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A Microbial Carbon-Phosphorus Bond Cleavage Enzyme Requires Two Protein Components for Activity

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***Enterobacter aerogenes* IFO 12010 contains a carbon-phosphorus (C-P) bond cleavage enzyme catalyzing the liberation of inorganic phosphate from various alkyl- and phenylphosphonic acids. The enzyme in the bacterium was found to be composed of two physically different protein components, E2 and E3. The molecular weights of E2 and E3 were 560,000 and 110,000, respectively, and E3 was resolved into two apparently homogeneous subunits. Neither component alone could catalyze the C-P bond cleavage reaction, but the reaction was efficiently catalyzed when the components were mixed.**

Since the discovery of a natural phosphonic acid, 2-aminoethylphosphonic acid, in 1959 (8), various kinds of phosphonic acids containing a direct carbon-phosphorus (C-P) bond have been found in many living systems. Insecticides, herbicides, fungicides, nerve gases, flame retardants, and several other important synthetic chemicals also contain the C-P bond and have been used in large quantities. Since the C-P bond is highly resistant to chemical hydrolysis and thermal decomposition, the biological breakdown of phosphonic acids is important not only for biochemical studies but also for prevention of overaccumulation of these acids in nature. To date, however, the metabolic route of these unique biological and synthetic molecules has remained obscure because of a failure to detect the enzyme activity responsible for the C-P bond cleavage reaction in cell-free systems (1–7, 14, 17, 18).

Recently, we found a C-P bond cleavage enzyme in cell extracts of *Enterobacter aerogenes* IFO 12010 (15). The enzyme in the bacterium could cleave the C-P bond in various alkyl- and phenylphosphonic acids (such as methylphosphonic acid, phosphonoacetic acid, and phenylphosphonic acid) and was shown to be distinct from the enzyme phosphonatase, which hydrolyzes the C-P bond in phosphonoacetaldehyde (9–11).

In order to characterize the properties of the enzyme and elucidate the C-P bond cleavage mechanism, we attempted to purify the C-P bond cleavage enzyme from *E. aerogenes* IFO 12010. The cells were aerobically grown on a medium [0.5% glucose, 0.1% $(NH_4)_2SO_4$, 0.01% $MgSO_4 \cdot 7H_2O$, 0.02% yeast extract (P_i-free), and 0.4% phosphonoacetic acid (pH 7.2)] at 30°C for 20 h. The cells (38 g [wet weight]) from a 15-liter culture were suspended in 80 ml of 10 mM Tris hydrochloride buffer (pH 7.5) containing 10 mM $MgCl_2$ and 0.2 mM dithiothreitol (buffer A); cell extract was prepared as described previously (15). P_i-free yeast extract was prepared as described previously (15). Phosphonic acids were used after filtration with a membrane filter (Millipore Corp., Bedford, Mass.), and glasswear was rinsed with nitric acid before use.

The dialyzed cell extract (protein, 5,600 mg; 130 ml) was applied to a DEAE-cellulose column (4 by 60 cm) equilibrated with buffer A, and proteins were eluted with a linear gradient of KCl (0 to 0.6 M, 4,000 ml) in buffer A. A 20-ml portion was collected every 9 min. The enzyme eluted as a single active peak at about 0.05 M KCl. The active fractions were combined and concentrated by ultrafiltration with a PM-10 membrane (Amicon Corp., Lexington, Mass.), and the concentrate (protein, 1,400 mg; 20 ml) was loaded onto a Sephadex G-150 column (5 by 70 cm) equilibrated with buffer A. Proteins were eluted with buffer A, and a 7-ml portion was collected every 10 min. Almost all the activity was recovered in the void fraction of the column, and the fraction was concentrated as described above. The enzyme solution (protein, 485 mg; 5 ml) was then applied to the first TSK HW65 column (2 by 70 cm) equilibrated with buffer A. A molecular sieve gel of TSK HW65 was purchased from Toyo Soda MFG, Tokyo, Japan. Proteins were eluted with buffer A, and a 4-ml portion was collected every 10 min. When the enzyme in each fraction was assayed, only one active peak (P1) was observed. However, the activity in P1 was low (about 0.1% of that applied to the column), and virtually all activity had disappeared. The column fractions were then combined into three pools (Fig. 1), and the activities in each pool and in all possible combinations were determined. It was found that pool 2 contained the C-P bond cleavage enzyme activity, and virtually all of the activity from the Sephadex G-150 column was restored.

Our interest was then focused on the protein components in pool 2. Pool 2 (fractions 55 to 60) was concentrated to 3 ml (protein, 110 mg) as described above and fractionated again on the TSK HW65 column (Fig. 1) under the same conditions as those employed for the first TSK HW65 column. A 2.5-ml portion of eluate was collected every 5 min. The C-P bond cleavage enzyme activity was not found when each fraction was assayed. To locate the fractions required for the enzyme activity, a portion from each fraction (fractions 50 to 70) was taken and mixed with samples from other fractions. As a result, we could separate two protein peaks (P2 and P3) that exhibited the C-P bond cleavage activity when they were mixed (Fig. 1). The protein components in P2 and P3 were named E2 and E3, respectively, and further fractionated by determining the activity with P2 or P3.

Fractions in P3 (fractions 62 to 66) were combined (protein, 32 mg; 12 ml), saturated with ammonium sulfate (30%), and applied to a butyl Toyopearl 650M column (1 by 5 cm) equilibrated with buffer A saturated with ammonium sulfate (30%). The enzyme was eluted with a linear gradient of ammonium sulfate (30 to 0%, 50 ml) in buffer A, and a 1-ml portion was collected every 4 min. The enzyme was eluted at about 15% ammonium sulfate, and then the activity in each

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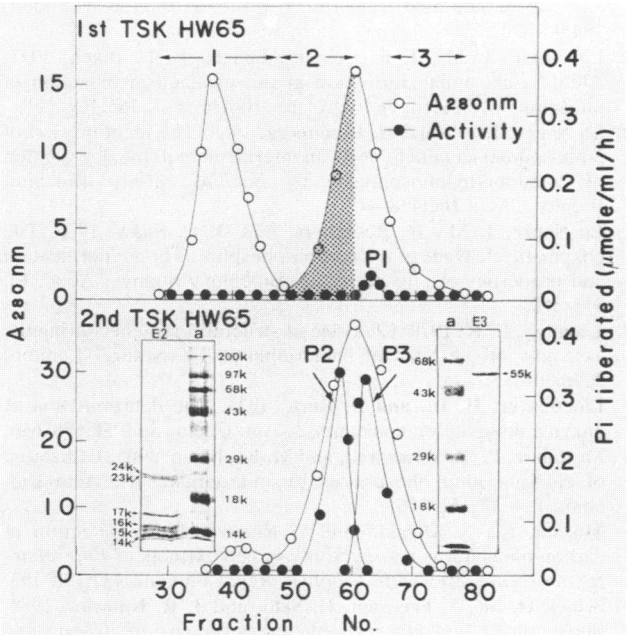

FIG. 1. Separation of two protein components required for the expression of C-P bond cleavage reaction. The enzyme was assayed at 37°C by determining P_i liberated in a reaction mixture consisting of 50 mM phosphonoacetic acid, 20 mM $MgCl_2$, 50 mM Tris hydrochloride buffer (pH 7.5), and the enzyme sample, as described previously (15). The first (top) and second (bottom) TSK HW65 column chromatographies were conducted as described in the text. Pool 2 (shaded area) was subjected to the second TSK HW65 column. Insets show sodium dodecyl sulfate-polyacryl-amide gel electrophoresis of the purified components E2 (left) and E3 (right) (lane a, molecular weight markers in kilodaltons [k]).

fraction was determined by using a sample of P2. The active fractions were concentrated to about 1.5 ml (protein, 4.8 mg) as described above and applied to a Sephadex G-200 column (1 by 50 cm) equilibrated with buffer A. Proteins were eluted with buffer A, and a 1-ml portion was collected every 5 min. Through these column chromatographies, E3 was purified approximately 130-fold with 3.5% yield from a step of the second TSK HW65 column. E3 showed a molecular mass of 110 kilodaltons on Sephadex G-200 gel filtration (Fig. 2). E3 showed a subunit molecular mass of 55 kilodaltons when it was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Laemmli (12) (Fig. 1).

Fractions in P2 (fractions 53 to 58) were also combined (protein, 28 mg; 12 ml) and applied to a butyl Toyopearl 650M column under the same conditions as those employed for the E3 fractionation. Unlike E3, E2 was eluted at about 5% ammonium sulfate, and then activity in each fraction was assayed by using a portion of P3. Active fractions were pooled, dialyzed overnight against buffer A at 4°C, and concentrated to 1.3 ml (protein, 4.5 mg) as described above. The enzyme solution was applied to a DEAE-Sepharose CL-6B column (1 by 90 cm) equilibrated with buffer A. Proteins were eluted with a linear gradient of KCl (0 to 0.8 M, 100 ml), and a 1.5-ml portion was collected every 5 min. The active fractions eluted at about 0.3 M KCl were concentrated to 1.2 ml (protein, 1.8 mg) and were then applied to a Sephadex G-200 column (1 by 50 cm) equilibrated with buffer A. Proteins were eluted with buffer A, and a 1-ml portion was collected every 6 min. When the activity in each fraction was determined by using a portion of P3 on Sephadex G-200 column chromatography, E2 showed only one protein peak that was closely superimposed on the C-P bond cleavage activity. The result indicates that E2 could be substantially purified. The final E2 preparation was purified about 90-fold with 4.5% yield from a step of the second TSK HW65 column and showed a molecular mass of 560 kilodaltons on Sephadex G-200 column chromatography (Fig. 2). Although the sodium dodecyl sulfate-polyacrylamide gel electrophoretic pattern of E2 was still quite complex and gave several protein bands with molecular masses of 24, 23, 17, 16, 15, and 14 kilodaltons (Fig. 1), most of the proteins appeared to be associated with the E2 component. Further purification may be required for the determination of the subunit structure of E2.

The final preparations of E2 and E3 after Sephadex G-200 column chromatography were used for the characterization of the C-P bond cleavage reaction. Neither purified component alone could catalyze the C-P bond cleavage reaction, but the reaction was efficiently catalyzed when the components were mixed. The greatest activity was found when six times as much E2 as E3 was used (data not shown), thus indicating that E2 and E3 coupled well at equimolar concentration; therefore, subsequent C-P bond cleavage reactions were performed by using 90 μg of E2 and 15 μg of E3. The reaction was most active at alkaline pH (pH 8 to 9). ATP and other cofactors (NAD, NADH, NADP, NADPH, and pyridoxal phosphate) were not required for the C-P bond cleavage reaction. The reaction was activated by divalent cations such as Mg^{2+}, Mn^{2+}, and Co^{2+} at almost the same efficiencies. Under the conditions specified in the legend to Fig. 1, the specific activity of the enzyme was 3.5 μmol/h per mg of protein, and K_m for phosphonoacetic acid was calculated to be 3.5 mM from a Lineweaver-Burk plot (13). The enzyme cleaved C-P bonds in alkyl- and phenylphosphonic acids (relative activities: methylphosphonic acid, 100; phosphonoacetic acid, 85; and phenylphosphonic acid, 32) (Aldrich Chemical Co., Inc., Milwaukee, Wis.), including a herbicide (glyphosate [relative activity, 15]; Monsanto Japan Co., Ryugasaki, Ibaragi, Japan) and an antibiotic (fosfomycine [relative activity, 45]; Sigma Chemical Co., St. Louis, Mo.).

Thus, *E. aerogenes* IFO 12010 contains a C-P bond cleavage enzyme, and the enzyme seems to require two protein components for its activity expression, although the function of each component is not yet clear. Recently, an enzyme catalyzing the formation of the C-P bond was purified by Seidel et al. (16). Our finding of the C-P bond cleavage enzyme, in combination with the C-P bond-forming

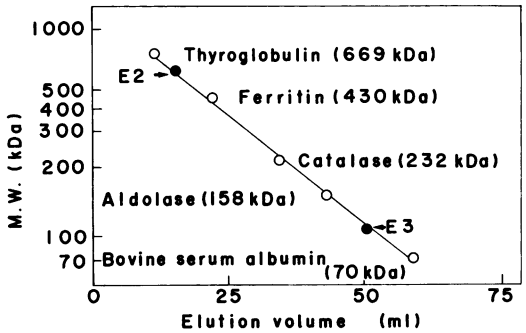

FIG. 2. Molecular weight determination of the purified E2 and E3 components. The purified E2 and E3 components were passed through the Sephadex G-200 column (1 by 50 cm) equilibrated with buffer A. Proteins were eluted with buffer A, and molecular weights were plotted as functions of elution volume.

enzyme, may facilitate the resolution of physiological functions of phosphonic acids and their metabolic route in living systems. The uncoupling of the C-P bond cleavage enzyme reaction attained here by the physical separation of two protein components appears to elucidate an aspect of the reaction mechanism.

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