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Purification and Properties of 4-Hydroxy-2-Ketopimelate Aldolase from *Acinetobacter*

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The chemical synthesis of 4-hydroxy-2-ketopimelic acid is described. An aldolase that cleaves this compound to succinic semialdehyde and pyruvate has been purified from *Acinetobacter* grown at the expense of 4-hydroxyphenylacetic acid. The molecular weight of the enzyme was about 158,000 from sedimentation equilibrium data; other physical determinations gave values in reasonable agreement. The protein was globular and was dissociated in sodium dodecyl sulfate to give a species of molecular weight 25,700. The enzyme attacked both enantiomers of synthetic 4-hydroxy-2-ketopimelate and was stimulated by Mg^{2+} and Mn^{2+} ions.

A reaction pathway has been proposed for the degradation of 4-hydroxyphenylacetic acid in which 4-hydroxy-2-ketopimelic acid (HKP) is formed and then undergoes aldol fission to give succinic semialdehyde and pyruvate (11). There is one previous reference to HKP as a metabolite (15). Its presence in a plant, *Asplenium septentrionale*, was detected by means of paper chromatography, but its chemical synthesis has not been reported. This paper describes the synthesis of HKP and also the properties of HKP aldolase which has been purified to homogeneity from *Acinetobacter* grown at the expense of 4-hydroxyphenylacetic acid.

MATERIALS AND METHODS

Preparation of cell extracts and assay of HKP aldolase. The characteristics of the organism, a species of *Acinetobacter*, and its growth with 4-hydroxyphenylacetic acid as sole carbon source have been described (11). To furnish sufficient material for enzyme purification, cells were grown in 16-liter batches in 20-liter bottles provided with vigorous forced aeration at 30 C. After overnight growth, 8 g of sodium 4-hydroxyphenylacetate was added, and the culture was harvested 2 h later (yield, 40 to 50 g [wet weight] of cells). The crude cell extract used as starting material for the procedure of Table 1 was obtained by suspending 108 g of cell paste in 324 ml of 0.1 M phosphate buffer, pH 7. Portions (200 ml) of the suspension were cooled in an ice bath and exposed for 30 min to the output of a Branson sonifier (20 kc/s). After treatment with ribonuclease and deoxyribonuclease, the suspension was centrifuged at 30,000 × *g* for 30 min to give 360 ml of a clear cell extract.

HKP aldolase was assayed at its pH optimum, 8.0. The initial rate of reaction was obtained from the decrease in absorbancy at 340 nm when reduced nicotinamide adenine dinucleotide (NADH) was oxidized in the presence of lactate dehydrogenase by the pyruvate formed from HKP by aldol fission. Cuvettes contained 1.2 ml of 0.05 M tris(hydroxymethyl)aminomethane(Tris)-hydrochloride buffer (pH 8.0), 10 μmol of $MgCl_2$, 0.1 μmol of NADH, 0.25 μmol of HKP, and 4 U of lactate dehydrogenase. After 1 min, to allow for reduction of any pyruvate present initially, enzyme was added and the initial rate of decrease in absorbance was measured. For crude extracts, a small correction was usually required for NADH oxidized in the absence of substrate. One unit of activity is defined as the amount of enzyme that formed 1 μmol of pyruvate per min under the conditions of assay. Protein concentrations at various stages in purification were determined as described previously (13).

Preparation of 4-hydroxy-2-ketopimelic acid solutions. An aqueous solution of 0.0153 mol of succinic semialdehyde (11) in 120 ml of 0.2 N HCl was cooled in an ice bath and brought to pH 6 by addition of 2 N NaOH. Oxaloacetic acid (0.034 mol) was added with stirring over a period of 5 min, at which time the pH was adjusted from 3 to 7.05 by adding NaOH. Stirring was continued for 5 h at 22 C, and the mixture was then allowed to stand for a further 19 h. The progress of the reaction was conveniently monitored by using ammonium sulfate-treated fractions of *Acinetobacter*. To a cuvette containing 1.2 ml of 0.1 M K^+-Na^+PO_4 buffer (pH 7.0) were added successively 2 μliters of the reaction mixture, 20 μliters of 0.1 M nicotinamide adenine dinucleotide phosphate, (NADP), and 20 μliters of a cell extract that had been collected at 50 to 80% saturation with $(NH_4)_2SO_4$. This extract contained succinic semialdehyde dehydrogenase but no HKP aldolase activity, and from the increase in absorbance at 340 nm the amount of unreacted succinic semialdehyde could be determined. Upon addition of 10 μliters of 1.0 M $MgCl_2$ and 20 μliters of cell extract precipitated at 20 to 80% saturation, the HKP that had been synthesized was cleaved by its aldolase, and the succinic semialdehyde

TABLE 1. *Purification of HKP aldolase*

Fraction	Protein (mg)	Total activity (U)	Sp act (U/mg)	Purification (fold)	Yield (%)
Crude extract	4,860	1,240	0.255	(1)	(100)
Heat treatment	780	1,050	1.36	5.4	85
Ammonium sulfate precipitation	127	706	5.6	22	56
Diethylaminoethyl-cellulose chromatography	5.7	242	41.0	174	19
Sephadex G-150 chromatography	3.86	164	42.5	181	13

thereby released was determined from the further increase in absorbance at 340 nm. From these determinations, a 77% yield of HKP was indicated for the reaction as formulated in Fig. 1. Solutions contained pyruvate as an impurity formed by decarboxylation of the oxaloacetate used, and HKP was separated from the mixture by the procedure which Marcus and Shannon (7) employed to remove pyruvate from 4-hydroxy-4-methyl-2-oxoglutarate, namely, chromatography on Dowex-1-chloride with elution by 0.04 N HCl. Fractions containing HKP were pooled, neutralized with NaOH, and concentrated under reduced pressure. The chemical structure of HKP is assigned on the basis of its mode of synthesis, enzymatic cleavage to succinic semialdehyde and pyruvate, and the proof of structure of its lactone.

Chemical structure of the lactone of HKP. When acidic solutions of HKP eluted from Dowex-1-chloride were lyophilized, a solid was obtained which gave colorless crystals (mp 116 to 117 C) upon recrystallization from ethyl acetate. The ultraviolet spectrum of an aqueous solution showed a λ_{max} at 226 nm (ϵ = 4,400) shifted to λ_{max} at 260 nm upon addition of NaOH. This alkali shift has been demonstrated for other 2-oxobutyrolactones (1, 4, 5, 12) and is consistent with displacement of the equilibrium between structures II and III which are proposed in Fig. 1. When the compound was chromatographed on a thin layer of silica gel (11) with ether-acetic acid-water (13:3:2, vol/vol) as solvent, two spots were located, by ultraviolet light, having R_f values 0.71 and 0.58. The structure assigned to the lactone was confirmed by measurements made with the gas chromatograph-mass spectrometer described previously (11). The mass spectrum of the compound (direct probe; 20 eV) showed a parent ion at *m/e* 172. Other prominent fragment ions were given at *m/e* (relative intensity): 154 (50), 127 (44), 126 (25), 116 (16), 113 (10), 100 (15), 99 (45), 98 (100), 85 (31), 84 (27), 71 (32), 57 (21), 56 (25), 55 (21), and 43 (45). Four metastable peaks were located at *m/e* 138 (transition from 172 to 154 with loss of water), 103 (transition from 154 to 126), 94 (transition from 172 to 127), and 76.8 (transition from 127 to 99). When the lactone was treated with excess of ethereal diazomethane, two positions were methylated (R = Me in IV of Fig. 1), as shown by a parent ion at *m/e* 200 (70 eV; retention time 2.5 min at 150 C). Treatment with Tri-Sil (11) gave a derivative for which the mass spectrum (70 eV; retention time 4.5 min at 120 C with increments of 5 C per min) showed a parent ion at *m/e* 316 consistent with silylation of two groups (R = $SiMe_3$ in IV of Fig. 1).

Physiocochemical examination of purified HKP aldolase. Sedimentation velocity experiments (52,000 rpm) were performed in a Spinco model E analytical ultracentrifuge with an AnD rotor having ultraviolet optics. The meniscus depletion method of Yphantis (17) was used in sedimentation equilibrium experiments. Centrifugation experiments were carried out in a 12-mm double-sector cell equipped with interference window holders and sapphire windows. At equilibrium, the Raleigh interferograph was taken and measurements of vertical fringe displacements were made with a Gaertner Comparator for a series of values of *r*, the radial distance. The partial specific volume of the protein was calculated from its amino acid analysis (2). This was obtained for a sample of enzyme (0.5 mg) that had been freed from salt by dialysis against water, lyophilized, and then hydrolyzed in 6 N HCl at 110 C in a sealed tube for 22 h. After removal of HCl, the sample was analyzed in a Beckman-Spinco amino acid analyzer by the method of Moore and Stein (9). An additional determination of cystine was made by treatment with performic acid before hydrolysis (8).

Analytical disc-gel electrophoresis was carried out at room temperature by the method of Davis (6), with 7.5% polyacrylamide gel at pH 8.9. Gels were removed from their tubes, stained with protein by immersion overnight in 0.05% amido black in 7% acetic acid, and destained with a gel destainer (Canalco). Electrophoresis in the presence of sodium dodecyl sulfate was performed as described by Weber and Osborn (16); gels were stained for protein with Coomassie brilliant blue (Sigma c-8127) for 16 h and were destained in 18% methanol-9% acetic acid. To determine whether subunits were joined by disulfide bridges that could be cleaved on reduction with β-mercaptoethanol, reduced and nonreduced samples of enzyme were prepared and examined. This procedure and also the use of electrophoresis on polyacrylamide gels containing urea and acetic acid were as described previously (13).

Purification of HKP aldolase. The crude cell extract (360 ml) was divided into three portions, and each was held at 70 C for 6 min, at which time the precipitate that had formed was discarded. To the solution (305 ml) at 4 C, 53.3 g of powdered $(NH_4)_2SO_4$ was added slowly with stirring; the precipitate was allowed to stand for 1 h, centrifuged, and discarded. A second addition of 42 g of $(NH_4)_2SO_4$ gave a precipitate that was collected by centrifuging, dissolved in 8 ml of 0.05 M Tris-hydrochloride buffer (pH 8), and dialyzed for 19 h against four changes of

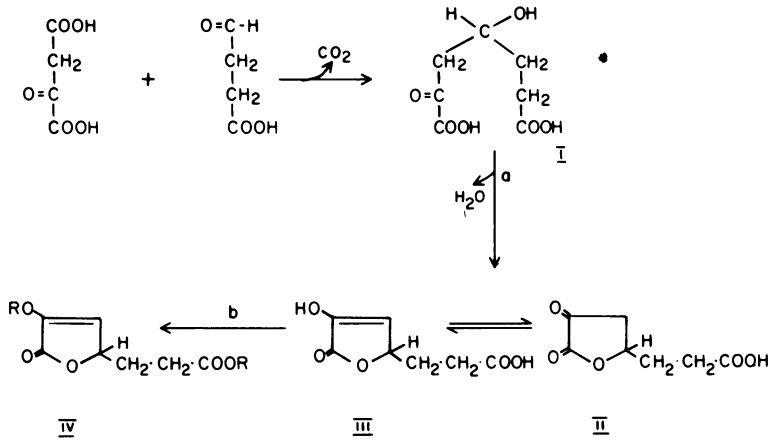

FIG. 1. *Chemical synthesis of 4-hydroxy-2-ketopimelic acid (I) from oxaloacetic acid and succinic semialdehyde. (a) Treatment with acid gave a lactone, keto-form II, and enol-form III. (b) Derivatives of the lactone were prepared (IV) having R = Me or $SiMe_3$.*

the same buffer. The solution was then clarified by centrifugation, made up to 14 ml with Tris buffer, and applied to a diethylaminoethyl-cellulose column (4.0 by 37 cm) that had equilibrated previously with 0.05 M Tris-hydrochloride buffer (pH 8) containing 0.05 M NaCl. Protein was then eluted with a linear gradient of 0.05 to 0.4 M NaCl in a total of 4 liters of Tris buffer at a flow rate of 2.2 ml/min, and 20-ml fractions were collected. Fractions 105 through 111 contained the enzyme and eluted at 0.2 to 0.25 M NaCl; they were pooled, concentrated by pressure dialysis with a Diaflo PM-30 membrane (Amico Corporation, Lexington, Mass.), and dialyzed against Tris buffer. The enzyme (1 ml) was then layered on top of a Sephadex G-150 column (1.7 by 96 cm) and eluted with 0.05 M Tris buffer (pH 8) at a constant flow rate of 0.15 ml/min. Protein and enzyme activity appeared only in fractions 30 through 35 (samples of 3 ml), and their elution profiles were coincident. These fractions were pooled and concentrated by pressure dialysis to a volume of 1 ml. Samples showed one band upon disc electrophoresis in acrylamide gel and also in urea-acetic acid acrylamide gel (Fig. 2).

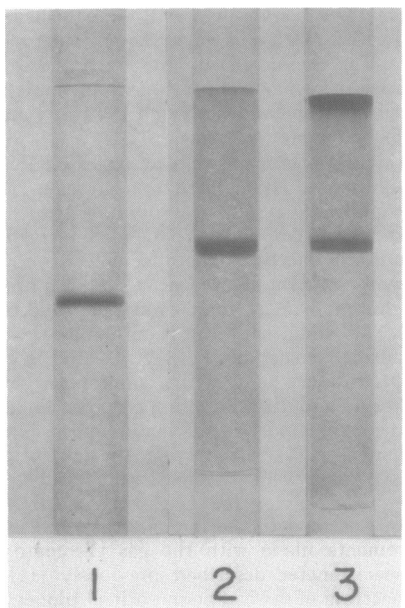

FIG. 2. *Electrophoresis of purified HKP aldolase in polyacrylamide gels. (1) Buffer at pH 8.9. (2, 3) Urea-acetic acid. The enzyme did not receive prior treatment with urea-acetic acid in 2. In 3, 20 μliters of aldolase (1.6 mg/ml) was incubated for 24 h at room temperature with 10 μliters of 10 M urea plus 10 μliters of 0.9 M acetic acid before application to the gel. The top band of 3 indicates precipitation of some of the protein during this treatment.*

RESULTS AND DISCUSSION

Ultracentrifugation of purified HKP aldolase. The sedimentation velocity, measured at 0.4 mg of protein, gave $s^0_{20,w} = 7.96 \times 10^{-13}$ s. Assuming the same dependence of sedimentation velocity on concentration which was established for a very similar protein, 4-hydroxy-4-methyl-2-ketoglutarate aldolase (13), HKP aldolase has $s^0_{20,w} = 7.99 \times 10^{-13}$ s. Table 2 gives the amino acid composition of an acid hydrolysate and also the number of residues for a subunit of molecular weight 25,700 found by sodium dodecyl sulfate electrophoresis. Assuming six subunits, the minimal molecular weight of the aldolase from amino acid analysis is approximately 154,000. The calculated partial specific volume, $\bar{v}$, is 0.751; tryptophan ($\bar{v}$ = 0.74) was not determined. The values for $\bar{v}$ and $s^0_{20,w}$ were used to calculate a frictional ratio, f/f_0, of 1.21 which lies within the range indica-

TABLE 2. *Amino acid analysis of HKP aldolase*

Amino acid	Composition (μmol/ml)	Residues[a] per 25,700 molecular weight
Aspartic acid	0.144	21
Threonine	0.120	17
Serine	0.072	10
Glutamic acid	0.264	38
Proline	0.056	8
Glycine	0.155	22
Alanine	0.208	30
Half-cystine	0.010	2
Valine	0.128	18
Methionine	0.037	5
Isoleucine	0.125	18
Leucine	0.197	28
Tyrosine	0.051	7
Phenylalanine	0.024	3
Histidine	0.021	3
Lysine	0.093	13
Arginine	0.048	7

[a] Nearest integer.

tive of a roughly spherical shape for the protein (14). For globular proteins, an approximate molecular weight, M, is given by $s^0_{20,w} = k \cdot M^{2/3}$. The proportionality constant, k, was calculated by using the published values (14) of $s^0_{20,w}$ and M for hemoglobin, ovalbumin, and catalase with frictional ratios of 1.14, 1.17 and 1.25, respectively. Values of k were in the range 2.806×10^{-3} to 2.848×10^{-3} and gave an average estimate of 150,000 for the molecular weight of HKP aldolase, using the value of $s^0_{20,w}$ that was previously determined.

A sedimentation (11,000 rpm) equilibrium experiment, performed with 0.4 mg of protein per ml, gave a linear relationship between the logarithm of fringe displacement and the square of the radial distance. From the slope of this line a molecular weight of 158,700 was calculated.

Sodium dodecyl sulfate electrophoresis of purified HKP aldolase. When submitted to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, the pure enzyme showed one major band of mobility 0.47, with a minor component of mobility 0.24. After treatment with β-mercaptoethanol, only the major band was observed (Fig. 3). Bovine serum albumin, ovalbumin, chymotrypsinogen, and cytochrome *c* were also run as standards, and from the linear plot of electrophoretic mobilities against the logarithms of the known molecular weights of these proteins that of the main subunit was calculated to be 25,700. The minor component observed before treatment with β-mercaptoethanol had a molecular weight of 57,800. It is suggested that the native enzyme consists of six similar subunits joined by noncovalent linkages and that small amounts of a dimer, arising from subunits linked through disulfide bridges, may have arisen during the purification of the enzyme.

Properties of the enzyme. HKP aldolase was rather heat stable. When held at 60 C for 20 min, there was no appreciable loss of activity; 85% of the activity was retained at 70 C for 6 min, and 30% was retained at 80 C for 5 min. When the enzyme was stored at 4 C in 0.05 M Tris-hydrochloride buffer (pH 8.0) or in 0.1 M phosphate buffer (pH 7.0), 90% of its activity remained after 1 week. When solutions also contained either 0.1 M $(NH_4)_2SO_4$ or 0.01 M ethylenediaminetetraacetic acid (pH 7.2), 80% of enzymatic activity was retained after 2 months at 4 C.

HKP was decomposed by the aldolase to give equimolar amounts of pyruvate and succinic semialdehyde which, in turn, could be converted enzymatically into lactate and succinate respectively. By this procedure, all of the synthetic HKP taken could be shown by chemical tests to have been decomposed enzymatically; accordingly, HKP aldolase cleaves both substrate enantiomers. Similar behavior has been reported as follows. 4-Hydroxy-2-methyl-2-ketoglutarate aldolase cleaves both enantiomers

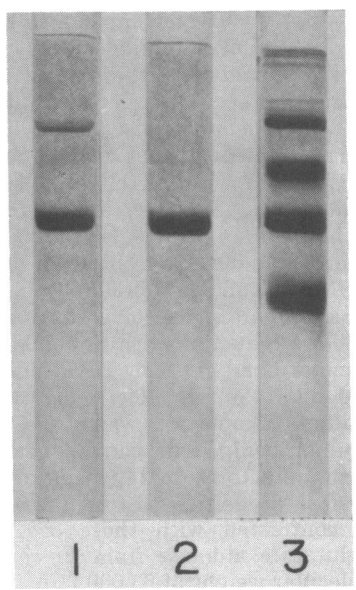

FIG. 3. *Electrophoresis of purified HKP aldolase on sodium dodecyl sulfate polyacrylamide gels. (1) Before treatment with β-mercaptoethanol. (2) After treatment. Standard proteins (3) were (from top to bottom): bovine serum albumin, ovalbumin, reduced HKP aldolase, and cytochrome c.*

of 4-carboxy-4-hydroxy-2-ketoadipate, although it attacks only one enantiomer of 4-hydroxy-2-methyl-2-ketoglutarate (13); and whereas 2-keto-4-hydroxyglutarate aldolase from *Escherichia coli* attacks L-2-keto-4-hydroxyglutarate preferentially, the enzyme from liver readily degrades both enantiomers (10). Initial rates of aldol fission were measured at pH 8.0 for a concentration range of 0.07 to 0.28 mM HKP. The Lineweaver-Burk plot was linear and gave K_m = 0.067 mM HKP. The enzyme showed optimal activity at pH 8.0; at pH 7.0 the rate of reaction was 70% of the optimal rate, and at pH values greater than 8.8 HKP underwent increasingly rapid nonenzymic fission. HKP aldolase exhibited narrow substrate specificity and had little or no action upon compounds with close structural resemblances which are attacked by other aldolases (3, 13). Thus, 4-hydroxy-4-methyl-2-ketoglutarate and 4-carboxy-4-hydroxy-2-ketoadipate were not attacked, and 4-hydroxy-2-ketovalerate was cleaved at only 3% of the rate for HKP.

Despite this difference in substrate specificity, HKP aldolase from *Acinetobacter* shows a strong resemblance in physical properties to 4-hydroxy-4-methyl-2-ketoglutarate aldolase from *Pseudomonas putida*. The last named enzyme has a pH optimum of 8.0, is also an approximately spherical protein with a molecular weight of 150,000, and appears to be composed of six similar subunits (13). The main differences observed were the extensive disulfide linkages that occur between subunits of the aldolase from *Pseudomonas putida* and the absolute requirement of that enzyme for Mg^{2+} or Mn^{2+} ions (13). Pure HKP aldolase from *Acinetobacter* was still able to cleave its substrate without added cations. However, the rate of reaction was doubled on addition of 1 mM $MgCl_2$ or $MnCl_2$, and it is probable that metal ions were only partially resolved during purification. Before reduction with β-mercaptoethanol, the major band given by HKP aldolase on sodium dodecyl sulfate electrophoresis indicated subunits of molecular weight 25,700, with only a minor component corresponding to a dimer of subunits, presumably disulfide-linked. The physical properties of the two aldolases may be contrasted with those of 2-keto-4-hydroxyglutarate aldolase from *E. coli* which has a molecular weight of 63,000 (10).

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