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Crystallization and Characteristics of 2-Keto-3-deoxy-6-phosphogluconic Aldolase*

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2-Keto-3-deoxy-6-phosphogluconic aldolase has been purified from *Pseudomonas fluorescens* (1) and *Pseudomonas saccharophila* (2), and a number of its properties have been documented. Investigation of the reaction mechanism has shown properties typical of an aldolase, that is, a reversible cleavage of 2-keto-3-deoxy-6-phosphogluconate and enolization of pyruvate (3). It was further shown that borohydride reductively bound approximately 1 mole of pyruvate per mole of aldolase (4). By analogy with the experiments of Horecker *et al.* (5) and Grazi, Cheng, and Horecker (6), this constitutes evidence for the formation of a pyruvyl-lysyl azomethine as the enzyme-substrate complex. For these and other studies, particularly on structure of the enzyme, it has been necessary to work with crystalline material and to ascertain some of its physical properties. The procedure for crystallization and some additional characteristics of 2-keto-3-deoxy-6-phosphogluconic aldolase are reported herein.

EXPERIMENTAL PROCEDURE

Molecular weight determinations were made in a Spinco model E ultracentrifuge according to the short column sedimentation equilibrium technique of Van Holde and Baldwin (7). Sedimentation velocity was measured with a synthetic boundary cell.

The ammonium sulfate content of fractions obtained during purification was determined with a precalibrated Barnstead purity meter on samples diluted 2×10^4-fold. Absorbance changes were measured at 30° in a Beckman model DU spectrophotometer fitted with a Gilford automatic cuvette changer (8) and a Gilford log converter (9) attached to a recorder.

2-Keto-3-deoxy-6-phosphogluconic aldolase was assayed with microcuvettes ($b = 1$) which contained (in a volume of 0.15 ml) imidazole buffer, 10 μmoles; DPNH, 0.08 μmole; KDP-gluconate,[1] 1 μmole; and excess lactic dehydrogenase. One unit of aldolase was defined as an absorbance change of 1.0 per minute, or the cleavage of 0.024 μmole of KDP-gluconate per minute. The assay was linear over a range of velocities in excess of 25 absorbance units per minute. All other materials, assays, and measurements were as described in the preceding papers (3, 10).

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[1] The abbreviation used is: KDP-gluconate, 2-keto-3-deoxy-6-phosphogluconate.

RESULTS AND DISCUSSION

Crystallization of 2-Keto-3-deoxy-6-phosphogluconic Aldolase

Preparation of Extract—Cell paste (50 g) was suspended in cold water to a volume of 100 ml and disrupted by treatment in a Raytheon 10-kc oscillator for 10 to 15 minutes. Cell debris was removed by centrifugation at 20,000 × *g*.

Heat and Protamine Sulfate Treatment—The extract, containing 30 to 60 mg of protein per ml, was heated with stirring to 52° and immediately chilled in ice. When the temperature had dropped to 10°, solid ammonium sulfate was added to a concentration of 0.2 M, followed by 0.2 volume of 2% protamine sulfate; the debris was removed by centrifugation. Frequently, the precipitate at this stage did not separate cleanly upon centrifugation, making optical estimation of protein invalid. However, in instances when protein could be estimated, this step yielded a 1.5- to 2-fold purification.

Ammonium Sulfate Fractionation in HCl—Solid ammonium sulfate was added to the supernatant solution to 1.5 M, and the precipitate was removed by centrifugation. The ammonium sulfate level of the supernatant solution was adjusted to 1.0 M with water, and 0.1 volume of 1.0 N HCl was added. After standing in an ice bath for 15 to 30 minutes, the denatured protein was removed by centrifugation. Solid ammonium sulfate was added to the supernatant solution to 2.0 M. The precipitate was collected and dissolved in 0.02 volume of 0.1 M sodium phosphate, pH 6.0, and the denatured protein was removed by centrifugation.

Chromatography on DEAE-cellulose—The preparation was passed through a DEAE-cellulose column (3 × 5.5 cm) that had been previously equilibrated against 0.1 M phosphate, pH 6.0. Under these conditions the aldolase was not retained. After the aldolase fraction had passed completely through the column, the resin was washed with approximately 1.5 volumes of phosphate buffer, added in portions. The treated fraction and washings were pooled. Care must be taken to assure a final protein level in the pooled eluates of more than 1 mg per ml. The volume of the wash is adjusted to achieve this. Chromatography on DEAE-cellulose, performed as described, facilitated further fractionation by removing high molecular weight materials. Attempts to chromatograph the aldolase under conditions which result in binding (0.03 M initial salt content) gave a higher purification, but with a loss of 50 to 70% of the activity.

Ammonium Sulfate Fractionation—Ammonium sulfate was added to the eluates to a final concentration of 2.0 M, and the precipitate was discarded. The supernatant solution was then

TABLE I

Crystallization of 2-keto-3-deoxy-6-phosphogluconic aldolase from P. fluorescens extracts

Step	Total activity	Recovery	Specific activity	Purification
	units × 10^3	%	*units/mg protein*	*-fold*
Extract	288	100	26	1
Heat and protamine sulfate	257			
Acid-ammonium sulfate	218	75	1,540	59
DEAE-cellulose	221	77	2,170	84
Ammonium sulfate	166	57	7,350	283
Calcium phosphate	105	40	9,740	378
Dialysis and lyophilization	78	26		
First crystals	46	16	13,300	511
Second crystals	27.2	9.5	13,000	500

made 2.5 M with ammonium sulfate, and the precipitate was dissolved in 0.2 volume of 0.1 M phosphate buffer, pH 6.0.

Calcium Phosphate Gel Adsorption and Elution—The preparation was diluted with cold water to give an ammonium sulfate level of 0.02 M, and the activity was adsorbed on calcium phosphate gel; 15 to 20 mg of gel were required per mg of protein. The activity was eluted from the gel with 0.1 M phosphate, pH 6.0; 1 ml of buffer per 2000 units of aldolase adsorbed was used. Sufficient buffer was added to elute 75% of the activity. At this point the protein level was about 0.2 mg per ml. Consequently the preparation was dialyzed for 3 to 4 hours against 20 volumes of cold water, lyophilized, and dissolved in a small volume (approximately 0.1 the original volume) of cold water to give a protein level of 2 mg per ml.

Crystallization—Ammonium sulfate was added to 1.5 M, and the precipitate was discarded. The enzyme was precipitated from the supernatant solution by adding ammonium sulfate to 3.0 M. The pellet was then extracted for about 10 minutes with 1.8 M ammonium sulfate solution, with the use of 1 ml per calculated 40,000 units of activity in the pellet. After centrifugation, saturated ammonium sulfate was cautiously added to the extract until a slight turbidity was visible. Within a few hours, crystals were evident, and crystallization was usually complete upon overnight storage in the refrigerator.

Recrystallization—The crystals were dissolved in cold water to a level of more than 5 mg of protein per ml. An approximately equal volume of cold, saturated ammonium sulfate was carefully added until a faint turbidity occurred. After this mixture was allowed to stand in the cold, crystals were obtained.

A summary of these steps is shown in Table I. On a second crystallization, crystals were obtained with a yield of 9.5% and a 500-fold purification. The specific activity was 13,000. The specific activity of other crystalline preparations ranged from 13,000 to 13,500.

Characteristics of 2-Keto-3-deoxy-6-phosphogluconic Aldolase

Physical Properties—As shown in Fig. 1, the crystals were well formed rhombohedrons measuring 10 to 20 μ across their long axis. No other crystal forms have been observed.

As seen in Fig. 2, the sedimentation velocity pattern of twice crystallized aldolase[2] revealed a single symmetrical peak with an s_{20} value of 4.5. The same sedimentation coefficient was obtained with noncrystalline aldolase (4) by the procedure of Martin and Ames (11).

The molecular weight of twice crystalline aldolase was determined from sedimentation equilibrium experiments and from the amount of pyruvate-1-^{14}C reductively bound to the aldolase in the presence of borohydride (Table II). Assuming a partial specific volume of 0.75 ml per g for the aldolase (7), the z-average molecular weight was calculated to be 106,750, and the weight average molecular weight from the linear portion of the plot of $(1/r)(dn/dr)$ *versus* n_c was 89,500. The weight average molecular weight was calculated to be 86,150. The higher z-average molecular weight suggests that a small amount of aggregate was present in the crystalline enzyme preparation, which was not evident in the velocity run. The molecular weights obtained are considered minimal since extrapolation to zero protein concentration was not attempted.

Grazi *et al.* (4) reported that 0.73 μmole of pyruvate was reductively bound by borohydride per μmole of aldolase. In their calculations the molecular weight, based upon an s_{20} value of 4.5, was assumed to be 50,000. However, on the assumption that a specific activity of 13,500 represents pure aldolase, a recalculation of these data has been undertaken. It was found that 3.78×10^2 μg of aldolase bound pyruvate-^{14}C to the extent of 3.48×10^4 c.p.m. From a specific activity of 4×10^6 c.p.m. per μmole, this radioactivity corresponds to 8.7×10^{-3} μmole of pyruvate. Thus, as shown in Table II, 1 mole of pyruvate was bound per aldolase unit of molecular weight 43,500 ($3.78 \times 10^2/8.7 \times 10^{-3}$). This value is about one-half of the observed molecular weight and suggests that the enzyme contains two active sites. This observation is consistent either with the existence of 1 aldolase molecule containing two active sites, or with a molecule consisting of two subunits of equal molecular weight. These possibilities were partially resolved by sedimentation equilibrium analysis of the enzyme (3 mg per ml) carried out in 0.02 M citrate, pH 7.6, 5.7 M guanidine-HCl, 0.1 M NaCl, and 0.1 M EDTA. Calculations showed the z-average molecular weight to be 94,000 and the weight average molecular weight from the linear portion of the plot of $(1/r)(dn/dr)$ *versus* n_c to be 49,500. This provides preliminary evidence for dissociation of the enzyme into two subunits by guanidine-HCl. Since high molecular weight components were also present, this suggests that guanidine-HCl either (*a*) dissociates a part of the enzyme and aggregates another portion or (*b*) completely dissociates the enzyme into subunits, some of which then aggregate to form the high molecular weight material observed.

The adsorption spectrum and difference spectrum with respect to bovine serum albumin are shown in Fig. 3. There is appreciably more adsorption in the region of 290 mμ, as is visible in both recordings. The *dashed line* in the figure shows the position of a base-line corresponding to equal absorbances at 280 mμ for each cuvette.

The specific activity of the crystals was 13,500 units per mg of protein. This corresponds to a turnover number of 3.2×10^4 moles of KDP-gluconate cleaved per minute per mole of aldolase.

2-Keto-3-deoxy-6-phosphogluconic aldolase crystallized from *P. saccharophila*[3] has essentially the same specific activity in the assay used in these experiments (3), but differs with respect to sedimentation constant, the ability to produce antibodies

[2] We are indebted to Dr. William Deal for performing the centrifugation studies and to Dr. Robert Brunner for the use of the Spinco model E centrifuge.

[3] C. W. Shuster and M. J. Doudoroff, personal communication.

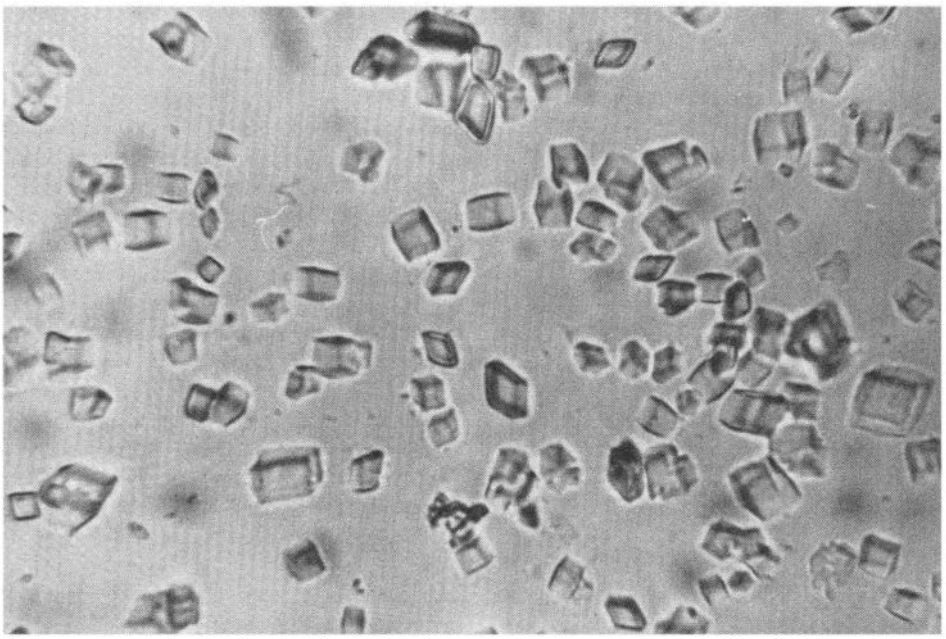

FIG. 1. 2-Keto-3-deoxy-6-phosphogluconic aldolase as crystallized from ammonium sulfate. Total magnification, 500-fold; phase contrast microscopy.

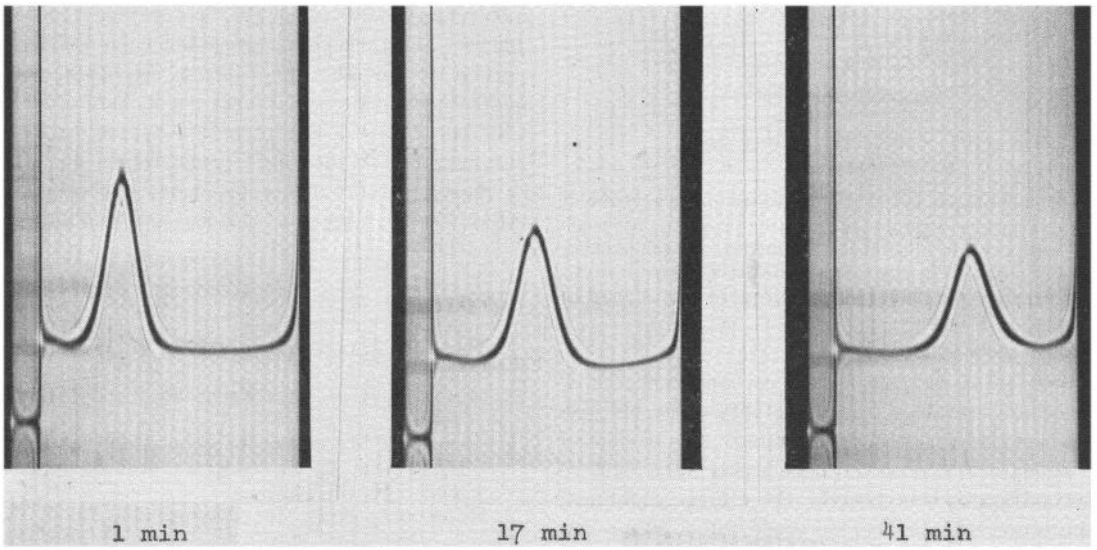

FIG. 2. Sedimentation velocity pattern of 2-keto-3-deoxy-6-phosphogluconic aldolase after 1, 17, and 41 minutes at 59,780 r.p.m. The enzyme (10 mg per ml) was dissolved in 0.05 M phosphate, pH 6.0, and 0.15 M NaCl. Direction of sedimentation is to the *right*.

which do not react with KDP-gluconic aldolase from *P. fluorescens*, and a different crystal structure (needles *versus* rhombohedrons).

Stability—The aldolase is stable in 0.1 N HCl for 4 hours. After incubation under these conditions, immediate neutralization was accomplished by addition of 0.0002 ml directly to an otherwise complete assay system. The rate of cleavage was immediately linear from the first time of observation, which was less then 5 seconds after neutralization. This is in contrast to the slow recovery of activity of muscle aldolase resulting from the reassociation of subunits formed in acid (12).

The crystalline enzyme dissolved in water was stable for 48 hours at room temperature. Subsequent activity loss was associated with the growth of microorganisms. At pH 9, activity was lost rapidly, but could be partially restored by acidifying the enzyme to pH 1 with HCl, followed by neutralization. This suggests that the conformation of the active site of KDP-gluconic aldolase is dependent upon the protonation of amino acid residues.

Substrate Specificity—As was previously demonstrated (3), the aldolase catalyzes the reversible cleavage of 2-keto-3-deoxy-6-phosphogluconate to form pyruvate and D-glyceraldehyde 3-phosphate, as well as the enolization of pyruvate. Similarly, muscle aldolase enolizes dihydroxyacetone phosphate (13).

TABLE II

Molecular weight of 2-keto-3-deoxy-6-phosphogluconic aldolase

Equilibrium experiments were carried out in a short column sedimentation equilibrium cell on twice crystalline enzyme (10 mg per ml) dissolved in and dialyzed against 0.05 M phosphate, pH 6.0, and 0.15 M NaCl. The pyruvate binding data were recalculated from the data of Grazi *et al.* (4) with a molecular weight of 87,825 and a specific activity of 13,500 units per mg of protein for pure aldolase.

	Molecular weight
z-Average	106,750
Weight average (linear plot of $(1/r)(dn/dr)$ *versus* n_c)	89,500
Weight average	86,150
Pyruvate-1-^{14}C + $NaBH_4$	43,500

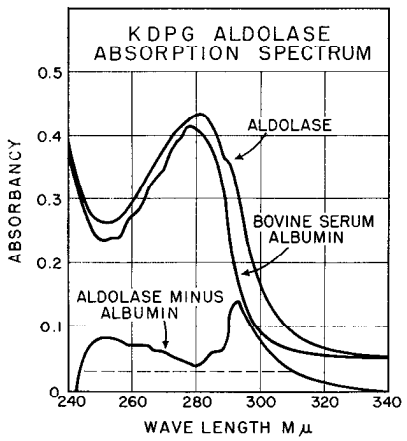

FIG. 3. Comparison of the ultraviolet absorption spectrum of crystalline 2-keto-3-deoxy-6-phosphogluconic (*KDPG*) aldolase and crystalline bovine serum albumin. A solution of each protein (0.5 mg per ml) was scanned in the Cary model 15 spectrophotometer. For the difference spectrum, serum albumin was placed in the reference beam.

Thus the specificity of an aldolase for the carbonyl acceptor of the aldehyde may be ascertained from the ability of the aldolase to bind and enolize the acceptor. Accordingly, the ability of KDP-gluconic aldolase to enolize the next higher homologue of pyruvate, α-ketobutyrate, was tested. In a total volume of 0.2 ml, α-ketobutyrate, 8.25 μmoles; imidazole buffer, 50 μmoles, pH 8.0; and aldolase, 140 units, were incubated in T_2O (5 μc) for 3 hours at 20°. The reaction mixture was quantitatively transferred to a Dowex 1-Cl column (0.75 × 5 cm), exhaustively washed with water, and eluted with a linear gradient (0 to 0.05 N HCl). Fractions (5 ml each) were assayed for α-keto acid with semicarbazide and also for radioactivity. It was found that 0.074 μg atom of hydrogen was incorporated per μmole of α-ketobutyrate isolated. Since the amount of enzyme was sufficient to cleave and presumably enolize 3.36 μmoles of substrate per minute, it is concluded that the aldolase cannot enolize α-ketobutyrate and hence cannot utilize it as a substrate.

The specificity of the aldolase for the aldehyde was also tested in an experiment in which pyruvate was incubated with glycolaldehyde phosphate. The reaction mixture, containing pyruvate-1-^{14}C, 4.92 μmoles (9.95 × 10^5 c.p.m. per μmole); glycolaldehyde phosphate, 4.6 μmoles; imidazole buffer, 150 μmoles, pH 8.0; and aldolase, 360 units, in a total volume of 1.0 ml, was incubated at room temperature for 5 hours. The reaction mixture was then chromatographed on Dowex 1-Cl as described above, and the fractions were assayed for radioactivity. Only a single radioactive peak corresponding to the elution position of pyruvate was found. Under similar conditions, pyruvate and glyceraldehyde 3-phosphate yielded 2-keto-3-deoxy-6-phosphogluconate (3). Since the amount of enzyme used presumably was sufficient to enolize about 8 μmoles of pyruvate per minute, it is concluded that glycolaldehyde phosphate, as a carbonium ion, cannot compete in the condensation reaction.

In view of the fact that 2-keto-3-deoxy-gluconic acid is not cleaved (2), it appears that high specificity exists for all substrates and reactants.

SUMMARY

2-Keto-3-deoxy-6-phosphogluconic aldolase has been crystallized by conventional procedures from extracts of *Pseudomonas fluorescens* grown on glucose. A single homogeneous peak (s_{20} = 4.5) was displayed in a sedimentation velocity run. Sedimentation equilibrium experiments showed a weight average molecular weight of 86,150, a z-average molecular weight of 106,750, and a weight average molecular weight from the linear portion of the plot, $(1/r)(dn/dr)$ *versus* n_c, of 89,500. Two moles of pyruvate were bound per mole of enzyme by reduction with borohydride, and guanidine hydrochloride dissociated the enzyme into subunits of molecular weight 49,500.

The aldolase did not enolize α-ketobutyrate or form a condensation product with pyruvate and glycolaldehyde phosphate.

REFERENCES

1. KOVACHEVICH, R., AND WOOD, W. A., *J. Biol. Chem.*, **213**, 757 (1955).
2. MACGEE, J., AND DOUDOROFF, M., *J. Biol. Chem.*, **210**, 617 (1954).
3. MELOCHE, H. P., AND WOOD, W. A., *J. Biol. Chem.*, **239**, 3511 (1964).
4. GRAZI, E., MELOCHE, H., MARTINEZ, G., WOOD, W. A., AND HORECKER, B. L., *Biochem. and Biophys. Research Communs.*, **10**, 4 (1963).
5. HORECKER, B. L., PONTREMOLI, S., RICCI, C., AND CHENG, T., *Proc. Natl. Acad. Sci. U. S.*, **47**, 1949 (1961).
6. GRAZI, E., CHENG, T., AND HORECKER, B. L., *Biochem. and Biophys. Research Communs.*, **7**, 250 (1962).
7. VAN HOLDE, K. E., AND BALDWIN, R. L., *J. Phys. Chem.*, **62**, 734 (1958).
8. WOOD, W. A., AND GILFORD, S. R., *Anal. Chem.*, **2**, 601 (1961).
9. WOOD, W. A., AND GILFORD, S. R., *Anal. Chem.*, **2**, 599 (1961).
10. MELOCHE, H. P., AND WOOD, W. A., *J. Biol. Chem.*, **239**, 3505 (1964).
11. MARTIN, R. G., AND AMES, B. N., *J. Biol. Chem.*, **236**, 1372 (1961).
12. DEAL, W. C., RUTTER, W. J., AND VAN HOLDE, K. E., *Biochemistry*, **2**, 246 (1963).
13. ROSE, I. A., AND RIEDER, S. V., *J. Biol. Chem.*, **231**, 315 (1958).