.3 M potassium phosphate buffer (*p*H 7) containing 10^{-3} M isoleucine. The total volume of the eluting solution was 500 ml. The enzyme was eluted in a single position at a concentration of approximately 0.2 M phosphate. These and all subsequent fractions were devoid of threonine dehydrogenase activity. The active fractions were combined and dialyzed overnight at 4 C against 20 to 40 volumes of IL buffer.

Chromatography on diethyl aminoethyl (DEAE) cellulose. The dialyzed fraction was adsorbed to a 70-ml column (1.6 by 35 cm) of DEAE cellulose, equilibrated with the same IL buffer, and eluted with 500 ml of a linear gradient of 0 to 0.5 M NaCl in the same buffer. The enzyme was again eluted in a single position corresponding to a concentration of 0.2 M NaCl. The active fractions were combined and dialyzed overnight against 20 to 40 volumes of IL buffer at 4 C. This preparation was concentrated with Aquacide (Calbiochem) and was stored unfrozen at 0 C. The final preparation had a specific activity of 850 (a 3400-fold increase in specific activity relative to the crude extract) with a recovery of 35% (Table 1).

Addition of isoleucine was essential in the above steps to prevent inactivation of the enzyme during heat treatment and elution from hydroxylapatite and DEAE columns. It was possible to

TABLE 1. *Purification of Pseudomonas multivorans threonine deaminase*[a]

Step	Fraction	Volume (ml)	Protein (mg/ml)	Specific activity (μmoles/min per mg of protein)	Per cent activity recovered
1	Crude extract	240	28.5	0.25	100
2	Protamine supernatant fluid, $(NH_4)_2SO_4$ fraction	472	5.4	1.03	154
	0 to 35% saturation	10	2.2	1.95	
3	35 to 40% saturation	20	19.5	5.35	122
	40 to 50% saturation	20	40.0	0.13	
4	Heat-treated 35 to 40% fraction	17	7.0	15.7	109
5	Hydroxylapatite eluate	74	0.065	250.0	70
6	DEAE-cellulose eluate (concentrated)	1.3	0.53	850.0	34

[a] Activities indicated were obtained in standard mixtures containing between 0.1 and 0.2 units of enzyme from the fraction specified.

omit isoleucine in the preceding steps without loss of activity.

Purity of the final preparation. When samples from step 6 (Table 1) of the above procedure were sedimented through 5 to 20% linear sucrose gradients containing 0.01 M potassium phosphate buffer (pH 7), protein was detected at a single region of the gradient which coincided with the region containing enzyme activity. The specific activity of fractions in this region was 1,000. If this value approximates the specific activity of pure enzyme, the purity of the preparations from step 6 was about 85%. As will be discussed later, the rate of sedimentation of the enzyme indicated a molecular weight of about 192,000.

Results with threonine-grown bacteria. When threonine deaminase was purified from extracts of *P. multivorans* grown on threonine as sole carbon source, the behavior of the enzyme during the above fractionation steps was identical to that described above for the enzyme from extracts of citrate-grown bacteria, and the levels of enzyme in crude extracts from both types of cells were also the same.

Enzyme stability. The 20- and 40-fold purified preparations obtained by fractionation with ammonium sulfate and by heat treatment (steps 3 and 4 of Table 1) retained complete activity for at least 4 months when stored unfrozen at 0 C. These fractions were also stable to freezing and thawing in the absence of mercaptoethanol, but they were completely inactivated by this treatment if 10^{-2} M mercaptoethanol was added prior to freezing.

The more highly purified fractions (steps 5 and 6 of Table 1) lost 25 and 50% of their activity, respectively, after storage for 1 week unfrozen at 0 C. In the presence of 1 M glycerol, full activity was retained for at least 2 weeks under these conditions. Fractions from step 6 (Table 1) were completely inactivated by freezing and thawing even in the absence of mercaptoethanol; 1 M glycerol or 0.5 M potassium phosphate (pH 7) protected against such inactivation.

P. multivorans threonine deaminase pH optimum as a function of salt concentration. At pH values below about 8.5, the activity of *P. multivorans* threonine deaminase was markedly affected by alterations in salt concentration, even in the presence of high concentrations of threonine. For example, Fig. 1 shows that activity at the standard concentration of Tris buffer (0.2 M) with 0.1 M L-threonine was maximal at pH 8.2 to 9.8 and that it decreased to nearly zero as the pH was decreased to 7. At comparatively low concentrations of buffer (0.01 M), the range of optimal activity was shifted to values between pH 8.6 and 9.8, and the activity decreased markedly below this range. In contrast to the results at higher con-

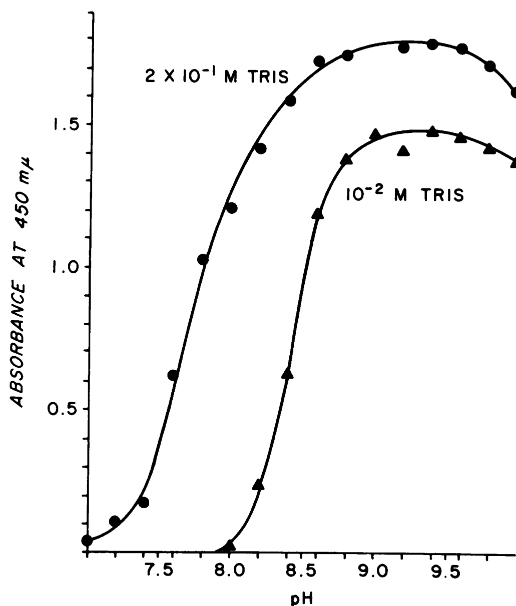

FIG. 1. *Activity of P. multivorans threonine deaminase as a function of pH. Reaction mixtures contained 0.1 M L-threonine adjusted to the pH of the buffer for each reaction mixture, standard concentrations of pyridoxal phosphate and mercaptoethanol, 50 μg of protein from a preparation equivalent to step 3 oj Table 1, and Tris-hydrochloride buffer at a final concentration of 0.01 or 0.2 M.*

centrations of buffer, there was no significant activity below pH 8. Other agents, as well as high concentrations of Tris, promoted enzyme activation over the range of pH 7 to 8. The most effective agents were divalent cations. For example, the levels of $CaCl_2$, $MgSO_4$, and $MnCl_2$ required for 50% maximal activation were, respectively, 2×8^{-4}, 4×8^{-4}, and 8×10^{-4} M. Increasing the ionic strength by addition of potassium phosphate, sodium chloride, or Tris buffer likewise increased activity (Fig. 2). One effect of lowering the salt concentration not apparent in these experiments is a decrease in the stability of the enzyme at low threonine concentrations. A number of agents, including high concentrations of threonine, were effective in overcoming this instability.

Kinetic properties with respect to threonine. The results in Fig. 3 indicate a sigmoidal response to increasing concentrations of threonine under standard assay conditions (0.2 M Tris-hydrochloride, pH 8.5). Hill plots of the data obtained with increasing concentrations of threonine (Fig. 3a) under standard assay conditions (0.2 M Tris-hydrochloride, pH 8.5) gave n values between 1.3 and 1.4 (Fig. 3b). The shape of the curve became markedly sigmoidal in the presence of relatively

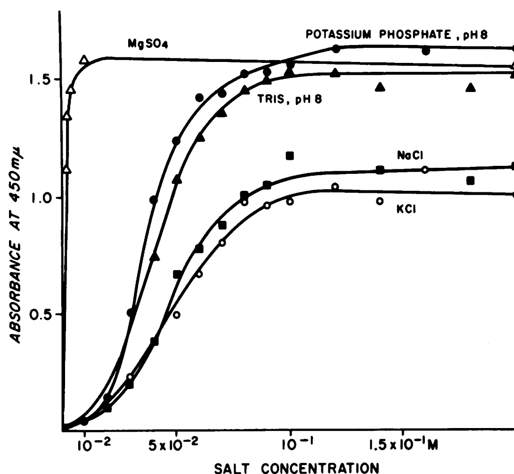

FIG. 2. *Activity of P. multivorans threonine deaminase as a function of salt concentration. The assay mixtures contained 0.01 M Tris-hydrochloride buffer (pH 8.0), 0.01 M L-threonine (pH 8.0), pyridoxal phosphate and mercaptoethanol at standard concentrations, the indicated concentrations of Tris-hydrochloride (pH 8.0), potassium phosphate (pH 8.0), NaCl, KCl, or $MgCl_2$, and 50 μg of protein from a preparation equivalent to step 3 of Table 1.*

low concentrations of isoleucine. For example, with 2×10^{-4} M L-isoleucine, the value of n was 2.5. The increase in n in the presence of isoleucine was accompanied by an increase in the level of threonine required for 50% maximal activity from 2×10^{-2} M to 10^{-1} M.

Under the conditions indicated in Fig. 3, the activity of *P. multivorans* threonine deaminase was linear with time with or without isoleucine and at both high and low concentrations of threonine. Substrate-dependent changes in the pre-steady-state rates of deamination have been reported in studies of threonine deaminases isolated from other organisms (4).

Results similar to those in Fig. 3 were obtained when L-serine was substituted for L-threonine, provided the reactions were carried out at *p*H 8. No activity was detected when D-threonine or D-serine were substituted for the L-isomers of these amino acids. At *p*H 8 (in the absence of isoleucine), deamination of either L-threonine or L-serine followed Michaelis-Menton kinetics (n was 1), but the activity with threonine was seven to eight times greater than that with serine. The respective K_m values for threonine and serine were 2×10^{-2} and 6×10^{-2} M. At a more alkaline *p*H, the enzyme tended to be irreversibly inactivated in the presence of serine (*results not shown*). Similar inactivation has been reported for the degradative threonine deaminase from *E. coli* (27).

Effects of different ligands on the stability of the enzyme at low threonine concentrations. At concentrations of threonine between 0.1 and 0.2 M, formation of the product was linear with time to a concentration of at least 4 μmoles of α-ketobutyr-

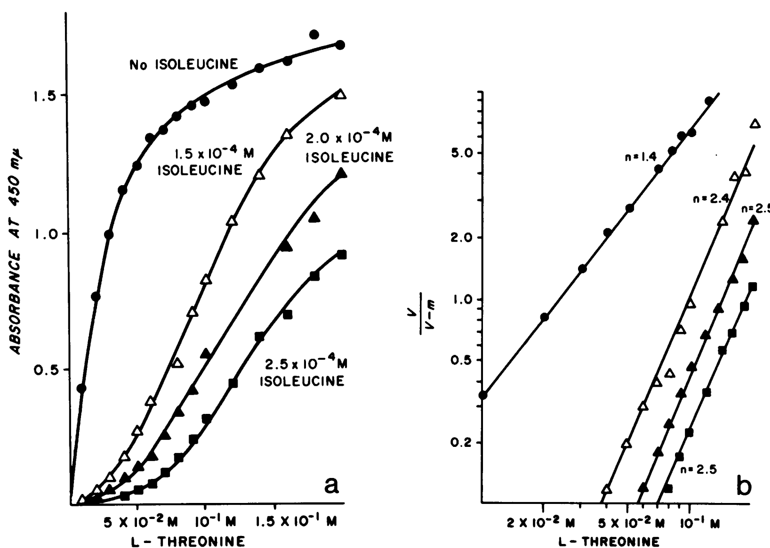

FIG. 3. *Activity of P. multivorans threonine deaminase as a function of the concentration of L-threonine. Reaction mixtures contained standard concentrations of pyridoxal phosphate and mercaptoethanol, 0.2 M Tris-hydrochloride buffer (pH 8.5), L-threonine at the indicated final concentrations (all at pH 8.5), and 50 μg of protein from a preparation equivalent to step 3 of Table 1.*

ate per ml. This was true under all the conditions tested in the present investigation. However, at lower threonine concentrations (for example, at 0.02 M), the enzyme was unstable when assayed at low ionic strength or at high *p*H. Under these conditions, the rate of threonine deamination rapidly became nonlinear, i.e., when less than 5% of the threonine was converted to product. It is obvious that measurements of substrate saturation under these conditions would yield erroneously sigmoid curves.

A number of different agents protected against loss of activity under the above conditions. These included amino acids with hydrophobic side chains, such as L-phenylalanine, L-methionine and L-tryptophan (D-isomers not tested), divalent cations, guanosine monophosphate (GMP), and phosphate. L-Leucine or L-valine afforded strong protection of the enzyme at *p*H 9.5, but they inhibited enzyme activity at *p*H 8.5 in assay mixtures of low ionic strength. The relative effectiveness of the different ligands affording protection against inactivation at low threonine concentrations and alkaline *p*H or low salt concentrations is indicated in Table 2.

Inhibition by isoleucine. The activity of the enzyme with threonine or serine was strongly inhibited by L-isoleucine. At *p*H 8.5, half-maximal inhibition occurred at approximately 1.5×10^{-4} M L-isoleucine (Fig. 4). Plots of data obtained from measurements of the reaction in the presence of increasing amounts of isoleucine yielded sigmoidal curves. Hill plots of these data gave *n* values of 3.5 and 3.6. At *p*H 8.0, *n* for isoleucine was 4.0; the corresponding value of $S_{0.5}$ was 1.3×10^{-4} M. As noted in the section describing the kinetics for threonine, the extent of inhibition by isoleucine did not increase during the course of the reaction.

Inhibition by isoleucine could be decreased by the addition of divalent cations or by increasing the *p*H. For example, at *p*H 9.5 (0.2 M Tris) or in the presence of 10^{-2} M $CaCl_2$ at *p*H 8.5, there was a 40- to 80-fold increase in the level of isoleucine required for 50% maximal inhibition, and *n* for isoleucine decreased from 3.5 to 1.5. These changes in the response of the *P. multivorans* enzyme to isoleucine are similar to the reported "desensitization" of the biosynthetic *E. coli* threonine deaminase at high *p*H (2).

Inhibition by valine or leucine. At low ionic strength, the *P. multivorans* threonine deaminase was inhibited by L-valine and L-leucine, as well as by L-isoleucine (Fig. 5). For example, in assay mixtures containing 0.01 M Tris-hydrochloride (*p*H 8.5) and 0.1 M L-threonine, 8×10^{-3} M L-valine or 1.8×10^{-2} M L-leucine inhibited the enzyme approximately 50%. As shown in Fig. 5,

TABLE 2. *Protection of Pseudomonas multivorans threonine deaminase against inactivation under conditions of low ionic strength or high pH*[b]

Ligand	Concn giving 50% maximal protection during incubation for 20 min
Tris buffer, pH 8.5	3×10^{-2} M
NaCl	2×10^{-2} M
Potassium phosphate buffer (*p*H 8.5)	7×10^{-3} M
$MgCl_2$	1×10^{-3} M
$MnCl_2$	3×10^{-4} M
$CaCl_2$	2×10^{-4} M
Guanosine monophosphate (GMP)	6×10^{-3} M
L-Leucine	6×10^{-5} M
L-Valine	7×10^{-4} M
L-Norleucine	1×10^{-3} M
L-Methionine	2×10^{-3} M
L-Phenylalanine	4×10^{-3} M
L-Tryptophan	6×10^{-3} M

[a] Effects of salts and GMP were determined in assay mixtures containing 0.01 M Tris-hydrochloride (*p*H 8.5), 0.01 M L-threonine (*p*H 8.5), and the usual concentration of pyridoxal phosphate and mercaptoethanol. The effects of amino acids were determined in assay mixtures containing 0.2 M Tris-hydrochloride (*p*H 9.5), 0.01 M L-threonine (*p*H 9.5), mercaptoethanol, and pyridoxal phosphate at the usual concentrations. The assay mixtures contained 40 μg of protein from a preparation equivalent to that of step 3 of Table 1.

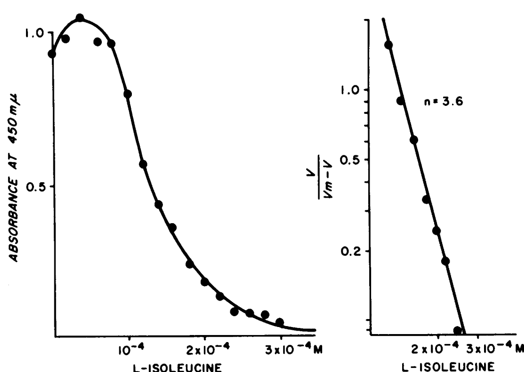

FIG. 4. *Inhibition of P. multivorans threonine deaminase by L-isoleucine. Standard assay mixtures were supplemented with the indicated amounts of L-isoleucine and 50 μg of protein from a preparation equivalent to step 3 of Table 1.*

the same degree of inhibition was found on adding 4×10^{-5} M L-isoleucine. Under these conditions of assay, adding any one of the three amino acids resulted in decreases in the rates of reaction. Plots

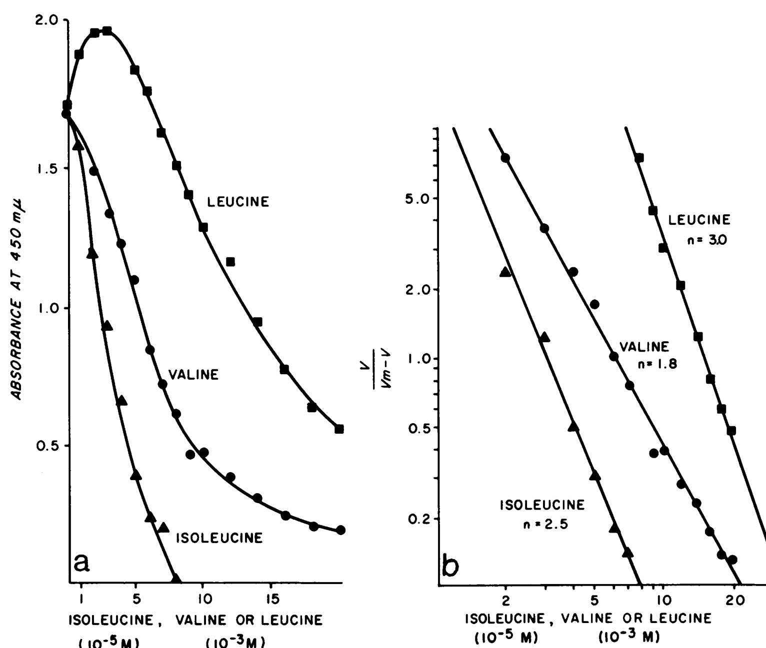

FIG. 5. *Inhibition of P. multivorans threonine deaminase by valine, leucine, and isoleucine. The reaction mixtures contained 0.01 M Tris-hydrochloride buffer (pH 8.5), 0.1 M L-threonine (pH 8.5), pyridoxal phosphate and mercaptoethanol at standard concentrations, the indicated amounts of L-valine, L-leucine, or L-isoleucine (all adjusted from a to pH 8.5), and 10 μg of protein from a preparation equivalent to step 3 of Table 1.*

of these data gave sigmoidal curves with n values of 3.0, 1.8, and 2.5, respectively, for L-leucine, L-valine, and L-isoleucine. Whether the binding sites for these three amino acids are identical remains to be established.

Heat lability of the enzyme. *P. multivorans* enzyme preparations in 0.01 M Tris buffer at pH 7.0 retained full activity after exposure for 20 min to 55 C. However, if the concentration of Tris buffer was increased to equal that used in the standard assay (0.2 M), there was a rapid loss of activity (Fig. 6). A similar rapid loss at 55 C was noted upon the addition of 0.01 M sodium or potassium phosphate, 0.2 M sodium chloride, or 0.01 M zinc sulfate (effect of the latter two compounds not shown), or upon increasing the pH of the 0.01 M Tris buffer to 8.5. It will be recalled that phosphate, sodium chloride, and Tris activate threonine deaminase (Fig. 2). However, addition of other compounds capable of activating the enzyme (L-norleucine, GMP, $MgCl_2$, $MnCl_2$, or $CaCl_2$), all at 0.01 M, did not labilize the enzyme to heat.

Loss of catalytic activity under conditions promoting inactivation could be prevented by the addition of isoleucine. Thus, 10^{-3} M isoleucine completely stabilized the enzyme against the inactivation promoted by 0.01 M phosphate, 0.01 M $ZnSO_4$, 0.2 M NaCl, or 0.2 M Tris buffer, pH 7.0 (results not shown). As stated earlier, leucine and valine were found to inhibit the enzyme under conditions of low ionic strength at pH 8.5. Under these conditions, both of the amino acids protected the enzyme against heat inactivation. For example, when the enzyme was heated for 20 min at 55 C in 0.01 or 0.05 M Tris buffer (pH 8.5), it was completely inactivated in the absence of amino acids. In the presence of 10^{-2} M L-leucine or L-valine, the enzyme was completely protected against inactivation under these conditions. None of the other amino acids listed in Table 2 was effective in this regard.

These experiments show a correlation between ability of ligands to inhibit the enzyme and to protect it against heat inactivation. On the other hand, a strict correlation was not found between the ability of various compounds to activate the enzyme and to increase its susceptibility to heat inactivation.

Effect of adenosine triphosphate (ATP) on enzyme activity. A strong inhibition of *P. multivorans* threonine deaminase was previously noted when ATP adjusted to pH 7 was added at a final concentration of 10^{-2} M in assay mixtures containing 0.01 M Tris (pH 8.5) and 0.1 M L-threonine (pH 8.5; Bacteriol. Proc. p. 119, 1968). Under these conditions, the amount of ATP added was sufficient to decrease the pH to 8.1. On the basis

of the behavior of the enzyme with respect to *p*H in assay mixtures of high ionic strength, this decrease would not have been expected to affect enzyme activity. However, as shown in Fig. 1, the enzyme displays a higher *p*H optimum at low salt concentrations, suggesting that the inhibition by ATP might be a consequence of an effect on the *p*H of the reaction mixture. This was confirmed by the finding that there was no inhibition when the ATP solution was adjusted to *p*H 8.5 immediately before addition to the reaction mixtures or when solutions of ATP adjusted to *p*H 7.0 were added to assay mixtures containing 0.2 M Tris and 0.1 M L-threonine (each at *p*H 8.0 or 8.5).

Sedimentation behavior of the enzyme. When preparations from steps 3 or 6 of Table 1, containing, respectively, 1.5 mg or 10 μg of protein per ml, were sedimented through sucrose gradients containing 0.05 M Tris-hydrochloride (*p*H 7.5), a sedimentation constant (*S*) of 8.8 was found. Gel filtration of similar amounts of the same preparations on Sephadex G-200 columns gave a single peak of enzyme activity corresponding to a molecular weight of about 192,000. When decreasing concentrations of enzyme were applied to gradients, there was a progressive decrease in the apparent rate of sedimentation of the enzyme (Fig. 7). At extremely low concentrations (about 15 μg per ml from a preparation equivalent to step 6) the enzyme sedimented as a species of approximately 3.4 *S*, corresponding to an apparent molecular weight of about 49,000. Isoleucine or components of the assay mixture tended to prevent dissociation of the enzyme (Fig. 7). The sedimentation constant of the enzyme in the presence of isoleucine was 9.2, corresponding to a molecular weight slightly greater than 200,000.

Dissociation of the enzyme and reaggregation to the native form. Dissociation could be achieved by dialysis against 0.05 to 0.2 M Tris buffer at *p*H 9.5 to 10.5. Under these conditions, preparations containing 2 to 10 mg of protein/ml (step 3 of Table 1) yielded several molecular species, with a 4.1 *S* form predominating. After 4 to 12 hr of dialysis, no activity could be detected in the absence of pyridoxal phosphate. It is of interest that comparable dialysis against alkaline solutions of phosphate buffer did not inactivate or dissociate the enzyme, indicating that Tris buffer contributed to the inactivation and dissociation. Similar results have been reported for the tryptophanase from *Bacillus alvei* (7).

Reactivation of dissociated preparations of *P. multivorans* by pyridoxal phosphate required 20 to 60 min of incubation at 30 C, and was optimal in the presence of a reducing agent and phosphate buffer at *p*H 7.0. Incubation under the latter

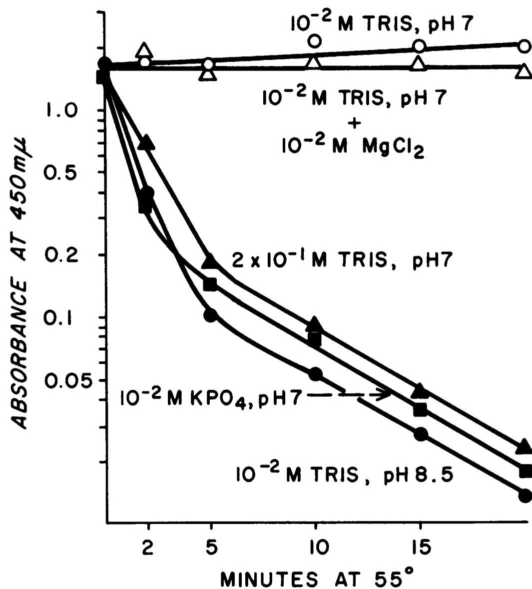

FIG. 6. *Effect of different ligands on the heat lability of P. multivorans threonine deaminase. Enzyme from a preparation equivalent to step 3 of Table 1 (80 μg of protein) was heated at 55 C in 0.2 ml of 0.01 M Tris-hydrochloride buffer (pH 7.0, except where indicated) containing the ligands indicated. After the specified time of heating, the samples were chilled and then assayed for threonine deaminase activity under standard conditions.*

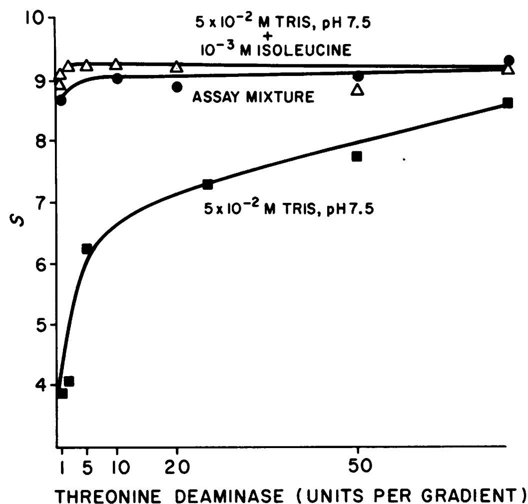

FIG. 7. *Sedimentation properties of P. multivorans threonine deaminase as a function of enzyme concentration. The specified amounts of protein from a preparation equivalent to step 6 of Table 1 were layered in 0.2 ml on 5 to 20% linear sucrose gradients (4.8 ml) containing the indicated additions and were centrifuged at 130,000 × g for 17 to 20 hr at 4 C. Sedimentation values were calculated using hemoglobin (4.1 S) as a marker.*

conditions also promoted association to higher molecular weight forms, including small amounts of materials having *S* values greater than 8.8 (Table 3). Incubation with threonine and isoleucine alone resulted in partial association. Clearly, reactivation and reaggregation of various dissociated forms could occur under the conditions employed for assay, thereby accounting for the apparent enzymatic activity of the various species listed in Table 3. It is not known at the present time whether partially dissociated forms of this enzyme can be reactivated without aggregation.

It should be noted that only 10 to 20% of the initial activity could be recovered when dissociated preparations (experiments 1–4 in Table 3) were sedimented and allowed to reaggregate prior to assay. In contrast, 40 to 80% recovery was obtained with preparations allowed to reaggregate prior to sedimentation (experiments 5–7 of Table 3), indicating that dissociated preparations were less stable than associated forms. Similar results had been obtained earlier with threonine deaminase from *C. tetanomorphum* (26).

When a dilute preparation of a more highly purified enzyme fraction (0.5 mg of protein/ml of a fraction corresponding to step 6 of Table 1) was dialyzed at alkaline *p*H, a complete loss of enzyme activity and an almost complete conversion to a 3.4 *S* form were observed. A similar conversion to an enzymatically inactive 3.4 *S* species was found with other preparations dialyzed at an alkaline *p*H in the presence of 8 M urea or 4 M guanidine. These findings suggest that the native enzyme (molecular weight, approximately 192,000) consists of four subunits, each of approximately 49,000 molecular weight (3.4 *S*).

Constitutive formation of the enzyme. *P. multivorans* grew approximately four times more rapidly with citrate than with threonine as sole carbon source (Table 4). However, the level of threonine deaminase was not markedly different under the two conditions. Furthermore, the activities were not affected by the presence of isoleucine, leucine, and valine in the media. Similar activities were also noted when cultures of a leucine auxotroph, strain 249-4, were grown with a limiting concentration of leucine (10 μg/ml) and were assayed 30, 60, and 90 min after leucine depletion. Furthermore, no increase in specific activity was noted when another leucine auxotroph, strain 249-10, was grown in a chemostat (at a specific growth rate of 0.1 hr^{-1}) with citrate as carbon source and a limiting supply of leucine (5 μg/ml; *results not shown*). It is evident, therefore, that *P. multivorans* threonine deaminase is

TABLE 3. *Aggregation of dissociated threonine deaminase*[a]

Expt	Conditions of 1 hr incubation after dialysis and filtration	*S* values of enzymatically active forms and percentage of total enzyme in each form
1	0.05 M Tris, *p*H 7.0-0.1 M mercaptoethanol	5.1 (5%), 4.1 (80%), 3.4 (15%)
2	0.1 M phosphate, *p*H 7.0-1.0 M mercaptoethanol	5.1 (20%), 4.1 (70%), 3.4 (10%)
3	As in expt 2 + 0.1 M L-threonine	9.2 (20%), 7.9 (30%), 5.1 (20%), 4.1 (30%)
4	0.1 M L-threonine	5.1 (20%), 4.1 (70%), 3.4 (10%)
5	As in expt 2 + 10^{-3} M pyridoxal phosphate	9.8 (10%), 9.2 (80%), 8.8 (10%)
6	As in expt 3 + 10^{-3} M pyridoxal phosphate	9.2 (90%), 8.8 (10%)
7	As in expt 2 + 10^{-3} M L-isoleucine	8.8 (20%), 7.9 (30%), 4.1 (50%)

[a] Enzyme preparation from step 3 of Table 1 was diluted 1:5 with 0.2 M Tris-hydrochloride buffer (*p*H 10.5) to give 4 mg of protein/ml and was dialyzed against 500 volumes of the same buffer at 4 C for 4 hr. The preparation was desalted by passage through a column of Sephadex G-50, supplemented with compounds shown above, and allowed to incubate in ice for 60 min. A 0.2-ml amount of each preparation was layered on a 5 to 20% sucrose gradient containing 0.05 M potassium phosphate buffer (*p*H 7.0) and centrifuged at 4 C for 17 hr at 130,000 × *g*. Fractions were collected and incubated for 1 hr at 30 C in the presence of 0.1 M potassium phosphate (*p*H 7.0), 10^{-3} M pyridoxal phosphate, and 0.1 M mercaptoethanol. A sample was then removed from each fraction for assay under standard conditions. The percentage of total enzyme in each molecular form was obtained by plotting the micromoles of α-ketobutyrate produced by each fraction and by estimating the areas under the peaks. The specific activity of the protein-containing fraction from the Sephadex column, determined in the standard assay in the presence of pyridoxal phosphate, was 2.1; in the absence of pyridoxal phosphate, the specific activity was 0.1. The initial specific activity was 5.7.

not subject to multivalent repression by leucine, isoleucine, and valine as reported for the isoleucine-inhibitable threonine deaminases from *E. coli* and *Salmonella typhimurium* (5).

Growth of threonine deaminase-deficient strains on threonine. In addition to the above cited differences in constitutive activity, the *P. multivorans* enzyme differs from the isoleucine-sensitive threonine deaminases of *E. coli* and *S. typhimurium* with respect to the number of binding sites for threonine and isoleucine, as estimated from kinetic studies and the ability of the enzyme to dissociate to subunits which can be reassociated to active enzyme. It seemed reasonable that these unusual properties might be related to the ability of *P. multivorans* to utilize threonine as sole carbon source and that possibly the enzyme might serve both biosynthetic and catabolic functions. To test this possibility, mutants deficient in threonine deaminase were isolated. Three independently isolated, threonine deaminase-deficient strains of *P. multivorans* 249, numbers 12, 13, and 14, grew as well or better than the wild type in media containing threonine as sole carbon source provided that such media were supplemented with isoleucine (Table 5). The mutants did not grow in either citrate or threonine media in the absence of isoleucine. It can be seen that the levels of threonine deaminase in mutants were less than 2% of that in the wild type. The results clearly indicate that threonine deaminase is not essential for growth of *P. multivorans* on threonine.

TABLE 4. *Constitutive formation of threonine deaminase during growth in different media*

Strain	Supplements added to basal salts medium[a]	Growth rate constant (hr⁻¹)[b]	Threonine deaminase (units/mg of protein)
249	Citrate	0.65	0.43
	Threonine	0.13	0.30
	Citrate + isoleucine, leucine, and valine	0.69	0.38
	Threonine + isoleucine, leucine, and valine	0.14	0.34
	Citrate + yeast extract	1.03	0.40
249-4	Citrate + leucine (50 μg/ml	0.70	0.42
	Citrate + leucine (10 μg/ml)	—	0.30

[a] Final concentrations of citrate or L-threonine were 0.3%. Isoleucine, leucine, and valine were each added to a concentration of 50 μg/ml.

[b] Determined as ln 2/mass doubling time. Growth was at 37 C. The culture of strain 4 containing 10 μg of L-leucine per ml was sampled 60 min after leucine depletion. The culture density was equivalent to 1.1 mg of protein per ml.

TABLE 5. *Growth of threonine deaminase-deficient strains on threonine*

Strain	Supplements added to basal salts medium[a]	Growth rate constant (hr⁻¹)[b]	Threonine deaminase (specific activity[c])
249	Citrate + isoleucine	0.50	0.450
	Threonine + isoleucine	0.13	0.402
249-12	Citrate + isoleucine	0.59	<0.005
	Threonine + isoleucine	0.15	<0.005
249-13	Citrate + isoleucine	0.55	<0.002
	Threonine + isoleucine	0.21	<0.003
249-14	Citrate + isoleucine	0.52	<0.005
	Threonine + isoleucine	0.18	<0.005

[a] Final concentration of L-isoleucine was 2×10^{-4} M.

[b] Determined as ln 2/mass doubling time at 35 C.

[c] Expressed as micromoles of α-ketobutyrate per minute per milligram of protein at 30 C.

Inability of threonine dehydrogenase-deficient strains of P. multivorans to grow on threonine. Adaptation of *P. multivorans* to growth on threonine was associated with the appearance of threonine dehydrogenase activity in the cultures. It can be seen from the results of Table 6 that the steady-state levels of threonine dehydrogenase in threonine-grown bacteria were approximately 12-fold higher than in citrate-grown bacteria. Enzyme activity was measured both in terms of nicotinamide adenine dinucleotide (NAD) reduction and the formation of aminoacetone. The latter was formed by decarboxylation of β-keto α-aminobutyrate, the immediate product of threonine dehydrogenase action. No activity was detected when nicotinamide adenine dinucleotide phosphate was substituted for NAD. The apparent K_m of the enzyme with respect to threonine was 2×10^{-3} M. It should be noted that this value is about 10% the concentration of threonine required to give half-maximal activity of threonine deaminase under similar conditions. However, at saturating concentrations of threonine for both enzymes, the dehydrogenase activity was fourfold lower than that of threonine deaminase.

When the cultures were grown in media containing both citrate and threonine, the levels of threonine dehydrogenase were intermediate be-

TABLE 6. *Levels of threonine dehydrogenase in strains 249, 249-17, and 18*

Strain	Supplements added to basal salts medium[a]	Threonine dehydrogenase specific activity[b]	
		I	II
249	Threonine	0.064	0.050
	Citrate	0.005	0.004
	Threonine + citrate	0.032	0.024
249-17	Threonine + citrate	0.003	0.003
249-18	Threonine + citrate	0.002	0.002

[a] Final concentrations of threonine and citrate were 0.2 and 0.3%, respectively.

[b] Expressed as micromoles of reduced nicotinamide adenine dinucleotide per minute per milligram of protein (I) or micromoles of aminoacetone per minute per milligram of protein (II), measured at 24 C.

tween those observed when either substrate was present alone. We interpret the results as indicating that threonine dehydrogenase is induced by threonine and that it is subject to catabolite repression in the presence of citrate.

The results suggested that threonine dehydrogenase, rather than threonine deaminase, might play the primary role in threonine catabolism. This was confirmed by the finding that two different threonine dehydrogenase-deficient mutants of *P. multivorans*, 249-17 and 249-18, were unable to utilize threonine as sole source of carbon. Table 6 shows that the levels of threonine dehydrogenase activity in these strains were less than 10% of the level found in the wild type grown under similar conditions. The levels of threonine deaminase were normal in the cultures of the mutants (*results not shown*), indicating that conversion of threonine to α-ketobutyrate by threonine deaminase does not serve as an alternative route of threonine catabolism.

It is not clear at present whether β-keto-α-amino butyrate might be further metabolized via acetyl coenzyme A and glycine, as found for *Arthrobacter* (14), or via aminoacetone, methylglyoxal, and pyruvate as postulated by Turner et al. for an unidentified Pseudomonad (10).

DISCUSSION

The general properties of threonine deaminase from *P. multivorans* resemble those of other isoleucine-inhibitable threonine deaminases which fit the description of a *K* system as outlined by Monod, Wyman, and Changeux (17). However, a number of features of the *P. multivorans* enzyme distinguish it from the other threonine deaminases of this type. First, the enzyme is relatively stable compared to the enzymes from *E. coli*, *S. typhimurium*, and *R. spheroides*, and can be reversibly dissociated to subunits and reassociated without appreciable loss of activity. Second, the apparent orders of binding of threonine and isoleucine to the *P. multivorans* enzyme are higher than those reported for the other enzymes. For example, kinetic data for the *Salmonella* enzyme (1) suggest two sites each for threonine and isoleucine, whereas data for the *P. multivorans* enzyme indicate at least three sites for threonine and four for isoleucine. Whether these values represent an actual difference in the number of binding sites for threonine and isoleucine or differences in the extent of nonexclusive binding of these ligands to different forms of the enzyme remains to be determined. It should be noted that the kinetic data for the isoleucine-inhibitable threonine deaminase from yeast also suggest more than two binding sites each for threonine and isoleucine (4).

Purified preparations of the *Samonella* enzyme were found to contain two pyridoxal phosphate residues per mole of enzyme (1), leading to the conclusion that there are two active sites on the enzyme. In this regard, it is of interest that the purest preparations of the *P. multivorans* enzyme had about twice the specific activity (850 to 1,000) of pure preparations of the *Salmonella* enzyme. (These values apparently represent the highest specific activities reported for a microbial threonine deaminase of the isoleucine inhibitable type.) Whether the high activity of the *P. multivorans* preparations represents a greater inherent activity or a difference in the number of active sites remains to be determined. In this regard, it will be of particular interest to determine the amount of pyridoxal phosphate in the *P. multivorans* enzyme.

The results of experiments describing the sedimentation properties of the *P. multivorans* threonine deaminase suggest that the enzyme is an oligomer of 192,000 molecular weight consisting of four subunits of about 49,000 molecular weight. The sedimentation behavior of the oligomeric and dissociated forms of the enzyme is similar to that reported for the associated and dissociated forms of *S. typhimurium* threonine deaminase (28). However, the *Salmonella* enzyme has not been reported to dissociate reversibly to subunits without loss of enzyme activity.

The studies of *Salmonella* threonine deaminase indicate that the enzyme consists of four identical subunits (28). As indicated above, only two of these appear to possess active sites, suggesting that the four subunits may not be functionally equivalent. In view of this possibility, further studies of the association and dissociation of the

P. multivorans enzyme may contribute to an understanding of whether the assembly of subunits influences the catalytic and regulatory properties of the fully associated enzyme.

It is not certain at the present time whether the differences in properties of the *P. multivorans* enzyme compared to other bacterial threonine deaminases are related to the ability of *P. multivorans* to use threonine as sole carbon source. It is clear, however, from the studies of threonine deaminase- and threonine dehydrogenase-deficient strains that the deaminase does not play a direct role in threonine catabolism and that threonine dehydrogenase is essential for the growth of *P. multivorans* on threonine. Our studies showed that the in vitro affinity of the latter enzyme for threonine was considerably greater than that of threonine deaminase. This suggests that threonine dehydrogenase might tend to divert threonine away from isoleucine biosynthesis in vivo. Possibly, the unusual properties of the *P. multivorans* threonine deaminase permit the enzyme to produce α-ketobutyrate in the presence of threonine dehydrogenase.

ACKNOWLEDGMENTS

H. R. Whiteley is a recipient of Research Career Award CM-K6-422.

During part of the work, T. G. Lessie held a Postdoctoral Fellowship in the Department of Microbiology, University of Washington, Seattle.

This investigation was supported by Public Health Service grants CA-03931 from the National Cancer Institute and AM-12163 from the National Institute of Arthritis and Metabolic Diseases, and by grant GB 7456 from the National Science Foundation.

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