Studies on the Metabolism of Biotin Vitamers by Microorganisms

Part III. Purification and Characterization of an Enzyme Synthesizing the Ureido Ring of Biotin Vitamers

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An enzyme which synthesizes ureido ring of biotin or desthiobiotin from biotin diaminocarboxylic acid or 7,8-diaminopelargonic acid, respectively, was purified about 2000-fold against the crude extract from *Pseudomonas graveolens* by a procedure involving ammonium sulfate fractionation, and DEAE-cellulose, hydroxylapatite and Sephadex G-200 column chromatographies. The final enzyme preparation showed a nearly symmetric peak upon ultracentrifugation.

The pH optimum of the enzyme was found to be at 7.0 to 8.0. On heating, the enzyme was stable up to 45°C, but, above 55°C, it was rapidly destroyed. Requirements for the enzyme reaction included bicarbonate, ATP and Mg^{2+} , as well as biotin diaminocarboxylic acid or 7,8-diaminopelargonic acid as substrate. The activity of the enzyme to form ureido ring from biotin diaminocarboxylic acid was one tenth of that from 7,8-diaminopelargonic acid. 7-Keto-8-aminopelargonic acid and 7-amino-8-ketopelargonic acid were inactive as the substrate.

In the previous paper,¹⁾ the authors have shown that the resting cells of *Bacillus sphaericus* convert biotin diaminocarboxylic acid into biotin, and that the cells of *Rhodotorula rubra* potentially convert it into bisnorbiotin *via* biotin. Moreover, the authors²⁾ reported that *Pseudomonas graveolens*, when grown in a 7,8-diaminopelargonic acid medium accumulated a large amount of desthiobiotin and bisnordesthiobiotin in the culture filtrate. These conversions could be assumed to proceed through a carboxylation to form ureido ring, and then β -oxidation. Recently, Eisenberg and Krell,³⁾ and Pai⁴⁾ demonstrated that

desthiobiotin was synthesized from 7,8-diaminopelargonic acid by the cell extract of *Escherichia coli*. However, the detailed studies on the nature of enzyme catalyzing the synthesis of ureido ring have not been reported.

The present paper described the purification and some properties of the ureido ring synthesizing enzyme of *P. graveolens*. A preliminary account of this work has been published.⁵⁾

EXPERIMENTAL

Chemicals. 7,8-Diaminopelargonic acid, 7-amino-8-ketopelargonic acid and 7-keto-8-aminopelargonic acid were a kind gift from Ajinomoto Co. Inc. Biotin diaminocarboxylic acid was synthesized from *d*-biotin by the method of Hofman *et al.*⁶⁾ The

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4) C. H. Pai, *J. Bacteriol.*, **99**, 696 (1969).

5) H. C. Yang, Y. Tani and K. Ogata, *Agr. Biol. Chem.*, **34**, 1748 (1970).

6) K. Hofman, D. Melvill and V. du Vigneaud, *J. Biol. Chem.*, **141**, 207 (1941).

synthesized sample was identified by measurement of its melting point and IR spectral analysis. DEAE-cellulose was a kind gift from Green Cross Corporation. Other chemicals used in this work were commercial products.

Organisms and medium. *P. graveolens* IFO 3460 was used throughout this work. The medium for the culture consisted of 10 g of peptone, 5 g of casamino acid, 20 g of glycerol, 1 g of K_2HPO_4 , 0.5 g of $MgSO_4 \cdot 7H_2O$, 1 g of $NaHCO_3$, 100 μ g of thiamine-HCl, 200 μ g of riboflavin, 400 μ g of pyridoxine, 200 μ g of nicotinic acid and 400 μ g of Ca-pantothenate in 1000 ml of tap water. The pH of the medium was adjusted to 7.2.

Analytical procedures

Enzyme assays and protein determination. The enzyme assays were performed by measuring the amount of biotin or desthiobiotin formed from biotin diamino-carboxylic acid or 7,8-diaminopelargonic acid, respectively. These reaction products were preliminarily identified by bioautographic techniques with several microorganisms. The reaction mixture contained the appropriate amount of enzyme, 100 μ moles of potassium phosphate buffer, pH 7.2, 0.1 μ moles of substrate, 50 μ moles of $NaHCO_3$, 10 μ moles of $MgCl_2$, 10 μ moles of ATP in a total volume of 1.2 ml. Incubation was carried out for 2 hr at 37°C. The reaction was arrested by heating the mixture for 2 min at 90°C. Specific activity was expressed as $m\mu$ moles of biotin or desthiobiotin synthesized per mg protein per 2 hr under conditions described. Protein was determined by the method of Lowry *et al.*⁷⁾ with bovine serum albumin as the standard and by measurement of absorbancy at 280 $m\mu$.

Microbiological assay. The vitamers synthesized were determined quantitatively by the microbiological methods with *Lactobacillus arabinosus*, *Saccharomyces cerevisiae* and *Bacillus subtilis* according to the methods reported previously.⁸⁾ Under conditions employed, biotin diamino-carboxylic acid supports the growth of

L. arabinosus (0.01% compared to *d*-biotin) and *S. cerevisiae* (10% compared to *d*-biotin), but in a considerably low extent,⁹⁻¹¹⁾ while it does not support the growth of *B. subtilis* at all. 7,8-Diaminopelargonic acid supports the growth of *S. cerevisiae* in lower extent than biotin and desthiobiotin as described previously,^{12,13)} while it does not support the growth of *L. arabinosus* and *B. subtilis* at all. Then, the microbiological assay for the measurement of enzyme activity was routinely performed using *B. subtilis*, with *d*-biotin as the standard.

RESULTS

Purification of enzyme

All operations were carried out around 5°C throughout the purification procedures.

Step 1. Enzyme extraction. *P. graveolens* was grown on 15 liters of the culture medium in 2 liters shaking flask containing each 500 ml medium at 28°C for 30 hr with reciprocal shaking. The cells were harvested by the continuous-flow centrifuge and washed with distilled water. The cells were suspended in 0.01 M potassium phosphate buffer, pH 7.0, containing 5×10^{-3} M 2-mercaptoethanol, to give a suspension of 1 g of wet cells per 3 ml of the buffer. The suspension was subjected to the action of the Kaijo-denki ultrasonic oscillator (20 kc) for 40 min. The cells and debris were removed by centrifugation at 10,000 $\times g$ for 20 min. The resultant supernatant solution was dialyzed overnight against two changes of 20 liters of the same buffer.

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Step 2. Ammonium sulfate fraction. To 600 ml of the dialyzed cell extract was added 91.5 g of solid ammonium sulfate to 0.20 saturation. After standing overnight, the precipitate was removed by centrifugation $20,000\times g$ for 30 min and discarded. The ammonium sulfate concentration was then increased to 0.50 saturation by the addition of 41.0 g of solid ammonium sulfate. After standing overnight, the precipitate was collected by centrifugation at $20,000\times g$ for 30 min and suspended in 0.01 M potassium phosphate buffer, pH 7.0, containing 5×10^{-3} M 2-mercaptoethanol. The solution was then dialyzed for 10 hr against three changes of 20 liters of the same buffer. An inactive precipitate formed was removed by centrifugation at $20,000\times g$ for 20 min. About 80 per cent of the activity of the crude extract was usually recovered in the step.

Step 3. DEAE-cellulose column chromatography. The dialyzed enzyme solution (240 ml) was subjected to DEAE-cellulose column chromatography. The adsorbent was packed into column (5 cm $\times$ 30 cm) and equilibrated with 0.01 M potassium phosphate buffer, pH 7.0, containing 5×10^{-3} M 2-mercaptoethanol. After the column was washed with 0.1 M potassium phosphate buffer, pH 7.0, containing 5×10^{-3} M 2-mercaptoethanol, the enzyme was eluted from the column with 0.1 M potassium phosphate buffer, pH 7.0, containing 5×10^{-3} M 2-mercaptoethanol and 0.2 M NaCl. The active fractions were pooled to give 1025 ml, concentrated with addition of ammonium sulfate to 0.60 saturation. The resultant precipitate was dissolved and dialyzed against 0.01 M potassium phosphate buffer, pH 7.0, containing 5×10^{-3} M 2-mercaptoethanol. The elution pattern is shown in Fig. 1.

Step 4. Hydroxylapatite column chromatography. The dialyzed enzyme solution (43 ml) was subjected to hydroxylapatite column (4 cm $\times$ 5 cm) equilibrated with 0.01 M potassium phosphate buffer, pH 7.0, containing 5×10^{-3} M 2-mer-

FIG. 1. DEAE-cellulose Column Chromatography.

Fractions of 20 ml were collected at a flow rate of 1 ml per min. Enzyme activity was shown in $m\mu$ moles of desthiobiotin formed from 7,8-diaminopelargonic acid per fraction under standard assay conditions.

FIG. 2. Hydroxylapatite Column Chromatography.

Fractions of 15 ml were collected at flow rate of 20 ml per hour. Enzyme activity was assayed as shown in Fig. 1.

captoethanol. After the column washed with 0.01 M potassium phosphate buffer, pH 7.0, containing 5×10^{-3} M 2-mer-

enzyme was eluted from the column with 0.03 M potassium phosphate buffer, pH 7.0, containing 5×10^{-3} M 2-mercaptoethanol. The active fractions were combined to give 785 ml and concentrated by the addition of ammonium sulfate to 0.60 saturation. The elution pattern is shown in Fig. 2.

Step 5. Sephadex G-200 filtration. The precipitate was dissolved in a minimum volume of 0.01 M potassium phosphate buffer, pH 7.0, containing 5×10^{-3} M 2-mercaptoethanol, and filtered on a Sephadex G-200 column (2.4 cm $\times$ 88 cm) equilibrated with 0.03 M potassium phosphate buffer, pH 7.0, containing 5×10^{-3} M 2-mercaptoethanol. The buffer was allowed

FIG. 3. Sephadex G-200 Gel Filtration (I).

Fractions of 5 ml were collected at a flow rate of 10 ml per hr. Enzyme activity was assayed as shown in Fig. 1.

FIG. 4. Sephadex G-200 Gel Filtration (II).

Fractions of 5 ml were collected at a flow rate 10 ml per hr. Enzyme activity was assayed as shown in Fig. 1.

to flow at a rate of 10 ml per hour and 5 ml fractions were collected. The elution pattern is shown in Fig. 3. Active fractions were combined and concentrated by ammonium sulfate at 0.60 saturation. The precipitate was taken by centrifugation and dissolved in 0.03 M potassium phosphate buffer, pH 7.0, containing 5×10^{-3} M 2-mercaptoethanol. Then, the above process was repeated. By the second Sephadex G-200 column chromatography, a single peak was obtained as shown in Fig. 4. Fractions from No. 35 to 50 were used as the purified enzyme. The enzyme was purified

TABLE I. SUMMARY OF PURIFICATION OF UREIDO RING SYNTHESIZING ENZYME FROM *Pseudomonas graveolens*

The enzyme activity was assayed under standard assay conditions.

Step	Total protein (mg)	Specific activity ^{a1} (DAPA)	Total activity (DAPA)	Yield (%)	Ratio (DAPA/BDC)
Crude extract	22,800.0	0.24	5,600	100	10.6
AmSO ₄ (20~50%)	13,440.0	0.33	4,480	80	10.2
DEAE-cellulose	717.5	10.66	7,650	136	12.1
Hydroxylapatite	66.7	76.91	5,130	90	12.5
Sephadex G-200 (I)	7.2	516.66	3,720	66	11.3
Sephadex G-200 (II)	6.3	542.55	3,417	61	10.8

^{a1} mμmoles of desthiobiotin or biotin formed/mg protein/2 hr.

Substrate: DAPA—7,8-diaminopelargonic acid, BDC—biotin diaminocarboxylic acid.

FIG. 5. Enzyme Activity as a Function of Incubation Time and of Enzyme Concentration.

The enzyme activity was assayed under the standard assay conditions. In Fig. 5(A), the purified enzyme of 12.5 μg of protein was used. In Fig. 5(B), the incubation was carried out for 2 hr at 37°C. Substrate; 7,8-diaminopelargonic acid (○—○), biotin diaminocarboxylic acid (●—●).

about 2,000-fold with about 60% recovery. A summary of the purification procedure is presented in Table I. The synthesis rate of biotin and desthiobiotin from biotin diaminocarboxylic acid was almost same through the purification steps.

With the purified enzyme preparation, ureido ring synthesizing enzyme activity was a linear function of both incubation time and protein concentration (Fig. 5).

Properties of enzyme

Ultracentrifugal analysis. The ureido ring synthesizing enzyme preparation dissolved in 0.03 M potassium phosphate buffer, pH 7.0, containing 5×10^{-3} M 2-mercaptoethanol was subjected to the measurements of purity and sedimentation constant using a Beckman-Spinco analytical ultracentrifuge model E. The typical sedimentation pattern is shown in Fig. 6. As seen in sedimentation pattern a single symmetrical moving peak was obtained, together with some disperse material of low molecular weight which produced an ill-defined peak. By the conventional area-method, it was calculated that main peak contained about 80~90 per cent of the total sedimentable molecule present in the sample. The sedimentation coefficient, $s_{20,w}$, was calculated to be 3.496×10^{-13} cm/sec.

FIG. 6. Sedimentation Patterns of Ureido Ring Synthesizing Enzyme.

The purified enzyme with a specific activity of 542.5 was used at a concentration of 0.30 per cent in 0.01 M potassium phosphate buffer (pH 7.0) containing 5×10^{-3} M 2-mercaptoethanol. The photographs were taken at 26(a), 42(b), 58(c) and 74(d) min after reaching 50,740.

FIG. 7. Stability of Enzyme against Heating.

The purified enzyme solution (0.5 ml) containing 0.06 mg of protein in 0.1 M potassium phosphate buffer, pH 7.0, was heated for 10 min at various temperatures as indicated. The remaining enzyme activity was assayed under the standard assay conditions. Substrate; 7,8-diaminopelargonic acid (O—O), biotin diaminocarboxylic acid (●—●).

FIG. 8. Effect of pH on Enzyme Activity.

The purified enzyme of 12 μg of protein was used.

The enzyme activity was determined under the standard assay conditions except that pH of the buffer was changed as indicated. The buffers were 0.2 M potassium phosphate (●—●) and 0.2 M Tris (O—O). Substrate; (A) 7,8-diaminopelargonic acid, (B) biotin diaminocarboxylic acid.

Stability. The purified enzyme preparation could be preserved at 5°C in 0.03 M potassium phosphate buffer of pH over the range of 7.0

TABLE II. EFFECT OF METAL ON UREIDO RING SYNTHESIZING ENZYME^{a)}

The purified enzyme of 12 μg of protein was used. The enzyme activity was determined under the standard assay conditions, except that the metal was changed as indicated.

Addition	Concentration			
	A ^{b)}		B ^{c)}	
	10 ⁻² M	10 ⁻³ M	10 ⁻² M	10 ⁻³ M
None	0.0	0.0	0.0	0.0
Ca ²⁺	5.5	2.2	3.5	2.0
Mn ²⁺	95.0	100.0	95.0	136.0
Fe ²⁺	89.0	72.0	90.0	91.0
Ni ²⁺	5.5	2.2	4.6	2.9
Zn ²⁺	3.3	2.2	3.8	1.8
Mg ²⁺	100.0	100.0	100.0	100.0

^{a)} Relative activity Mg²⁺=100%, ^{b)} desthiobiotin formed, ^{c)} biotin formed.

A; Substrate, 7,8-diaminopelargonic acid, B; biotin diaminocarboxylic acid.

TABLE III. COFACTOR REQUIREMENTS

The purified enzyme of 12.5 μg of protein was used. The enzyme activity was determined under the standard assay conditions, except that the cofactor was omitted as indicated.

Cofactor omitted	Biotin formed ^{a)}	Desthiobiotin formed ^{a)}
None	0.49	4.29
ATP	0.06	0.41
NaHCO ₃	0.06	0.37
Mg ²⁺	0.04	0.18

^{a)} mμmoles of biotin or desthiobiotin formed/12.5 μg protein/2 hr.

to 8.0, with little loss of activity. On heating, the enzyme was stable up to 45°C, but above 55°C, it was rapidly destroyed (Fig. 7).

Effect of pH on enzyme activity. The variation of the enzyme activity with pH is shown in Fig. 8. The pH optimum of the enzyme was found to be at 7.0 to 8.0.

Metal requirement. Effect of various metal ions on the enzyme activity were examined.

As shown in Table II, the requirement of Mg^{2+} , Mn^{2+} and Fe^{2+} for the enzyme activity was evident, but Ca^{2+} , Ni^{2+} and Zn^{2+} showed a slight activation of this enzyme.

Cofactor requirement. Table III shows the requirement of cofactors for the synthesis of ureido ring by purified enzyme. The results, quite similar to that by Eisenberg *et al.*³¹ with the crude extract of *E. coli*, ATP, Mg^{2+} and $NaHCO_3$ are essential factors or substrates for the synthesis.

Substrate specificity. The substrate specificity of the enzyme was tested by determining both biotin and desthiobiotin formed (Table IV). Biotin or desthiobiotin was formed only

TABLE IV. SUBSTRATE SPECIFICITY OF ENZYME

The purified enzyme of 12.5 μg of protein was used. The enzyme activity was determined under the standard assay conditions except that the substrate was changed as indicated.

Substrate	Biotin formed ^{a)}	Desthiobiotin formed ^{a)}
7-Keto-8-aminopelargonic acid	0	0
7-Amino-8-ketopelargonic acid	0	0
7,8-Diaminopelargonic acid	0	4.43
Biotin diaminocarboxylic acid	0.40	0

^{a)} m μ moles of biotin or desthiobiotin formed/12.5 μg protein/2 hr.

when biotin diaminocarboxylic acid or 7,8-diaminopelargonic acid was used as the substrates. The activity of the enzyme to form ureido ring formation from biotin diaminocarboxylic acid was one tenth of that from 7,8-diaminopelargonic acid. 7-Keto-8-aminopelargonic acid and 7-amino-8-ketopelargonic acid were inactive as the substrate.

DISCUSSION

Although a considerable information is now available on the biosynthesis and metabolic function of biotin, less is known about formation of ureido ring of biotin vitamers by the purified enzyme. The present study demonstrates for the first time for the formation of biotin or desthiobiotin from biotin diaminocarboxylic acid or 7,8-diaminopelargonic acid, respectively, by the purified enzyme from *P. graveolens*. The results, quite similar to that obtained with the crude extract of *E. coli* by Eisenberg *et al.*,³¹ indicated that ATP, Mg^{2+} and $NaHCO_3$ are essential factors or substrates for the synthesis. Throughout the purification procedures, ureido ring synthesizing enzyme activity to form biotin from biotin diaminocarboxylic acid was always associated with the activity to form desthiobiotin from 7,8-diaminopelargonic acid. It may be indicated that both activities are brought by a single enzyme. The substrate specificity of the enzyme was tested by determining both biotin and desthiobiotin formation. The results, biotin or desthiobiotin was formed only when biotin diaminocarboxylic acid or 7,8-diaminopelargonic acid was used as the substrates. The activity of the enzyme to form ureido ring formation biotin diaminocarboxylic acid was one tenth of that from 7,8-diaminopelargonic acid. Biotin was detected in the purified ureido ring synthesizing enzyme *P. graveolens* by preliminary experiment, suggesting that this reaction may proceed through activation of CO_2 by the mechanism proposed by Lynen.¹⁴⁾ However, a clarification of possible role of biotin as cofactor in this reaction must await further purification of the enzyme.

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