The Bacterial Degradation of Pantothenic Acid. II. Pantothenate Hydrolase*

Veikko Nurmikko, Esko Salo, Heikki Hakola, Kauko Mäkinen, and Esmond E. Snell

ABSTRACT: When grown with pantothenate as a sole source of carbon and nitrogen, *Pseudomonas* P-2 contains an inducible enzyme, pantothenate hydrolase, that hydrolyzes pantothenate to pantoic acid and β-alanine. By ammonium sulfate fractionation and gel filtration, the hydrolase was purified about 370-fold from disrupted cells of *Ps.* P-2. The enzyme functions optimally at 28° and is rapidly inactivated at temperatures above 30°. Its K_M value for pantothenate at its optimum pH of 7.4 is 5 mM. No diffusible cofactors are required. The activity of the enzyme in crude cell-free extracts is easily sufficient to justify the view that its action represents the first step in metabolic degradation of pantothenate by this organism.

The isolation by enrichment culture of a soil organism, *Pseudomonas* P-2, that utilizes pantothenate as a sole source of carbon and nitrogen has been described previously (Goodhue and Snell, 1966a,b). β-Alanine and pantoic acid were among the products found in the spent culture medium, and it was concluded that the first step in degradative metabolism of the vitamin was cleavage of the amide bond. This study shows that this cleavage results through simple hydrolysis by an inducible amidase, pantothenate hydrolase. The enzyme is unusual in its sensitivity to heat.

Experimental Procedure

Source of Enzyme. Ps. P-2 was cultured in 4.5–5.0 l. lots of pantothenate medium from 2% inoculum with aeration, as described earlier (Goodhue and Snell, 1966a,b). At the end of the exponential phase of growth (12–40 hr at 30°) cultures were cooled to 0–2°, and the cells harvested by centrifugation, washed twice at 0° with distilled water, suspended in 6.7 mM potassium phosphate (pH 7.4), and held at 0° until used. These cell suspensions, containing approximately 200 mg of cells (dry weight) per ml, were disrupted by treatment at 0° in 25-ml lots in the M. S. E. Ultrasonic Disintegrator at 18–20 kc (1.1–1.4 amp) for 20 min, then centrifuged for 10–15 min at 27,000–37,000 × *g*. The residue was resuspended in buffer and again treated in this same fashion. The procedure was repeated (up to six times) until no additional enzyme was extracted. The combined supernatant fluids (protein 8–20 mg/ml) were stored at −36° until used.

Assay of Pantothenate Hydrolase. Hydrolase activity was determined routinely by measuring the β-alanine formed after 1 hr at 28° in 1.0 ml of reaction mixture containing, at pH 7.4, 1.8–18 μmoles of potassium pantothenate, 0.92 μmole of potassium phosphate, and 0.1 ml of an appropriately diluted enzyme preparation. The reaction was stopped by heating at 100° for 3 min. One unit of enzyme is the amount which forms 1 μmole of β-alanine/min under these conditions.

Determination of β-Alanine. Initially β-alanine was separated from reaction mixtures by descending chromatography on paper with pyridine–water (65:35) as solvent. The ninhydrin-reactive zones were cut out and the color was extracted with methanol and estimated photometrically (Klett colorimeter, 565–630 mμ filter) against similarly treated standards of β-alanine. After it was established that β-alanine was the only significant ninhydrin-reactive compound formed, it was determined by the method of Yemm and Cocking (1955). Recoveries of β-alanine from the reaction mixture were between 96 and 105%.

Determination of Pantoate. Pantoic acid was determined by converting it quantitatively to pantolactone which was then determined as the corresponding hydroxamic acid by a slight modification of the procedure of Matsuyama (1957). Extensive trials to establish conditions necessary for quantitative lactone formation showed that at 20° pantolactone was formed rapidly only below pH 2.0; once formed, the lactone is stable at room temperatures in dilute aqueous solutions up to pH 9.0. Quantitative lactone formation was obtained in 4 hr at 20° in 0.47 N HCl. Blank reaction mixtures carried through the procedure permitted correction for the 8% or less of the pantothenate present in reaction mixtures that was hydrolyzed during this time. The procedure was as follows: to 1.15 ml of sample or standard containing 0–2.5 μmoles of pantoate was added 0.05 ml of 11.6 N HCl. After 4 hr at 20°, 0.05 ml of 11.6 N KOH and 0.75 ml of 0.75 N hydroxylamine, pH 9.4, were added. After 10 hr at 2°, 3 ml of ferric chloride reagent (6 ml of 1 N HCl, 10 ml of 40% trichloroacetic acid, 34 ml of water, and 10 ml of 1.48 M

* From the Departments of Biochemistry, University of Turku, Turku, Finland, and the University of California, Berkeley, California. *Received August 30, 1965; revised October 27, 1965.* This work was supported in part by grants (E-1575 and A-1448) from the National Institutes of Health, U. S. Public Health Service.

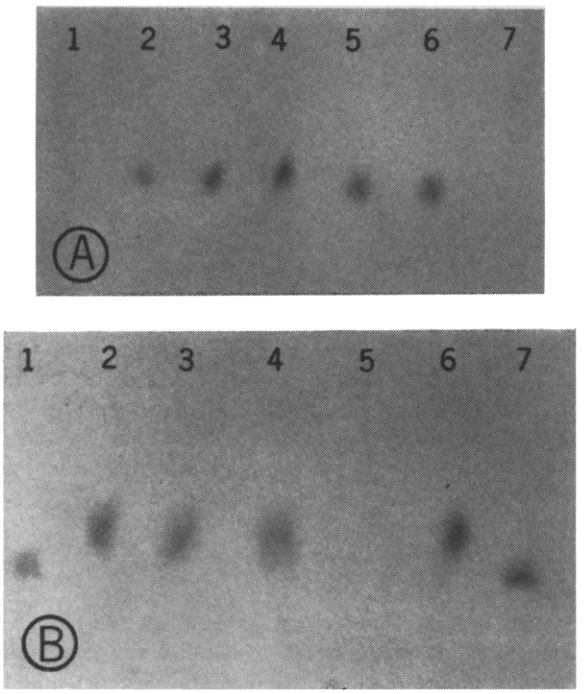

FIGURE 1: Formation of (A) β-alanine and (B) pantoic acid from pantothenate by an extract of *Ps.* P-2. Chromatogram of: (A) 1, pantoic acid (0.2 μmole); 2, 3, and 4, β-alanine (0.05, 0.1, and 0.2 μmole, respectively); 5 and 6, 0.1 ml of the enzyme reaction mixture; 7, control reaction mixture with heated enzyme; (B) 1 and 7, pantolactone (1 μmole); 2 and 6, pantoic acid (1 μmole); 3 and 4, enzymatic reaction mixture (0.5 ml); 5, heated control reaction mixture. The reaction mixture in each case (see text) contained per milliliter 1.8 μmoles of pantothenate and cell-free extract of *Ps.* P-2 to supply approximately 1.0 mg of protein, and was incubated 1 hr at 28°.

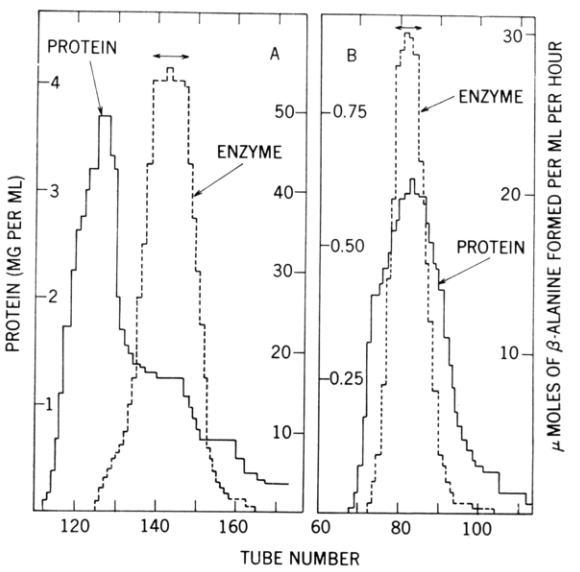

FIGURE 2: Gel filtration of partially purified pantothenate hydrolase. (A) Step d of purification procedure (see text). (B) Step f of purification procedure (see text). Fractions indicated by the horizontal arrows were combined for the subsequent operations.

$FeCl_3$ in 0.2 N HCl–KCl buffer, pH 1.2) was added. After 10 min, the absorbance of the ferric hydroxamate of pantoic acid was measured between 470 and 530 mμ and corrected for the reagent blank and hydrolysis of any pantothenate present. Recoveries of known amounts of added pantoate ranged from 91 to 104%.

Chromatography of Pantoate. Pantoic acid was readily separated from pantothenic acid and β-alanine by descending paper chromatography with 1-butanol–acetic acid–water (120:30:50, v/v) as solvent. After drying, chromatograms were sprayed with acid–acetone (10 ml of 1 N HCl in 90 ml of acetone), held at room temperature overnight to permit lactonization, then sprayed with hydroxylamine reagent and finally, after 10 min, with ferric chloride reagent. Pantolactone yields red-violet zones which disappear in about 30 min.

Miscellaneous Methods. Pantothenic acid was determined by assay with *Lactobacillus arabinosus* 8014 in Bacto Pantothenate assay medium. Pantoate, pantolactone, and β-alanine are inactive for this organism.

Protein was determined by the procedure of Lowry *et al.* (1951) with serum albumin as standard.

Results

Nature and Stoichiometry of Pantothenate Cleavage. When crude extract of *Ps.* P-2 was incubated with pantothenate, β-alanine was the only ninhydrin-reactive compound found on chromatograms (Figure 1A). Similarly, pantoic acid, but not pantolactone, was readily demonstrated by the ferric hydroxamate spray technique (Figure 1B). Quantitative determinations (Table I) showed that stoichiometry of the reaction

TABLE I: Stoichiometry of Pantothenate Cleavage by the Pantothenate Hydrolase of *Pseudomonas* P-2.[a]

Reaction Mixture	Pantothenic Acid Degraded (μmoles)	β-Alanine Formed (μmoles)	Pantoic Acid Formed (μmoles)
I	0.60	0.77	0.74
II	0.96	0.96	0.99
III	1.33	1.44	1.35

[a] Reaction mixtures (see text) contained 1.8–1.9 μmoles/ml of pantothenate and independent preparations of crude extract of *Ps.* P-2 to supply 0.06–0.078 mg of protein. Incubation was for 2 hr at 28°. Blank values were obtained from a control containing heated enzyme.

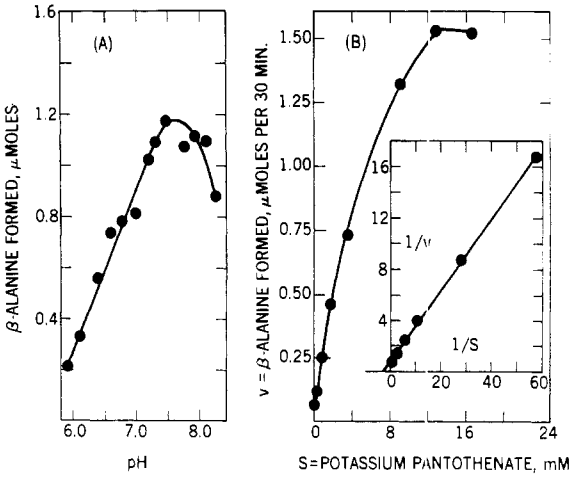

FIGURE 3: Effect of (A) pH and (B) concentration of substrate on rate of action of pantothenate hydrolase. The incubation mixture contained per milliliter 1.8 μmoles of pantothenate and cell-free extract of *Ps.* P-2 to supply 0.9 mg of protein, and was incubated for 1.0 hr at 28°.

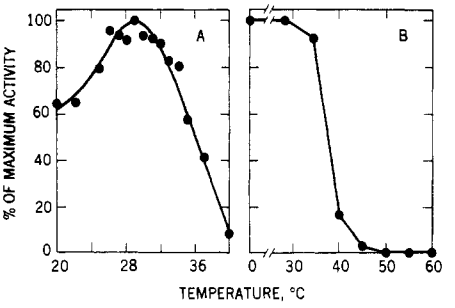

FIGURE 4: Effect of temperature on (A) hydrolysis of pantothenate by pantothenate hydrolase and (B) stability of pantothenate hydrolase. Reaction mixtures in A contained per milliliter 18 μmoles of pantothenate and cell-free extract of *Ps.* P-2 to supply 0.9 mg of protein. Incubation was for 1 hr prior to determination of β-alanine. In B, preparations of the enzyme were heated in buffer A at the indicated temperature for 5 min prior to determination of their enzyme activity at 28°.

in the crude extract corresponds to eq 1.

pantothenic acid + H_2O →
β-alanine + pantoic acid (1)

Partial Purification of Pantothenate Hydrolase. Partial purification of the enzyme was carried out to make certain that its action was not more complex than that represented by eq 1. Unless specified otherwise, all steps were carried out at 0–5°. Buffer A (6.7 mM potassium phosphate, pH 7.4) was used to dissolve all precipitates. The pH after each addition of ammonium sulfate was adjusted to 7.4 with 10 N potassium hydroxide and the solution allowed to stand for 1–2 hr before centrifuging out the precipitate. A protocol of the purification procedure is given in Table I.

A. PRECIPITATION WITH STREPTOMYCIN. To 500 ml of crude cell extract (derived from approximately 100 g of cells of *Ps.* P-2) 10 g of streptomycin sulfate was slowly added with stirring. The pH was adjusted to 7.4. After 15 min, the mixture was centrifuged and the precipitate discarded.

B. FIRST AMMONIUM SULFATE FRACTIONATION. To the solution from A was added with stirring 114 g of ammonium sulfate (37% saturation), and the pH was adjusted to 7.4. The insoluble material was discarded. To the supernatant solution was added an additional 23.4 g of ammonium sulfate (46% saturation), the pH was again adjusted to 7.4, and the active precipitate was collected by centrifugation.

C. SECOND AMMONIUM SULFATE FRACTIONATION. The active precipitate from B was dissolved in 75 ml of buffer A, ammonium sulfate (14.5 g) was added to 29% saturation, the pH readjusted to 7.4, and the precipitate discarded. Additional ammonium sulfate (8 g, 42% of saturation) was added, the pH adjusted to 7.4, and the active precipitate collected by centrifugation.

D. FRACTIONATION ON SEPHADEX G-100. The active precipitate from C was dissolved in 15 ml of buffer A, applied to a 105 × 6.7 cm column of Sephadex G-100 previously equilibrated with the same buffer, and washed through with additional buffer. The flow rate was 3.0 ml/min and 9.3-ml fractions were collected. Pantothenate hydrolase is retarded more than the bulk of the protein (Figure 2A).

E. THIRD AMMONIUM SULFATE FRACTIONATION. Fractions 138–146 of the column effluent were combined and diluted to 100 ml, and 85 ml of saturated ammonium sulfate solution, pH 7.4, was added with stirring. The precipitate was discarded, and the enzyme was precipitated with additional saturated ammonium sulfate solution (50 ml/100 ml of solution, pH 7.4).

F. SECOND FRACTIONATION ON SEPHADEX. The precipitated enzyme from E was dissolved in 9 ml of buffer A and applied to a column (80 × 3.7 cm) of Sephadex G-100, as described previously. Fractions 79–84 (Figure 2B) were combined (volume, 17 ml) and the enzyme was precipitated by addition of 30 ml of saturated ammonium sulfate (pH 7.4). The active precipitate was centrifuged out, redissolved in a small amount (<1.0 ml) of buffer A, and again precipitated by gradual addition of saturated ammonium sulfate until the solution became faintly turbid. After 16 hr the precipitate was collected by centrifugation and dissolved in 1 ml of buffer A. This material, purified 370-fold over the initial crude extract (Table I), produces pantoic acid and β-alanine from pantothenate in the same manner as crude extract, without evidence of formation of any intermediate compounds. The enzyme appears to act, therefore, as a true hydrolase, cleaving pantothenic acid according to reaction 1.

Effect of pH and Substrate Concentration. Panto-

TABLE II: Purification of Pantothenate Hydrolase from *Ps.* P-2.

Fractionation Step[a]	Volume (ml)	Protein (mg/ml)	Specific Activity (units/mg)	Yield (%)
Cell-free extract	500	25	0.37	(100)
AS I (0.37–0.46 sat.)	90	9	3.3	59
AS II (0.29–0.42 sat.)	25	17.5	6.0	57
Sephadex I + AS III	9	0.15	69	7.0
Sephadex II + AS	1	1.1	135	3.2

[a] AS = ammonium sulfate.

thenate hydrolase acts optimally between pH 7.3 and 8.0 (Figure 3A). Under the reaction conditions adopted, 18 mM pantothenate gives a maximum rate of reaction (Figure 3B); the K_M value calculated from Lineweaver–Burk plots (insert, Figure 3B) is 5 mM at pH 7.5.

Effect of Temperature and Time of Reaction. Hydrolysis of pantothenate by the hydrolase proceeds optimally at 28–29° (Figure 4A). Reduced activity above 30° results from heat denaturation of the enzyme (Figure 4B), which is almost complete in 5 min at 40°. At pH 7.4 and 28° and with 18 μmoles of substrate/ml of reaction mixture, the rate of β-alanine production with limiting amounts of the hydrolase is linear with time for 2–3 hr.

Effect of Activators and Inhibitors. No evidence for a dissociable metal ion or other activator was found. Inclusion of metal ion complexing agents in the assay mixture inhibits enzymatic activity, however, but only at relatively high concentrations. For example, 10 mM KCN, EDTA, or diethyldithiocarbamate inhibited to the extent of 42, 45, and 36%, respectively. The basis for this inhibition is not known; at present it does not provide adequate evidence that the hydrolase is a metalloenzyme.

Discussion

In a medium containing 10 μmoles of pantothenate/ml as carbon–nitrogen source, *Ps.* P-2 produces approximately 0.6 mg of cells/ml (dry weight) with a generation time of about 4.5 hr (Goodhue and Snell, 1966a). About 45% of the bacterial cell can be extracted as soluble protein, with a specific activity for pantothenate hydrolase of 0.37 (Table II). If the enzyme content per cell remains constant during exponential growth, it can be calculated (see Rahn, 1943) that about 53 μmoles of pantothenate could be hydrolyzed to β-alanine and pantoate by pantothenate hydrolase acting under optimal conditions during the 5.55 generations required to produce 0.6 mg of cells from the 2% inoculum used. This is over five times the amount of pantothenate actually supplied, and emphasizes the fact that the activity of the hydrolase is more than ample to fulfill its role as the first obligatory catalyst in the degradation of pantothenate.

In contrast to the findings in resting cells and concentrated cell-free extracts (Goodhue and Snell, 1966a), further degradation of the β-alanine and pantoic acid produced by the hydrolase does not occur in the diluted crude extract used for assay. It will be shown in a subsequent paper that the next step in pantoate degradation is an oxidative one, and requires addition of oxidized diphosphopyridine nucleotide to the diluted extract (Goodhue and Snell, 1966b).

References

Goodhue, C. T., and Snell, E. E. (1966a), *Biochemistry 5*, 393.

Goodhue, C. T., and Snell, E. E. (1966b), *Biochemistry*, *5*, 403.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Matsuyama, A. (1957), *Bull. Agr. Chem. Soc. Japan 21*, 47.

Rahn, O. (1943), Mathematics in Bacteriology, Minneapolis, Minn., Burgess Publishing Company.

Yemm, E. W., and Cocking, E. C. (1955), *Analyst 80*, 209.