L-Threonine Deaminase of *Rhodospirillum rubrum*

Purification and Characterization

Ross S. FELDBERG and Prasanta DATTA

Department of Biological Chemistry, The University of Michigan, Ann Arbor

A procedure for the purification of threonine deaminase from *Rhodospirillum rubrum* is described. The 2200-fold purified enzyme was judged to be pure by disc-gel electrophoresis and ultracentrifugation.

Sedimentation velocity centrifugation yielded an $s^{0}_{20,w}$ value of 8.1 S and a $D_{20,w}$ of 4.65×10^{-7} cm$^2 \times$ sec^{-1}. Assuming a partial specific volume of 0.74 g/ml the molecular weight of 164000 was calculated from the Svedberg equation. However, from calibrated Sephadex G-200 columns, a $D_{20,w}$ of 4.15×10^{-7} cm$^2 \times$ sec^{-1} was obtained and using this equation, the molecular weight was calculated to be 180000.

Electrophoresis of the enzyme on polyacrylamide gels in the presence of sodium dodecyl sulfate revealed that the native enzyme was composed of four subunits, each of about 46000 molecular weight. In addition to the native tetrameric molecule, an active octameric species was detected in polyacrylamide gel electrophoresis.

The spectrum of the pure enzyme showed absorption maxima at 279 nm and 412 nm, characteristic of pyridoxal 5'-phosphate-containing enzymes. Analysis of the cofactor content revealed the presence of 4.2 moles of pyridoxal phosphate per 180000 g of protein.

The enzyme displayed a normal Michaelis-Menten substrate saturation curve in the absence of isoleucine. High concentrations of isoleucine inhibited the enzyme activity somewhat only at low levels of the substrate; the inhibition was weak as compared to all other biosynthetic threonine deaminases. No other amino acid or any nucleotide tested produced any significant activation or inhibition.

In assays at pH values greater than 8.4, a transient build up of a 245 nm absorbing species was observed. It is suggested that this species corresponds to one of the two intermediates of the deamination reaction, α-aminocrotonate or α-iminobutyrate, and that at high pH the rate-limiting step in the reaction is the hydrolysis of the intermediate to the final products, 2-oxo-butyrate and ammonia.

Depending on the metabolic demands of the cell, the non-oxidative deamination of L-threonine to 2-oxobutyrate and ammonia can serve two physiological functions. In *Clostridium tetanomorphum* [1] and in anaerobically-grown *Escherichia coli* [2] the degradation of threonine to 2-oxobutyrate appears to be involved in energy production in the cell. The deaminases isolated from these organisms, activated by ADP and AMP respectively, are thus referred to as the "biodegradative" enzymes. In all other bacteria examined, the deamination of threonine is the first reaction unique to the biosynthesis of the branched-chain amino acid L-isoleucine, and the deaminase is subject to feedback inhibition by this end product. Accordingly, enzymes belonging to this class are designated as "biosynthetic" threonine deaminases. Although both biosynthetic and biodegradative enzymes catalyze the same over-all reaction, in view of their diverse metabolic roles it is not surprising that these deaminases differ in their structural and regulatory features as indicated by recent studies on the purified deaminases from *E. coli* [3,4], *C. tetanomorphum* [5], *Salmonella typhimurium* [6] and *Bacillus subtilis* [7].

Two important considerations prompted our study of the threonine deaminase from *Rhodospirillum rubrum*. First, as reported by Hughes *et al.* [8], this enzyme is neither activated by AMP or ADP nor inhibited significantly by L-isoleucine.

Enzymes. L-Threonine deaminase has also been referred to as threonine dehydrase and threonine dehydratase. The systematic name is L-threonine hydro-lyase (deaminating) (EC 4.2.1.16). Other enzymes used were malic dehydrogenase (EC 1.1.1.37); yeast alcohol dehydrogenase (EC 1.1.1.1.); catalase (EC 1.11.1.6); peroxidase (EC 1.11.1.7); fumarate hydratase or fumarase (EC 4.2.1.2); and xanthine oxidase (EC 1.2.3.2).

Thus, the *R. rubrum* threonine deaminase lacks the distinguishing features of either the biosynthetic or the biodegradative enzyme, raising some important questions as to its physiological role in the bacterium. Secondly, the enzyme from *R. rubrum* shows the unusual property of cold-inactivation. Insofar as the phenomenon of cold inactivation reflects the weakening of intersubunit bonds in the protein, an understanding of this property could lead to some insight into the subunit-subunit interactions implicated in regulatory enzymes. In this communication we report the purification and characterization of the threonine deaminase from *R. rubrum*. The accompanying paper describes the phenomenon of cold inactivation of this enzyme.

EXPERIMENTAL PROCEDURES

Materials

L-Threonine (*allo*-free), L-isoleucine and phenazine methosulfate were obtained from Calbiochem. Other amino acids, pyridoxal 5'-phosphate, 2-oxobutyrate, protamine sulfate, dithiothreitol, nitro blue tetrazolium, and sodium dodecyl sulfate were supplied by Sigma Chemical Company. L-Glutamic acid and enzyme-grade ammonium sulfate were purchased from Mann Laboratories. The reagents for polyacrylamide gel electrophoresis were obtained from Eastman Organic Chemicals. Bovine serum albumin, malic dehydrogenase and lactic dehydrogenase (Beef heart; Type III) were obtained from Sigma. Catalase, yeast alcohol dehydrogenase and peroxidase were supplied by Worthington. Fumarase and xanthine oxidase were gifts of Dr. V. Massey. Sephadex G-25 and G-200 were products of Pharmacia. Calcium phosphate gel, prepared by the method of Swingle and Tiselius [9] was a gift of Dr. V. Massey. All other chemicals were of reagent grade.

Bacteria

Rhodospirillum rubrum S1H (ATCC 25903), derived from the strain S1, was obtained from Dr. H. Gest. This organism produces 15- to 20-fold higher levels of threonine deaminase compared to the wild type strain S1 [10]. Cultures were maintained photosynthetically under anaerobic conditions, in the malate-ammonium sulfate medium of Ormerod *et al.* [11]. For large scale growth, bacteria were grown at 25 °C aerobically in the dark in the defined malate medium [11] with 0.1 % L-glutamate as the nitrogen source. The cells were harvested after 64 to 72 h and washed once with 0.10 M potassium phosphate buffer, pH 7.0, containing 1 mM L-isoleucine and 0.10 mM dithiothreitol. The cell pellet, with an overlayer of buffer, was stored at − 15 °C in 50-g portions.

Threonine Deaminase Assays

Threonine deaminase activity was routinely assayed by the spectrophotometric method of Davis [12]. The rate of production of keto acid was measured at 25 °C by the increase in absorbance at 230 nm in a cuvette with a 1 cm light path using a Gilford model 2000 spectrophotometer. The standard reaction mixture contained 100 μmoles potassium phosphate buffer, pH 7.8, 50 μmoles L-threonine and enzyme in a final volume of 1.0 ml. Under the conditions of the assay, the absorbance of an authentic sample of 2-oxobutyrate at 230 nm was 540 $M^{-1} \times cm^{-1}$, and that of pyruvate was 630 $M^{-1} \times cm^{-1}$. One unit of enzyme was defined as the amount of enzyme required to form 1 μmole of 2-oxo acid in 1 min at 25 °C. The specific activity was defined as the μmoles of product formed $\times min^{-1} \times mg$ of protein^{-1}.

When enzyme activity was measured in the presence of adenine nucleotides, a coupled assay with lactic dehydrogenase was employed [13]. The assay mixture contained, in μmoles: potassium phosphate buffer, pH 7.8, 100; L-threonine, 50; NADH, 3.3; 50 μg crystalline beef heart lactic dehydrogenase, and a rate-limiting amount of threonine deaminase in a final volume of 1.0 ml.

A third assay for the enzyme is based on the formation of the 2,4-dinitrophenylhydrazone of the oxo acid and is a modification [14] of the method of Friedemann and Haugen [15]. The reaction mixture was identical to that used for the spectrophotometric assay.

Polyacrylamide Gel Electrophoresis

Electrophoresis was carried out in 7.5 % acrylamide gels at pH 9.3 according to the method of Davis [16]. Protein was detected by staining the gels in 1 % amido black in 7 % acetic acid for 1 h. The gels were destained electrophoretically. Threonine deaminase activity on the gels was detected by the method of Feldberg and Datta [17].

Electrophoresis of sodium dodecyl sulfate-denatured enzyme was carried out on a 10 % acrylamide gel in the presence of the detergent by the method of Shapiro *et al.* [18], as given in detail by Weber and Osborn [19]. To shorten the electrophoresis time, the gel buffer was diluted to have the same concentration as the electrophoresis buffer. After electrophoresis, the gels were stained for 2 h in the Coomassie brilliant blue-methanol-acetic acid stain of Weber and Osborn [19], and destained by gentle shaking in the methanol-acetic acid destaining solution for several days.

Absorption Spectrum

The absorption spectrum of the enzyme was determined on a Cary 14 recording spectrophotometer with a 0−1.0 absorbance slidewire.

Analytical Centrifugation

Sedimentation and diffusion studies were carried out in the Spinco model E ultracentrifuge equipped with a RTIC temperature control unit and schlieren optics. All experiments were performed at 20 °C and the sedimentation and diffusion coefficients obtained were corrected to the "standard condition" of water. Sedimentation velocity centrifugation was performed in a single-sector cell with the AND rotor at a speed of 50740 rev./min. The diffusion coefficient was determined using the double-sector synthetic boundary cell with the AND rotor at a speed of 12590 rev./min.

Gel Filtration on Calibrated Sephadex G-200

The molecular weight of threonine deaminase was estimated on a calibrated Sephadex G-200 column by the method of Andrews [20]. Xanthine oxidase was assayed by the method of Kalckar [21], alcohol dehydrogenase, by the method of Vallee and Hoch [22], fumarase, as described by Racker [23], catalase as given by Beers and Sizer [24], and peroxidase, by the rate of color development at 460 nm using o-dianisidine as the hydrogen donor [25].

Pyridoxal Phosphate Determination

The pyridoxal phosphate content of the enzyme was determined by the fluorimetric method as described by Adams [26].

Protein Determination

Throughout the enzyme purification procedure, the concentration of protein was estimated by the spectrophotometric method of Warburg and Christian [27]. The concentration of the pure enzyme, determined by the method of Lowry et al. [28], with the modification that the solution B contained 1% sodium citrate rather than 1% sodium tartrate, gave a value 2.5-fold higher than that obtained by the method of Warburg and Christian.

Enzyme Purification

Unless otherwise noted, all operations were carried out at room temperature, except for centrifugations which were performed at 4° C. The buffers used were: buffer A, 50 mM potassium phosphate buffer, pH 7.0; buffer B, 3 mM potassium phosphate buffer, pH 6.15, and buffer C, 3 mM potassium phosphate buffer, pH 6.95. All three buffers also contained 1 mM L-isoleucine and 0.10 mM dithiothreitol.

Extraction. Frozen cell paste (350 g) was allowed to thaw and was suspended in buffer A in a total volume of 700 ml. After adding 28 ml of 1 N KOH, the cell suspension was chilled in a NaCl-ice bath and sonically disrupted for six 1-min pulses using a Branson Sonifier Cell Disruptor. The extract was adjusted to pH 6.8, and centrifuged at $45000 \times g$ for 40 min. The supernatant solution was used in the next step.

Protamine Sulfate Treatment. Protamine sulfate, dissolved in buffer A to give a 3—5% solution, was added dropwise to the extract over a 30-min period until a protamine sulfate to protein ratio of 0.15 mg per mg was attained. The suspension was centrifuged at $48000 \times g$ for 120 min and the clear supernatant fluid obtained was adjusted to pH 6.8.

Heat Treatment. The supernatant fluid from the previous step was heated in 125-ml portions to 54 °C and maintained at that temperature for 4 min. After heating the flasks were chilled rapidly and the suspension was centrifuged at $10000 \times g$ for 30 min. The supernatant fluid was usually frozen overnight at this stage with no loss in activity.

Ammonium Sulfate Fractionation. The supernatant fluid from the heat treatment step was diluted with buffer A to 1400 units of enzyme activity per ml and adjusted to pH 7.1. Solid ammonium sulfate (14.5 g per 100 ml of solution) was added to the solution at 4 °C, without pH adjustment, over a 60-min period. The suspension was centrifuged at $10000 \times g$ for 15 min and the precipitate was discarded. The pH of the supernatant fluid was adjusted to pH 6.0 with 1 M H_3PO_4 and the solution was allowed to warm up to 30 °C over a 45-min period. The precipitate formed was collected by centrifugation at $10000 \times g$ for 10 min and was dissolved in a small volume of buffer B. Insoluble material was removed by centrifugation at $30000 \times g$ for 10 min, and the clear solution was desalted immediately on a Sephadex G-25 column $(20 \times 2.8$ cm) equilibrated with buffer B. The fractions containing the bulk of the enzyme activity were pooled and stored frozen overnight.

Calcium Phosphate Gel Chromatography. Calcium phosphate gel (35 ml; 30 mg/ml) was mixed with 150 ml of a 7% suspension of Whatman CF-1 cellulose powder in buffer B. The slurry was deaerated and poured into a column 14×1.9 cm. The column was equilibrated with buffer B at 25 °C and the desalted enzyme was applied to the column at a flow rate of 75 ml per hour. The column was washed with buffer B until no further 280 nm absorbing material was eluted. Elution of the enzyme was carried out with buffer C. Fractions containing maximum enzyme activity were concentrated to 4.0 ml using a Schleicher & Schüll Collodion bag ultrafiltration apparatus and the enzyme was stored frozen overnight.

Preparative Gel Electrophoresis. The method used for analytical polyacrylamide gel electrophoresis was scaled up for the preparative step. The separating gel was 7 cm in height while the stacking gel was 2 cm in height. The gel was subjected to pre-electrophoresis for 30 min in the standard electrophoresis buffer containing 0.5 mM L-isoleucine. The enzyme from the

Table. *Purification of threonine deaminase from* R. rubrum

Step	Volume	Protein	Total activity	Specific activity
	ml	mg	units [b]	units/mg
Sonic extract	600	73 800	10 260	0.14
Protamine sulfate	650	13 000	11 560	0.89
Heat treatment	645	8 400	10 980	1.31
Ammonium sulfate	14	105	7 390	70.5
Calcium phosphate gel eluate (concentrated)	4.1	27	5 920	219
Preparative gel electrophoresis and DEAE column	5.0	11.5 [a]	3 400	309

[a] Protein concentration was estimated by the method of Lowry *et al.* [28].

[b] μmoles of 2-oxobutyrate formed per min at 25 °C.

previous step was mixed with 0.3 ml of bromophenol blue and 100—200 mg of sucrose, and was applied to the stacking gel. Electrophoresis was started at 4—5 mA until the dye had passed through the stacking gel and was continued at 10—15 mA for 4—6 h. Two yellow bands were visible on the gel: a major band, and a minor band migrating about 1 cm behind it. The section containing the major yellow band was sliced from the gel and was homogenized in 2 ml of buffer A. The resulting suspension was filtered through a layer of glass wool. The filtrate was centrifuged at 40 000 × *g* for 10 min.

Removal of Acrylamide. To remove the last traces of acrylamide, the supernatant fluid from the previous step was applied to a DEAE-cellulose column (2 × 2.2 cm) equilibrated with buffer A. The column was washed with the buffer until the absorbance at 225 nm was negligible, and the enzyme was eluted with buffer A containing 0.3 M KCl. The enzyme solution was concentrated to 5 ml using the Collodion bag apparatus and dialyzed overnight against 2 l of buffer A.

The purified enzyme was stored at −15 °C in small aliquots and was stable for 2—3 months. A summary of the purification procedure, given in the Table, shows that a 2200 purification was obtained with an over-all yield of 30%.

RESULTS

Criteria for Purity

Electrophoresis of the purified enzyme on analytical polyacrylamide gels revealed the presence of a major and a minor protein band in the same relative positions as the two yellow bands observed on the preparative gel. As shown in Fig.1, both bands were enzymatically active with either L-threonine or L-serine as substrate, indicating that the enzyme preparation was of high purity.
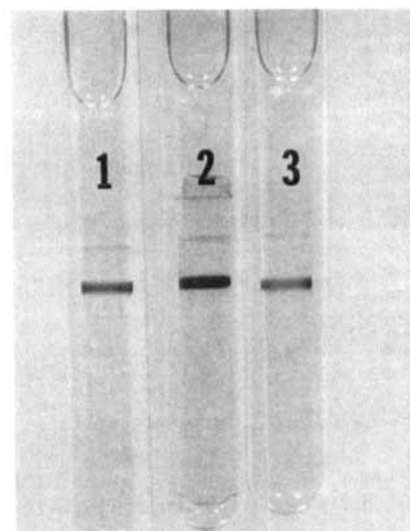

Fig. 1. *Polyacrylamide gel electrophoresis of the purified threonine deaminase.* Disc-gel electrophoresis was carried out in 7.5% polyacrylamide gels at pH 9.3. Each gel contained 45 μg of protein. Gel 1 was stained for threonine deaminase activity with serine as substrate; gel 2 was stained for protein with amido black and gel 3 was stained for activity with threonine as substrate
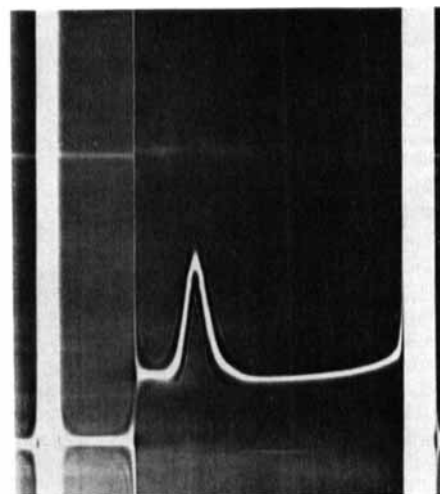

Fig. 2. *Sedimentation velocity pattern of threonine deaminase.* Purified threonine deaminase (4.1 mg/ml) in 50 mM potassium phosphate buffer, pH 6.8, supplemented with 1 mM L-isoleucine and 0.1 mM dithiothreitol was sedimented in a single sector cell at 20 °C. Photograph was taken after 25 min at 50 740 rev./min at a bar angle of 52 °C. Sedimentation is from left to right

Additional evidence for the purity of the enzyme is the schlieren pattern obtained during sedimentation velocity centrifugation. Fig.2 shows that the enzyme sedimented as a single, symmetrical component in the ultracentrifuge.
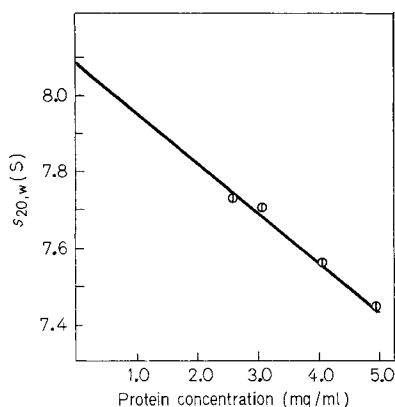
Fig.3. *Dependence of sedimentation coefficient on protein concentration.* Sedimentation velocity centrifugation was carried out under the conditions described in Fig.2 at four different protein concentrations
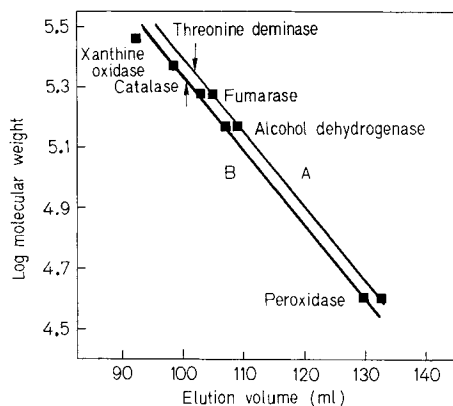

Fig.4. *Gel filtration on calibrated Sephadex G-200 columns.* The samples were applied to the columns (72 × 1.7 cm) in 1.0 ml total volumes containing 500 μg peroxidase, 600 μg yeast alcohol dehydrogenase, 350 μg fumarase, 250 μg threonine deaminase and, in experiment B, 60 μg catalase and 200 μg xanthine oxidase in addition. Column A was eluted with 25 mM potassium phosphate buffer, pH 7.5 supplemented with 1 mM EDTA and 0.5 mM dithiothreitol; column B, the same buffer supplemented with 0.2 M KCl. The enzymes were located by assaying for their activities

PHYSICAL PROPERTIES

Molecular Weight of the Enzyme

Sedimentation coefficients determined at four protein concentrations were plotted as a function of protein concentration (Fig.3), and the extrapolation of this line to zero protein concentration gave an $s^{\circ}_{20,w}$ of 8.1 S.

The diffusion coefficient, determined at a protein concentration of 4.7 mg/ml, was 4.65×10^{-7} cm^2 × sec^{-1}

From these two values, and an assumed partial specific volume for the protein of 0.74 ± 0.01 g/ml, the molecular weight of the enzyme was calculated to be 164000 ± 5000.

From the elution profiles of threonine deaminase and several standard proteins on Sephadex G-200 columns using two different buffers (Fig.4), a molecular weight of 220000 was determined for the *R. rubrum* threonine deaminase. This value was significantly higher than the 164000 molecular weight determined by centrifugation. Since the elution volume from gel filtration is actually dependent on the Stokes radius of a protein [29,30], the data from Fig.4 were employed to calculate a Stokes radius for the enzyme of approximately 5 nm. Using this value with the Stokes-Einstein equation [30], a $D_{20,w}$ for the protein was determined to be 4.15×10^{-7} cm^2 × sec^{-1}. Using this value for the diffusion coefficient, with the $s^{\circ}_{20,w}$ of 8.1 S, the molecular weight of the enzyme was calculated to be 180000.

One possible explanation for the higher apparent molecular weight of the enzyme on Sephadex is that there is a rapid equilibrium between the native enzyme and a higher molecular weight species.

In view of the above results, the exact molecular weight of the enzyme can not be unambiguously established. As a preliminary estimate, we have assumed a molecular weight of 180000; this value is in agreement with the results of sodium dodecyl sulfate-acrylamide gel electrophoresis given below.

Molecular Weight of the Subunit

To determine the number and molecular weight of its subunits, the purified threonine deaminase was subjected to electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate. The enzyme displayed a single band corresponding to a subunit molecular weight of approximately 46000 (Fig.5). This result indicates that the subunits of the enzyme are of equal size, that the native enzyme is composed of four subunits, and that the native enzyme has a molecular weight of approximately 184000. However, since the accuracy of this method is about $\pm 10^{0}/_{0}$, the molecular weight of the native tetramer could range from 165000 to 200000.

Characterization of the Two Enzyme Species on Acrylamide Gels

The reappearance of two catalytically active protein bands on polyacrylamide gels even though only the major band had been eluted from the preparative gel suggests that the minor slow moving species was derived from the major fast moving component. To determine the nature of the minor active species the enzyme was subjected to electrophoresis on poly-
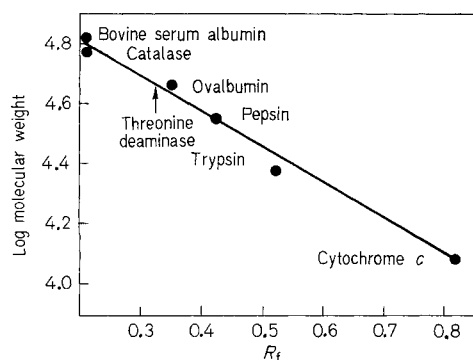
Fig.5. *Determination of the subunit molecular weight of threonine deaminase by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels.* All proteins were run on duplicate gels. The mobility of each protein was measured relative to the migration of the tracking dye, bromophenol blue. For details, see Methods section
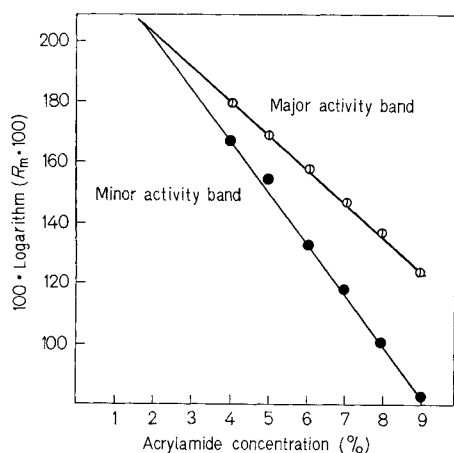

Fig.7. *Absorption spectrum of L-threonine deaminase.* The enzyme concentration was 1.6 mg/ml in 25 mM potassium phosphate buffer, pH 6.8. A 1.0 cm light path was used

Fig.6. *The effect of acrylamide concentration on the mobility of threonine deaminase.* Disc-gel electrophoresis was carried out using the standard stacking gel with separating gels of varying acrylamide concentration. 20 μg of threonine deaminase were applied to each gel and electrophoresis was carried out until the tracking dye had reached the bottom of the 4% gel. The dye position in each gel was marked with a of fine copper wire and the gels were stained for threonine deaminase activity. The mobilities of the activity bands were measured relative to the dye front

about 400000 molecular weight. It thus appears that under the conditions of electrophoresis it is possible for the native enzyme to dimerize to produce an active 400000 molecular weight species.

Absorption Spectrum

The absorption spectrum of threonine deaminase in 0.025 M potassium phosphate buffer, pH 6.8, is given in Fig.7. An identical spectrum was obtained at pH 8.0. The ratio of the absorbance at 278 nm to that at 412 nm was 2.9; this value is 3 to 4 times lower than that reported for the *S. typhimurium* enzyme. This finding is consistent with the observation that the spectrophotometric method of protein determination underestimated the protein concentration of pure enzyme when compared to the protein concentration determined by the method of Lowry *et al.*

The 412 nm absorption maximum is characteristic of enzymes containing pyridoxal phosphate as a hydrogen-bonded Schiff base, and has been found with all other threonine deaminases examined [3—7].

acrylamide gels of varying cross-linkage according to the method of Hedrick and Smith [31]. The dependence of the mobility of the two active bands on the acrylamide concentrations is shown in Fig.6. Using the standard curve given by Hedrick and Smith, it was found that the major active band corresponded to a species of approximately 200000 molecular weight. The minor active band migrated as a species of
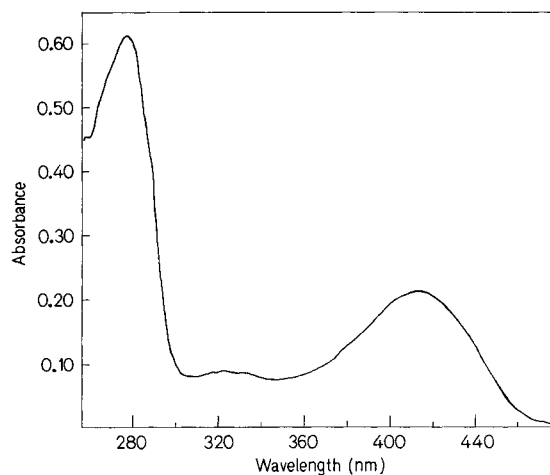
Pyridoxal Phosphate Content

The activity of the purified enzyme was found to be independent of added pyridoxal phosphate. Furthermore, dialysis against 0.05 M Tris-HCl pH 9.0, containing 0.01 M KCl and 1 mM L-isoleucine failed to resolve the cofactor.

Analysis of the pyridoxal phosphate content of the native enzyme revealed 4.2 moles of pyridoxal
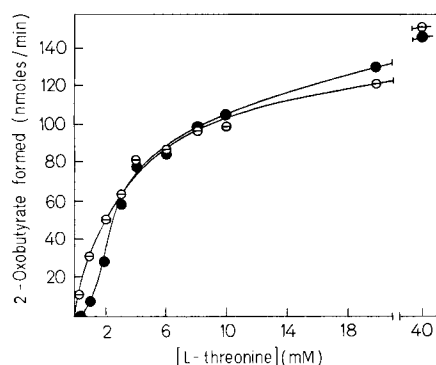
Fig.8. *Dependence of enzyme activity on L-threonine in the presence and absence of isoleucine.* Assays were carried out in 100 μmoles potassium phosphate buffer, pH 7.9 in a total volume of 1.0 ml. No isoleucine present (⊖), 10 mM L-iso-leucine present during assay (●)

5'-phosphate per 180000 g of protein. (Based on 164000 g, this value becomes 3.8 moles of cofactor.)

CATALYTIC PROPERTIES

Like all other threonine deaminases studied, the *R. rubrum* enzyme catalyzed the deamination of L-serine as well as of L-threonine, and the deamination of L-serine resulted in the inactivation of the enzyme.

Substrate Saturation

The substrate saturation curves for threonine in the presence and absence of 10 mM L-isoleucine are shown in Fig. 8. The Lineweaver-Burk plot (not shown) in the absence of isoleucine was linear and an apparent K_m for threonine of 6.8 mM was calculated. In agreement with the findings of Hughes *et al.* [8], a significant inhibition of enzyme activity by isoleucine was observed only at threonine concentrations below 3 mM. No differences in the sensitivity of the enzyme to isoleucine were observed when the threonine saturation curves were obtained in potassium phosphate buffer, pH 7.0, or Tris-HCl buffer, pH 8.5. It should be emphasized that the enzyme isolated from *R. rubrum* strain Sl also showed the same inhibition characteristics by L-isoleucine [8].

Effect of Amino Acids and Nucleotides on Activity

The following compounds did not inhibit the enzyme activity significantly (*i.e.*, less than 15%) when assayed with 5 mM L-threonine: L-isoleucine (10 mM), L-valine (10 mM), L-methionine (10 mM), L-aspartate (10 mM), L-lysine (10 mM), ATP (5 mM),
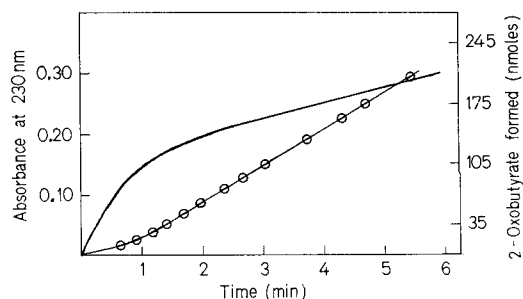

Fig.9. *Absorbance increase at 230 nm and 2-oxobutyrate formation at pH 8.9.* The increase in absorbance at 230 nm was followed in an assay mixture containing 100 μmoles potassium borate, pH 8.9, 20 μmoles L-threonine and enzyme in a volume of 1.0 ml. The linear secondary rate was equivalent to the formation of 48 nmoles 2-oxobutyrate per minute. The increase in 2-oxobutyrate was followed by the formation of the dinitrophenylhydrazone derivative. The assay mixture contained 200 μmoles of potassium borate buffer, pH 8.9, 40 μmoles L-threonine and enzyme in a total volume of 2.0 ml. Aliquots of 100 μl were removed from the assay mixture at various times and were assayed as described under Methods. The final slope was equivalent to the formation of 42 nmoles of 2-oxobutyrate per minute. ——, absorbance at 230 nm; O, 2-oxobutyrate formed

ADP (2.5 mM), AMP (5 mM), and the combinations, ATP + isoleucine, lysine + methionine + isoleucine, and valine + leucine + isoleucine.

High pH Kinetics

In Tris-phosphate or potassium borate buffers at pH values greater than 8.4, the enzyme displayed unusual kinetics when assayed spectrophotometrically. Upon initiation of the reaction by the enzyme, a rapid, transient increase in the absorbance at 230 nm, followed by a slower, linear increase, was observed. Simultaneous measurement of the rate of formation of 2-oxobutyrate by a modification [14] of the method of Friedemann and Haugen [15], revealed that this "burst" was associated with a lag in the production of 2-oxobutyrate (Fig.9). A similar lag in product fromation at high pH was noted by deRobichon-Szul-majster and Magee [32], using the lactic dehydro-genase-coupled assay with the enzyme from *Saccharomyces cerevisiae*.

The nature of this curve, showing an initial rapid increase in absorbance, followed by a slower linear rate, is typical of a pre-steady state build-up of an intermediate that has a higher absorbance at 230 nm than does the product of the reaction, 2-oxobutyrate. From the proposed mechanism for threonine deaminase [33], the increase in initial absorbance might be due to the accumulation of either α-iminobutyrate, or its tautomer, α-aminocrotonate. Indeed, measure-

ment of the extent of the initial "burst" at several wavelengths showed λ_{max} for the burst at 245 nm.

DISCUSSION

The characterization of the pure threonine deaminase from *R. rubrum* has enabled us to compare the properties of this enzyme with the other pure deaminases. The *R. rubrum* enzyme has a molecular weight of approximately 180000, in comparison to the biodegradative enzymes of *C. tetanomorphum* and *E. coli* which have molecular weights of about 150000 and the biosynthetic enzymes from *S. typhimurium* and *B. subtilis* with molecular weights of 190000 to 200000. All five enzymes are composed of four subunits, but the pyridoxal phosphate content of the *S. typhimurium* enzyme was only 2 moles of cofactor per mole of enzyme, whereas a value of 4 moles of cofactor per mole of enzyme was found with the *R. rubrum* and *C. tetanomorphum* enzymes. Unlike the biodegradative enzymes which show a ready interconversion between active protomeric and oligomeric forms, the *R. rubrum* threonine deaminase is dissociated only under conditions of cold-inactivation (see accompanying paper) or when the protein is denatured in sodium dodecyl sulfate. The *R. rubrum* enzyme is, however, the first threonine deaminase to show association of the native enzyme to higher molecular weight species (Fig. 6).

Studies on both crude extracts and on purified deaminase of *R. rubrum* have confirmed the relative insensitivity of the enzyme toward isoleucine inhibition. No other amino acid or nucleotide, under a variety of assay conditions, showed any inhibitory or stimulatory effect on the activity of this enzyme. Considering the position of threonine deaminase as the first enzyme unique to isoleucine biosynthesis, it is difficult to rationalize this lack of rigid feedback control. However, in the context of the over-all regulation of the aspartic acid pathway in *R. rubrum* this loss of sensitivity to isoleucine inhibition can be interpreted physiologically [34].

An important kinetic finding with the *R. rubrum* enzyme is the lag in the formation of 2-oxobutyrate at pH values higher than 8.4. Using the coupled assay with lactate dehydrogeanse, deRobichon-Szulmajster and Magee [32] found that the yeast threonine deaminase displayed an initial lag in the oxidation of NADH at high pH values. With the *R. rubrum* enzyme, a direct correlation was observed between the initial "burst" of absorbance at 230 nm and a lag in the 2-oxobutyrate formation (Fig.9). These data suggested the build-up of an intermediate followed by a steady-state condition of its formation and breakdown. The λ_{max} for this intermediate was 245 nm, suggesting some degree of unsaturation in the compound. It is conceivable that the α-iminobutyrate is the compound being observed; in water

this compound would hydrolyse to form 2-oxobutyrate. It is of interest that serine, which would produce α-iminopropionate, considered to be more unstable than α-iminobutyrate, did not reveal any "burst" phenomenon when used as substrate in assays at pH 8.8. If we assume that at high pH values the rate-limiting step is no longer the dissociation of α-iminobutyrate from the enzyme, but is rather due to hydrolysis of this compound to 2-oxobutyrate, then kinetics obtained with threonine deaminases at high pH values may require reevaluation.

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R. S. Feldberg's present address:
Department of Biochemistry, University of Aberdeen
Marischal College, Aberdeen, AB9 1AS, Great Britain

P. Datta
Department of Biological Chemistry, University of Michigan
Ann Arbor, Michigan 48104, U.S.A.