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Purification and Characterization of a B_6-Independent Threonine Dehydratase from *Pseudomonas putida*†

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ABSTRACT: L-Threonine dehydratase [EC 4.2.1.16] has been purified to electrophoretic and ultracentrifugal homogeneity from *Pseudomonas putida* in 9% yield. Although the enzyme is inhibited by several common carbonyl-attacking reagents, an exhaustive search for pyridoxal phosphate failed to demonstrate the presence of significant amounts of this coenzyme. The *Pseudomonas* enzyme also differs from threonine dehydratases isolated from other sources in that it possesses a much lower specific activity toward threonine and exhibits a more acid pH optimum. It is, however, able to utilize L-serine as an alternate substrate, is strongly inhibited by L-isoleucine, and undergoes a time-dependent inactivation with serine, properties shared by most established biosynthetic-type threonine dehydratases. Preliminary evidence suggests the existence of a dehydroalanyl residue as a component of the active site. This conclusion rests on the identification of tritiated alanine from hydrolysates of enzyme which had been inactivated by reduction with NaB^3H_4. The failure to obtain a satisfactory yield of [^{3}H]alanine, however, requires that the possibility of an alternate coenzyme form not be dismissed.

The dehydration and subsequent deamination of threonine to α-ketobutyrate by threonine dehydratase [L-threonine hydrolyase (deaminating), EC 4.2.1.16] has long been recognized to require the participation of pyridoxal 5′-phosphate as coenzyme. This requirement has been deemed absolute regardless of whether the enzyme is functioning in the biosynthesis of isoleucine or in threonine catabolism. In all instances where purified threonine dehydratases have been examined, an absorption maximum in the range of 404–415 nm has been observed, indicative of an aldimine linkage between pyridoxal-P and the ϵ-amino group of a lysine residue (Umbarger, 1973).

As part of a study concerned with the biosynthesis of the α-ketobutyrate prosthetic group of urocanase in *Pseudomonas putida*, it became necessary to characterize this organism's threonine dehydratase in order to determine if its properties were consistent with an involvement in the formation of the α-ketobutyrate coenzyme. The results presented here concern only the more general properties exhibited by this enzyme; a later report will present findings having to do with the question of urocanase biosynthesis. The *Pseudomonas putida* threonine dehydratase has been found to contain no pyridoxal-P, although it shares a number of other properties characteristic of most threonine dehydratases. Presumably as a direct result of this lack of pyridoxal-P, the enzyme possesses a rather low catalytic activity. Catalysis appears to be mediated through the participation of a different coenzyme which has been tentatively suggested to be a dehydroalanine residue.

Materials and Methods

Strains and Culture Conditions. Pseudomonas putida A.3.12 was obtained from the American Type Culture Collection

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(ATCC 12633) and was maintained on nutrient agar slants at 4°. Also obtained from this source was *Saccharomyces carlbergensis* 4228 (ATCC 9080) which was kept on malt agar slants at 4°. For large scale production, *P. putida* was grown in a New Brunswick Scientific Fermacell F-130 Fermenter at 30° in a medium containing 1% NZ-amine, 0.5% yeast extract, 0.5% dibasic potassium phosphate, and 0.2% L-histidine hydrochloride in 90 l. of distilled water. The inoculum consisted of 4 l. of *P. putida* which had been grown overnight in nutrient broth. After 15 hr of growth, the cells were harvested in a refrigerated Sharples centrifuge. Yield usually approximated 5.0 g of wet cells per liter of medium. Cell paste was stored at −20° until needed.

Assay Procedures. Threonine dehydratase was assayed by following the conversion of its reaction product, α-ketobutyrate, to α-hydroxybutyrate by lactic dehydrogenase, with the corresponding oxidation of NADH to NAD^+ monitored at 340 nm. Assays were performed in a Gilford Model 2000 recording spectrophotometer at 25° in silica microcuvettes of 1-cm light path. Each reaction contained 0.1 μmol of NADH, 1 μmol of dithiothreitol, 1.5 units of lactic dehydrogenase, 25 μmol of L-threonine, 20 μmol of potassium phosphate buffer (pH 7.5), and enzyme in a total volume of 0.2 ml. The same reaction mixture minus threonine was run as a control, for crude extracts of the organism contained an active NADH oxidase. Activity is expressed as μmoles of α-ketobutyrate formed per min.

Protein was estimated by the spectrophotometric method of Groves *et al.* (1968), standardized with bovine serum albumin.

Gel Electrophoresis. The procedure of Davis (1964) was employed, with ammonium persulfate used as a polymerizing agent in the separating gel and riboflavine used in the stacking gel. Protein was stained with a 1% solution of Coomassie Blue in 10% trichloroacetic acid. The activity stain developed by Feldberg and Datta (1970) was used to locate the band corresponding to threonine dehydratase.

Sedimentation Equilibrium Ultracentrifugation. Purified threonine dehydratase, dialyzed exhaustively against 100 mM potassium phosphate (pH 7.5), containing 1 mM dithiothreitol, was placed in charcoal-filled Epon double sector cells. The reference contained buffer minus the enzyme. Ultracentrifugation was performed at 20° and 13,000 rpm using a Spinco Model E ultracentrifuge equipped with ultraviolet scanning capabilities and operated at 280 nm.

Amino Acid Analysis. A Beckman Model 119 amino acid analyzer with the capability to split the stream emerging from fractionation was employed to detect radioactive amino acids. [^{14}C]Serine and [^{14}C]glutamate were used to align the peaks on the analyzer chart to the fractions collected and to determine the splitting ratio. Fractions (1 ml) were collected and 0.5 ml was counted in a Beckman LS-200B ambient liquid scintillation counter after the addition of 10 ml of toluene–Triton X-100, 2:1 v/v, containing 5 g of diphenyloxazole per liter.

Gas–Liquid Chromatography. A Barber-Colman Model 5000 gas chromatograph equipped with a tritium monitor and a flame ionization detector was used to search for tritiated α-hydroxy acids. Phenylboronate derivatives, made by adding 100 μl of 100 mM phenylboronic acid in methanol to 10 μl of trifluoroacetic acid and 10 μl of sample (or standard) in methanol, were separated on a 3% OV-17 column at 145°. Tritiated lactate, made by reducing pyruvate with NaB^3H_4, and unlabeled α-hydroxybutyrate were used as standards.

Special Materials. Hydroxylapatite was prepared according to the method of Levin (1962). Sources of other materials were as follows: NaB^3H_4 from New England Nuclear Corp.; [1-^{14}C]-L-tyrosine from Calatomic Corp.; protamine sulfate, enzyme-grade $(NH_4)_2SO_4$, and pyridoxal-P from Schwarz/Mann; NZ-amine, Type NAK, from Sheffield Chemical Co.; lactic dehydrogenase (type II), Coomassie Blue, pyridoxamine-P, glutamic oxalacetic transaminase (type I), and tyrosine apodecarboxylase from Sigma Chemical Co.; and OV-17 from Supelco, Inc. All other materials were obtained from various suppliers and were used without further purification.

Results

Purification of Threonine Dehydratase. Step 1. Crude Extract. Frozen *Pseudomonas putida* cells (120 g) were thawed in twice their weight of 0.05 M potassium phosphate (pH 7.5), containing 0.01 M 2-mercaptoethanol (PME buffer), and 100-ml portions of the suspension were subjected to sonification four times for 1 min each with cooling to 10° or below during treatment. A Branson Sonifier, Model W140, was operated at maximum output for disruption. The mixture was then centrifuged in the cold at 27,000*g* for 30 min, and the supernatant fluid was assayed for protein and diluted to 40 mg/ml if necessary with PME buffer. The enzyme was maintained between 0 and 4° during the entire purification.

Step 2. Protamine Sulfate Treatment. Nucleic acids were precipitated by the addition of two-tenths the total volume of a 4% protamine sulfate solution in the PME buffer. The solution was added slowly with stirring, and the resulting mixture was allowed to stir an additional 30 min. After centrifugation as described above, the supernatant fluid was placed in an ice bath.

Step 3. Ammonium Sulfate Fractionation. Solid enzyme grade ammonium sulfate was slowly added to a concentration of 33% saturation at 0°. During the addition the pH was maintained at 7.5 with 4 N potassium hydroxide, and afterwards the solution was allowed to stir overnight (16 hr). Centrifugation as above produced a supernatant fluid which was brought to 45% saturation with ammonium sulfate, with a similar pH control, overnight stirring, and centrifugation. This time the precipitate was collected and dissolved in 80 ml of PME buffer. Desalting was accomplished by the use of a Sephadex G-25 column (4 × 49 cm) equilibrated with PME buffer. The enzyme solution (100 ml) was applied and eluted in 25-ml fractions with buffer at the maximum flow rate of the column (2.5–3.0 ml/min). Eluted fractions were checked for the absence of ammonium sulfate with Nessler's reagent, and then pooled.

Step 4. Chromatography on Hydroxylapatite. A hydroxylapatite column (2.3 × 20 cm) was equilibrated with PME buffer, and the enzyme solution was applied to the top. After the enzyme had entered, the column was washed with buffer until all unbound protein had been removed. No threonine dehydratase activity was detected in these fractions. A gradient was prepared by placing 100 ml each of 0.08, 0.12, 0.15, 0.17, 0.20, 0.22, 0.25, 0.27, and 0.30 M potassium phosphate (pH 7.5), each containing 0.01 M 2-mercaptoethanol, in a Buchler Varigrad. The gradient was applied at the rate of 0.3–0.4 ml/min, and 10-ml fractions were collected. Fractions with the highest specific activities (usually found around 0.25 M phosphate) were pooled and concentrated by precipitation with 70% saturated ammonium sulfate, again maintaining the pH at 7.5. After stirring at 0° for 1 hr, the suspension was centrifuged as above and the precipitate was dissolved in a minimal amount of PME buffer.

TABLE I: Purification of Threonine Dehydratase.

Step	Volume (ml)	Total Units	Total Protein (mg)	Specific Activity	Yield (%)
1. Crude extract	225	222	8900	0.025	100
2. Protamine sulfate	248	219	5450	0.040	99
3. $(NH_4)_2SO_4$, 33–45%	100	197	2100	0.094	89
4. Hydroxylapatite	270	95	292	0.32	43
5. Sephadex G-200	190	51	45	1.1	23
6. DEAE-cellulose	160	19	9.0	2.1[a]	9

[a] An identical value was obtained after concentration on a small DEAE-cellulose column, indicating that no further purification was achieved by this procedure.

Step 5. Chromatography on Sephadex G-200. The enzyme was applied to the top of a PME buffer-equilibrated Sephadex G-200 column (4 × 100 cm) layered with 2 cm of Sephadex G-25. Elution was accomplished at the rate of 0.1–0.2 ml/min with PME buffer, and 10-ml fractions were collected. The enzyme eluted after 400 ml had passed through the column and those fractions containing the highest specific activity were pooled.

Step 6. Chromatography on DEAE-Cellulose. The enzyme was applied directly to a Whatman DE-52 column (2.3 × 40 cm) equilibrated with PME buffer. A linear gradient was prepared containing 500 ml of 0.05 M potassium phosphate in the elution chamber and 500 ml of 0.4 M potassium phosphate in a connecting chamber. Both buffers were pH 7.5 and contained 0.01 M 2-mercaptoethanol, and the eluting chamber was stirred constantly during elution of the column. Rate of elution was 0.8–1.0 ml/min, and 10-ml fractions were collected, examined for specific activity, and pooled accordingly. Threonine dehydratase was usually found in the 0.22–0.26 M phosphate fractions. A summary of the purification is given in Table I.

Concentration and Storage. An equal volume of distilled water containing 1.0 mM dithiothreitol was added to the pooled fractions, and the resulting solution was applied to a small DEAE-cellulose column (0.5 × 10 cm). The application was followed by a brief wash containing 0.05 M potassium phosphate (pH 7.5) and 1.0 mM dithiothreitol. Elution was effected by 0.4 M potassium phosphate (pH 7.5), containing 1.0 mM dithiothreitol, and the concentrated enzyme was stored under liquid nitrogen. Enzyme stored in this fashion lost no activity in periods up to 3 months.

Evidence for Homogeneity. Polyacrylamide disc gel electrophoresis revealed a homogeneous preparation as shown in Figure 1, which illustrates the results of both protein and activity stains. The purity seen in this figure was also found in similar runs halted after 2 or 3 hr. In no case were other protein components detected in concentrated step 6 preparations. Based on the sensitivity of staining, it appears that the enzyme is over 95% pure by electrophoretic analysis.

FIGURE 1: Polyacrylamide gel electrophoresis of purified threonine dehydratase. The protein (100 μg) was taken from the concentrated step 6 fraction. A 7.5% gel column was used with an electrode buffer of pH 8.3. Electrophoresis was conducted at 2 mA/gel for 4 hr. The gel on the right was stained for activity, while the left one was stained for protein. Bromophenol Blue tracking dye had reached the gel bottom at 3.5 hr and is not seen in the photographs.

Sedimentation equilibrium ultracentrifugation also confirmed the homogeneity of purified preparations, as evidenced by a linear ln concentration *vs.* (radial distance)^2 plot. A molecular weight of 185,000 was calculated from a slope of 1.852 ± 0.042, assuming a partial specific volume of 0.73 ml g^{-1} and based on a measured density of 1.01 g ml^{-1}.

Catalytic Properties of the Enzyme. Purified threonine dehydratase exhibited an abnormally low specific activity of 2.1 μmol per min per mg of protein at 25°. The marked deviation of this value from those of other threonine dehydratases can be seen in the following list of specific activities: *Escherichia coli* (degradative enzyme), 900 at 37° (Shizuta *et al.*, 1969); *Clostridium tetanomorphum*, 500 at 25° (Vanquickenborne and Phillips, 1968); *Bacillus subtilis*, 400 at 37° (Hatfield and Umbarger, 1970); *Salmonella typhimurium*, 450 at 34° (Burns and Zarlengo, 1968); and *Pseudomonas multivorans*, 1000 at 30° (Lessie and Whiteley, 1969).

An analysis of the pH–rate profile in phosphate buffer over the range 6.5–8.5 revealed a maximum activity at 7.5, with approximately $^2/_3$ of the maximum rate being exhibited at pH 7 or 8. Although comparison with optimum pH values reported for other threonine dehydratases is made difficult by the multitude of assay buffers and procedures employed, similar conditions (phosphate buffer, coupled assay technique) have been used in the assay of the *E. coli* degradative threonine dehydratase (Phillips and Wood, 1965) and *C. tetanomorphum* dehydratase (Vanquickenborne and Phillips, 1968). In these instances more alkaline pH optima, pH 8–8.2, have been reported. Lessie and Whiteley (1969) found that the enzyme from *P. multivorans* gave highest rates at pH 8.5–9.5 in 0.2 M Tris buffer, with only $^1/_5$ of the rate at pH 7.5.

The K_m for threonine, determined at pH 7.5, was 2.5 mM. As seen in Figure 2, no evidence for cooperative homotropic effects was noted. Isoleucine, however, produced a drastic change in the shape of the velocity–substrate relationship. A pronounced sigmoidicity was found when isoleucine was present in a 25 μM concentration or higher. Figure 3 illustrates the inhibition by isoleucine at constant (125 mM) threonine. Under these conditions, 50% inhibition is achieved at an isoleucine level of 40 μM. Determination of the Hill coefficient for isoleucine gave a value of 2.4. A similar set of observations was made in the case of the *P. multivorans*

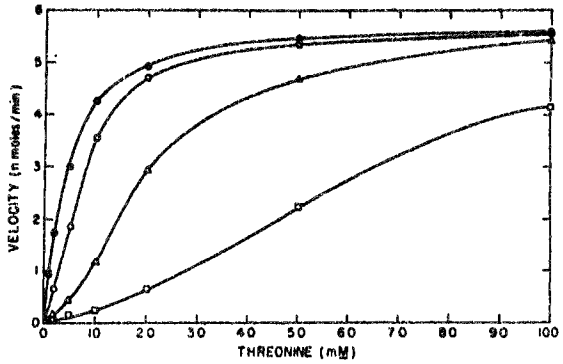

FIGURE 2: Dependence of velocity on threonine concentration in the presence and absence of isoleucine. Standard assay conditions were employed except for the additions of threonine and isoleucine at the stated concentrations: (●) no isoleucine; (○) 10 μM isoleucine; (△) 25 μM isoleucine; (□) 50 μM isoleucine.

threonine dehydratase (Lessie and Whiteley, 1969). In this instance, 150 μM isoleucine exerted half-maximal inhibition when assayed at pH 8.5. The Hill coefficient was 3.5.

Repression of threonine dehydratase by growth in amino acid media was not observed. Identical specific activities were obtained in crude extracts of cells grown on glucose–NH_3 minimal medium and the rich NZ amine–yeast extract medium.

P. putida threonine dehydratase also demonstrated activity toward L-serine, but with a large elevation in K_m to 40 mM. The characteristic inactivation of the enzyme during serine dehydration (Umbarger, 1973) proceeded in a time-dependent fashion as illustrated by Figure 4.

Inactivation of the enzyme by various carbonyl-attacking reagents was also noted, leading to the belief that the coenzyme pyridoxal-P was present. The degree of inhibition by these reagents after 10 min of incubation is displayed in Table II. It should be noted that cyanide, which is normally inhibitory toward pyridoxal-P containing enzymes, has no effect. Therefore, an exhaustive search for pyridoxal-P was undertaken to prove its presence or absence.

Effect of Pyridoxal-P Addition on Specific Activity. Purified threonine dehydratase, with a specific activity of 2.1 μmol min^{-1} mg, was incubated at 25° with 0.2 mM pyridoxal-P or

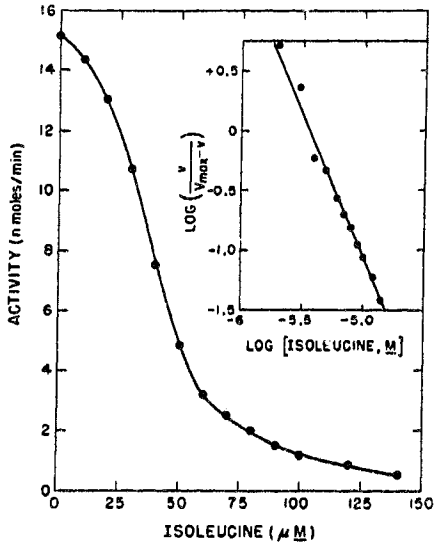

FIGURE 3: Inhibition of threonine dehydratase by isoleucine. Standard assay conditions were employed with a threonine concentration of 0.125 M. Inset: Hill plot of the same data.

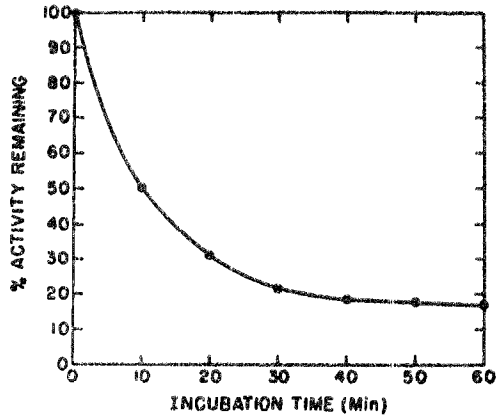

FIGURE 4: Time course of the inactivation of threonine dehydratase acting on serine. Serine (50 μmol) and enzyme (1 unit) were incubated at 25° in 0.5 M phosphate buffer (pH 7.5). At the indicated times, an aliquot was withdrawn, diluted 1:100, and 10 μl of this was assayed by the standard procedure (threonine as substrate) for the remaining enzyme activity.

0.2 mM pyridoxamine-P and aliquots were assayed at various times up to 1 hr. No increase in specific activity was noted in either case, and the addition of either compound during the assay itself also resulted in no change of specific activity.

Absorption Spectrum. The active enzyme at a concentration of 2 mg/ml in 0.05 M potassium phosphate and 1 mM dithiothreitol (pH 7.2) was placed in a Cary Model 17 recording spectrophotometer and blanked against the same buffer. The absorption spectrum revealed no peaks in the region from 340 to 415 nm, where most pyridoxal-P enzymes normally absorb. Figure 5 shows the spectrum compared with two known pyridoxal-P-containing enzymes.

Phenylhydrazine Assay for Pyridoxal-P. The colorimetric assay procedure of Wada and Snell (1962) was employed to test for the presence of pyridoxal-P. A control reaction with glutamic-oxalacetic transaminase revealed 1.8 nmol of pyridoxal-P phenylhydrazone per nmole of enzyme. This is near the expected value of 2 mol of pyridoxal-P per 110,000 g reported by Lis *et al.* (1960); 1 nmol of threonine dehydratase, however, formed color corresponding to less than 0.05 nmol of the phenylhydrazone.

Assay with Tyrosine Apodecarboxylase. The decarboxylation of tyrosine to tyramine by tyrosine apodecarboxylase requires the addition of pyridoxal-P for activity. Samples of threonine

TABLE II: Sensitivity of Threonine Dehydratase to Various Carbonyl-Attacking Reagents.

Inhibitor	Concn (mM)	% Inhibn[a]
Hydroxylamine	1	95
Phenylhydrazine	1	71
Potassium borohydride	1	51
Semicarbazide	10	64
Nitromethane	100	0
Potassium cyanide	10	0

[a] Determined after incubation at 25° in 0.05 M potassium phosphate buffer (pH 7.5) plus 1 mM dithiothreitol for 10 min. Enzyme (+ inhibitor) was diluted 100-fold before addition to the normal assay mixture.

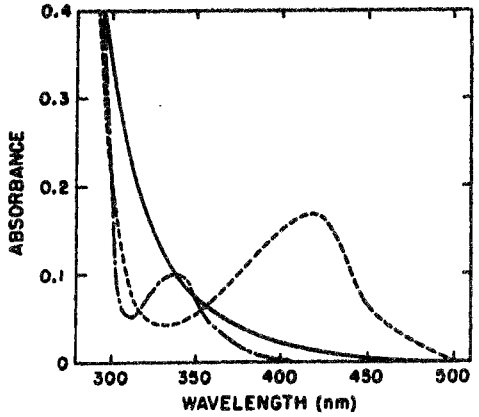

FIGURE 5: Absorption spectra of *P. putida* threonine dehydratase (——), glutamic–oxalacetic transaminase (-·-·-), and *C. tetanomorphum* threonine dehydratase (------). The protein concentrations were 2.0, 1.0, and 1.2 mg/ml, respectively. All scans were taken in 0.05 M potassium phosphate (pH 7.2) containing 1 mM dithiothreitol.

dehydratase boiled for 5 min in 0.2 N H_2SO_4 were assayed for pyridoxal phosphate (Sundaresan and Coursin, 1970), by measuring their ability to provide the coenzyme to the apodecarboxylase. Released $^{14}CO_2$ was trapped in 0.3 ml of ethanolamine and this solution was counted in a Beckman LS-200B liquid scintillation counter in 10 ml of toluene containing 0.5% of diphenyloxazole. Glutamic–oxalacetic transaminase (10 pmol) gave counts corresponding to 20 pmol of pyridoxal-P, as shown in Table III. However, 10 pmol of threonine dehydratase released no $^{14}CO_2$ and 400 pmol gave counts corresponding to only 8 pmol of pyridoxal-P.

Microbiological Assay with S. carlsbergensis. Haskell and Snell (1970) have described a microbiological assay which will detect any natural form of B_6. *S. carlsbergensis*, a B-requiring yeast, will maintain a linear growth response when supplemented with B_6 in concentrations between 0.5 and 5 pmol/ml. Samples to be assayed for B_6 were autoclaved at 121° for 5 hr in 0.055 N HCl prior to their addition to the culture medium.

TABLE III: Pyridoxal-P Estimation by the Tyrosine Apodecarboxylase Assay.

Compound	nmol Added	$^{14}CO_2$ Released (cpm/8 min)[a]	Pyridoxal-P (nmol)
GOT[b]	0.005	5,000	0.0105
	0.010	10,100	0.0205
	0.015	14,700	0.0300
TDH[c]	0.010	<100	
	0.10	850	0.0015
	0.20	2,100	0.0040
	0.40	3,400	0.0075
Pyridoxal-P	0.012	5,900	0.012
Pyridoxal-P + TDH	0.012 + 0.2	7,900	0.016

[a] [1-^{14}C]-L-Tyrosine with a specific activity of 10 μCi/mmol was used for each assay. Other conditions were precisely as described by Sundaresan and Coursin (1970). Values cited are corrected for endogenous activity. [b] GOT, glutamic–oxalacetic transaminase, mol wt 110,000. [c] TDH, threonine dehydratase, mol wt 185,000.

TABLE IV: Vitamin B_6 Assay with *S. carlsbergensis*.[a]

Enzyme	pmol Added/10 ml	pmol of B_6 Found[b]	Mol of Estimated/mol of Enzyme
GOT[c]	13	14	1.1
	25	32	1.3
	38	46	1.2
TDH[d]	50	8	0.16
	100	16	0.16
	200	33	0.16

[a] Growth as estimated by turbidity measurements in a Bausch and Lomb Spectronic 20 at 650 nm. [b] Standard growth curves were prepared with pyridoxine hydrochloride (5–50 pmol/10 ml) and results are expressed on this basis. Values are averages of duplicate determinations. [c] GOT, glutamic–oxalacetic transaminase. [d] TDH, threonine dehydratase.

The results of these experiments are presented in Table IV. Growth of the yeast corresponded to 1.2 pmol of B_6 (as pyridoxine) per pmole of glutamic–oxalacetic transaminase, which is to say that the hydrolysis procedure was approximately 60% efficient; since this value was quite reproducible over many assays, it was assumed that threonine dehydratase would also release any B_6 coenzyme in 60% yield, although this is not necessarily an accurate assumption. But since 100 pmol of threonine dehydratase yielded growth corresponding to 16 pmol of B_6, the factor of 60% would indicate only 0.27 pmol of B_6 per picomole of enzyme. This result is interpreted as evidence for the absence of pyridoxal-P as the normal coenzyme.

Preliminary Identification of the Coenzyme for Threonine Dehydratase. The foregoing data clearly argue that the *P. putida* threonine dehydratase contains no appreciable quantity of pyridoxal-P. Despite this, the enzyme was active, albeit less than usually noted for this enzyme from other sources, and could be inhibited by certain carbonyl-attacking reagents. This situation has been encountered in other instances, the most notable being the histidine decarboxylase of *Lactobacillus* (Riley and Snell, 1970), urocanase and histidase from *P. putida* (George and Phillips, 1970; Givot *et al.*, 1969), and the *S*-adenosylmethionine decarboxylase of *E. coli* (Wickner *et al.*, 1970). Several different coenzymes have been identified. Pyruvate replaces pyridoxal-P in the two decarboxylases, while α-ketobutyrate and dehydroalanine have been postulated to be coenzymes for urocanase and histidase, respectively. In order to clarify the situation somewhat in the case of threonine dehydratase, the purified enzyme (0.5 mg) was reduced with NaB^3H_4 (170 μmol, 37 mCi) according to the procedure of George and Phillips (1970). The enzyme after reduction was dialyzed against four 1-l. changes of 0.05 M potassium phosphate (pH 7.5) and passed through a 1 × 25 cm column of Sephadex G-10 in the same buffer. Collection of the protein fraction, and determination of protein and tritium content, allowed the calculation of the number of ^3H atoms bound per mole of enzyme. This value was found to be 6 g-atoms of H incorporated per mole of threonine dehydratase.

The labeled enzyme was then subjected to hydrolysis in 6 N HCl for 24 hr and the hydrolysate was dried over NaOH. The residue was dissolved in water, and 0.15 ml containing 4.5×10^5 dpm of ^3H was passed through a 1 × 10 cm column of

Dowex 50 (H^+). The column was washed with 10 ml of water and all effluent was pooled and evaporated over NaOH. This material was then taken up in methanol and derivatized with phenylboronic acid to permit identification of any α-hydroxy acids which might be present. Although some radioactivity was present in the Dowex 50 column wash (20% of the amount applied), the identification of any [^{3}H]lactate or [^{3}H]-α-hydroxybutyrate was completely negative. No ^{3}H compounds were eluted from the gas chromatograph column, although standard [^{3}H]lactate could be easily identified and was well separated from standard α-hydroxybutyrate.

A second portion of the hydrolyzed, labeled enzyme (approximately 3.0×10^5 dpm of ^{3}H) was applied to the long column of the amino acid analyzer. As shown in Figure 6, three regions of ^{3}H radioactivity were detected. Of these, only one corresponded to a known amino acid, namely alanine. Radioactivity recovered in the alanine fraction was calculated to be only 5% of that applied, the remaining amounts being found in the unretarded fractions (20%), the two unidentified components (5–10%), and the NaOH regeneration of the column (roughly 50%). Despite the obvious fact that alanine was identified as a minor component in the labeled mixture, it seems reasonable to draw the preliminary conclusion that the coenzyme of threonine dehydratase is a dehydroalanyl residue which upon reduction with NaB^3H_4 yields [^{3}H]alanine as one of the labeled products.

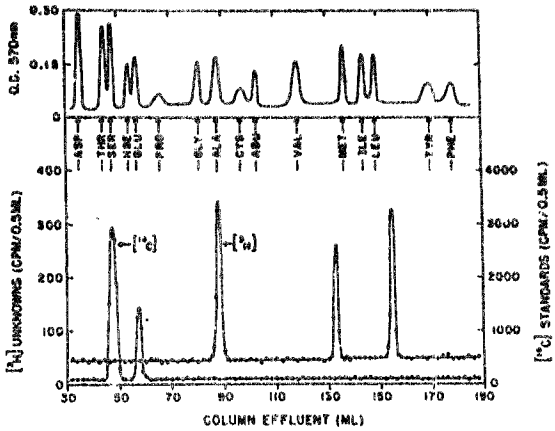

FIGURE 6: Ion exchange chromatography of the hydrolysate of NaB^3H_4 reduced threonine dehydratase. Chromatography was conducted on a Spinco 119 amino acid analyzer, utilizing the column designed for neutral and acidic amino acids. To the hydrolysate of [^{3}H]threonine dehydratase was added a mixture of unlabeled amino acids plus small amounts of [^{14}C]glutamate and serine to serve as internal markers. Efficiency for tritium counting was 20%. Radioactivity appearing in the tritium window during the elution of [^{14}C]glutamate and serine has been assumed to be all due to the carbon-labeled compound. The stream splitting ratio, determined from the recovery of [^{14}C]glutamate and serine, was 50% to the collector. The optical densities of the ninhydrin color were measured at 570 nm except in the area of proline where the values shown are those from the 440-nm tracing. HSE = homoserine, ABU = 2-aminobutyrate, CYS = cystine.

Discussion

The thorough reviews by Wood (1969) and Umbarger (1973) fully describe the properties exhibited by microbial threonine dehydratases. Based on their summarization of these characteristics, it is concluded that the *P. putida* dehydratase is of the biosynthetic type, possessing sensitivity to feedback inhibition by isoleucine. Cooperativity in threonine binding was seen only when isoleucine was present, the usual observation that is encountered in other systems. Moreover, the absence of repression by branched chain amino acids was also found in the case of the *P. multivorans* enzyme (Lessie and Whiteley, 1969). Thus from a standpoint of control, there seems little to distinguish the enzymes from the two species of *Pseudomonas*. There is presently no indication that more than one form of threonine dehydratase exists in soluble extracts of *P. putida*.

The purified dehydratase from *P. putida* was subjected to a thorough examination for the presence of pyridoxal-P. The sensitivity of the assays for pyridoxal-P was sufficiently high that it was possible to detect low levels of this compound in amounts ranging from 0.02 mole/mole of protein in the tyrosine apodecarboxylase assay to 0.27 mol in the microbiological assay. These quantities are, however, believed to be due either to contaminants in the threonine dehydratase or to nonspecificity in the case of the microbiological assay rather than to the presence of pyridoxal-P in the enzyme itself. This conclusion is based on the belief that if the enzyme as isolated were composed of 2–27% native enzyme plus the remainder as denatured apoenzyme, inhomogeneity should have been detected on disc gel electrophoresis. In addition, no dramatic loss in activity was observed at any step of the purification, as might be anticipated were coenzyme resolution encountered. Furthermore, an examination of Table I reveals that threonine dehydratase is present as roughly 1% of the total soluble cell protein. This would be in keeping with a severely reduced native catalytic effectiveness and a consequent derepression in order to provide sufficient activity for its function in isoleucine biosynthesis. Were the low activity seen in the purified enzyme an artifact of partial coenzyme resolution, such a high level of enzyme protein would not be expected. It is thus concluded that pyridoxal phosphate is essentially absent from the enzyme, adding threonine dehydratase to the growing list of enzymes which carry out a B_6-type catalysis by other means. One must, therefore, be cautious in the assignment of a role for pyridoxal-P based solely on studies with inhibitors or by analogy to other enzymes of the same class.

The fact remains, however, that threonine dehydratase of *P. putida* is strongly inhibited by carbonyl-attacking reagents. The most reasonable alternative to pyridoxal-P as a coenzyme would be something analogous to the covalently bound pyruvoyl groups found in histidine decarboxylase of *Lactobacillus* (Riley and Snell, 1968, 1970). In this instance, pyruvate assumes the role of pyridoxal-P by forming a Schiff base with the α-amino group of histidine, thereby facilitating decarboxylation (Recsei and Snell, 1970). Although a brief examination of the products of hydrolysis from NaB^3H_4-reduced threonine dehydratase failed to reveal any tritiated lactate (the expected product from reduction of a pyruvoyl residue), a more detailed search must be undertaken to rule out conclusively this type of carbonyl compound being present in native threonine dehydratase.

A somewhat less attractive possibility is the presence of a dehydroamino acid bound in peptide linkage. Dehydroalanine has been identified as critical to catalysis in two enzymes: histidine ammonia-lyase (histidase) by Givot *et al.* (1969) and Wickner (1969), and phenylalanine ammonia-lyase, reported by Hansen and Havir (1970). Both enzymes exhibit inhibition by a variety of carbonyl-attacking reagents, reflecting the close similarity of reactivity seen for dehydroamino acids and α-keto acids. From a mechanistic point of view, however, the substitution of a dehydroamino acid for pyridoxal-P presents some difficulty. Both histidase and phenylalanine ammonia-lyase involve α,β-elimination reactions in amino acids but neither has ever been formally treated as a pyridoxal-type of

reaction. Moreover, in neither case has a complete mechanistic interpretation of the function of dehydroalanine been offered.

The identification of [^{3}H]alanine from hydrolysates of NaB^{3}H$_4$-treated threonine dehydratase, albeit in low yield, and the failure to detect either pyridoxal-P or an α-keto acid derivative, requires that the coenzyme be tentatively identified as a dehydroalanyl residue. More obscure possibilities are yet to be eliminated. For example, one might argue for the existence of an α-keto acid coenzyme in a Schiff base linkage with an amino donor, by analogy to the situation often found for pyridoxal-P enzymes. Depending on the nature of the amino group donor, it might be possible to form substantial amounts of [^{3}H]alanine from reduction with NaB^{3}H$_4$ in place of [^{3}H]lactate.

Finally, it must be remembered that dehydroalanyl residues can be adventitiously generated in glycoproteins containing glycosidic linkages to serine hydroxyl groups (Payza *et al.*, 1969). This usually occurs upon alkaline elimination of the glycosyl moiety and borohydride treatment of such proteins can produce new alanyl residues. Future investigations of threonine dehydratase must be conducted to establish conclusively that this enzyme is not a glycoprotein. For the present it can only be stated that no indication of carbohydrate presence has been noted and, moreover, the borohydride reduction procedure employed carefully controls the pH to guard against the artificial generation of dehydroalanyl residues.

There still remain unsettled questions regarding the absolute identity of the coenzyme of this threonine dehydratase. The identification of the other labeled components seen in the amino acid analysis pattern (Figure 6) and a more detailed examination of the position of tritium incorporated during reduction are necessary to solidify any further claims. Nevertheless, this enzyme seems clearly to involve a different type of coenzyme from that in any other threonine dehydratase studied to this time.

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