PURIFICATION AND CHARACTERIZATION OF 2-KETO-3-DEOXY-6-PHOSPHOGLUCONATE ALDOLASE FROM *AZOTOBACTER VINELANDII*: EVIDENCE THAT THE ENZYME IS BIFUNCTIONAL TOWARDS 2-KETO-4-HYDROXY GLUTARATE CLEAVAGE

Taha S.M. Taha and Thomas L. Deits*

Department of Biochemistry
Michigan State University
East Lansing, Michigan 48824

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2-keto-3-deoxy-6-phosphogluconate aldolase (E.C. 4.1.2.14) has been purified in two chromatographic steps to 99% purity in 73% overall yield from *Azotobacter vinelandii*. The pure enzyme is a 70 kD trimeric Class I aldolase, inhibitable by bromopyruvate or pyruvate plus sodium borohydride, with a specific activity of 625 μmol per min per mg protein and a K_m of 38 μM for 2-keto-3-deoxy-6-phosphogluconate. The enzyme also has 2-keto-4-hydroxy glutarate aldolase (E.C. 4.1.3.16) activity, with a specific activity of 4.8 μmol per min per mg protein and a K_m of 39 μM. 2-keto-4-hydroxy glutarate inhibits the 2-keto-3-deoxy-6-phosphogluconate aldolase activity of the enzyme with an apparent K_i of 0.17 mM. Slow steps following formation of the Schiff base intermediate between KHG and the enzyme are responsible for both the slower turnover of this substrate and for its inhibitory effect.

The Entner-Doudoroff pathway is widely distributed among prokaryotes and eukaryotes (1) and provides the only significant glycolytic pathway in *Azotobacter* (2,3). The high energy demands of nitrogen auxotrophy and the air sensitivity of the enzymes of nitrogen fixation place special regulatory burdens on *Azotobacter* metabolism (4), and so an understanding of the structure and function of the enzymes of this pathway is important to understanding the physiology of this organism.

In its most common form, the Entner-Doudoroff pathway involves the dehydration of 6-phosphogluconate to produce 2-keto-3-deoxy-6-phosphogluconate (KDPG) which is then cleaved to produce glyceraldehyde-3-phosphate and pyruvate. 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase (E.C. 4.1.2.14) catalyses the KDPG cleavage step in the

*Corresponding author. Fax: (517) 353-9334.

Abbreviations used: KDPG, 2-keto-3-deoxy-6-phosphogluconic acid; KHG, 2-keto-4-hydroxy glutarate; DSS, dissuccinimidyl suberate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; Mes, 4-morpholine-ethanesulfonic acid; Bicine, N,N-bis(2-hydroxyethyl)-glycine.

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pathway. KDPG aldolase has been purified from *Pseudomonas fluorescens* (5), *Pseudomonas putida* (6) and *Zymomonas mobilis* (7). The *eda* gene coding for KDPG aldolase has been cloned from *Z. mobilis* (8) and *Escherichia coli* (9). The three-dimensional structure of the *P. putida* KDPG aldolase has been reported to 2.8 Å resolution (10).

It was recently reported that in *E. coli*, the *eda* gene is the same gene, termed *hga*, encoding the enzyme previously identified as 2-keto-4-hydroxy glutarate (KHG) aldolase (E.C. 4.1.3.16) (9). Given the modest similarity of the deduced protein sequences of *eda* genes from the *E. coli* and *Z. mobilis* (approximately 50% identity, 65% similarity (9)), it is at present uncertain whether bifunctionality for KHG and KDPG is a common feature of this enzyme. We present evidence that *A. vinelandii* KDPG aldolase is bifunctional for cleavage of KDPG and KHG with a specific activity for KDPG comparable to that of other KDPG aldolases (5,6,7) and a specific activity for KHG comparable to that reported for the *E. coli* KHG aldolase (12). We also provide preliminary kinetic evidence which suggests that KHG and KDPG share the same active site.

Experimental Procedures

Materials. DSS and the Micro BCA (bicinchoninic acid) protein assay kit were purchased from Pierce Chemical Co. 3-bromopyruvate, fructose-1,6-bisphosphate, α-glycerophosphate dehydrogenase, triose phosphate isomerase, fructose-1,6-bisphosphate aldolase, lactic dehydrogenase and column materials were from Sigma Chemical Company (St. Louis, MO). KDPG was synthesized enzymatically and purified as described by O'Connell and Meloche (13). KHG was synthesized by the method of Dekker and Maitra (14) from *threo*-D,L-4-hydroxy glutamate generously provided by E. Dekker.

Enzyme purification. KDPG aldolase was purified from *A. vinelandii* (ATCC strain 13705) grown at 37° C on ATCC Medium 13 containing 20g/L glucose as the only carbon source. The purification was carried out in 25 mM Tris-HCl buffer pH 8.0 containing 50 mM sodium fluoride, 2 mM $MnCl_2$, 10 mM β-mercaptoethanol, and 10% glycerol (isolation buffer). β-mercaptoethanol and $MnCl_2$ were added to the buffer after deareation by repeated vacuum degassing and argon sparging. It is not necessary to deareate the buffer or add sodium fluoride, $MnCl_2$, and β-mercaptoethanol for aldolase purification; they are required for the parallel purification of 6-phosphogluconate dehydratase from the same extract (details to be published elsewhere).

30 g of bacteria were suspended in isolation buffer containing 1 mM phenylmethylsulfonyl fluoride at a ratio of 1 gm bacteria per 2 ml buffer. The bacterial suspension was French pressed at 16,000 psi and centrifuged at 20,000*g* for 20 minutes to remove unbroken cells and cell debris. The supernatant from this step was centrifuged at 40,000*g* for 30 minutes and the supernatant loaded onto a Q-Sepharose column (2 x 20 cm) previously equilibrated with isolation buffer. The aldolase is only slightly retained on the column and elutes after the column is washed with approximately 100 mL of isolation buffer.

The fractions containing aldolase activity were combined and 50 mM citrate buffer pH 3.5 containing 1M sodium chloride was added to bring the sodium chloride concentration to 0.1 M. The pH was adjusted to 2.0 with hydrochloric acid and the solution incubated with stirring at 2° C for one hour. The pH was then adjusted to 3.5 with sodium hydroxide and the precipitated proteins removed by centrifugation. The supernatant was loaded onto an S-Sepharose column (2 x 20 cm) equilibrated with 50 mM citrate buffer pH 3.5 containing 0.1

M sodium chloride. The column was washed with 200 mL of the same buffer and the aldolase eluted with a 0.1-1.0 sodium chloride gradient (400 ml total volume) in the same buffer. The fractions with aldolase activity were combined and the pH adjusted to 6.0 with sodium hydroxide. If further purification was necessary, as for crosslinking studies, the enzyme was concentrated 10-fold using an Amicon concentrator and applied to a Sephadex S-200 column (2 x 40 cm) equilibrated with 25 mM Tris-HCl buffer pH 8.0 containing 0.1 M sodium chloride. The fractions with aldolase activity were combined, dialyzed against the appropriate buffer (see below) and stored in liquid nitrogen.

Assay of KDPG aldolase activity. KDPG aldolase activity was determined using a coupled assay with lactic dehydrogenase (15). The assay mix contained 10 mM Tris-HCl, pH 8.0, 0.15 mM NADH, 50 μM KDPG, and 10 units of lactic dehydrogenase. Initial rates of reaction were monitored at 340 nm. One unit of enzyme activity produces 1 μmole of NADH per minute. For studies on salt inhibition of KDPG aldolase, lactic dehydrogenase and KDPG aldolase were dialyzed versus 10 mM Tris-HCl, pH 8.0. All reported values are the averages of at least duplicate determinations.

Assay of KHG aldolase activity. KHG aldolase activity was determined by a modification of the method of Kobes and Dekker (16). The assay mix contained 10 mM bicine, pH 8.5, 0.15 mM NADH, 50 μM KHG, and 10 units of lactic dehydrogenase in a final volume of 1 ml. Initial rates of reaction were monitored at 340 nm.

Determination of the quaternary structure of KDPG aldolase.

Crosslinking studies were carried out on purified aldolase dialyzed overnight against 10 mM HEPES buffer pH 8.0 or 10 mM MES buffer, pH 5.5. The enzyme solution was adjusted to a final protein concentration of 1 mg/mL and treated with a 20 fold molar excess of DSS. The crosslinker was prepared as a 10.5 mM stock in dimethyl sulfoxide. 5 μl of this stock were added to the aldolase solution and the volume was adjusted to 100 μL by addition of the appropriate buffer. The reaction was allowed to proceed for 30 minutes at room temperature and then quenched by addition of 100 mM lysine to a final concentration of 10 mM. Control experiments were conducted in parallel using fructose-1,6-diphosphate aldolase from rabbit muscle.

Other methods. Fructose-1,6-bisphosphate aldolase activity was determined by a coupled assay involving α-glycerophosphate dehydrogenase and triose phosphate isomerase (17). KHG concentration was determined according to the method of Dekker and Maitra (14), with the exceptions that: 1. All volumes were reduced 10-fold. 2. The alkaline 2,4-dinitrophenylhydrazone solution was centrifuged (5 min at 18,000*g*) prior to measurement. 3. Derivative concentration was determined at 530 nm using α-ketoglutarate (Sigma) as a gravimetric standard.

Protein concentration was determined by the bicinchoninic acid method (18) using the procedures supplied by the manufacturer (Pierce Chemical Co.) with bovine serum albumin as a standard. Proteins were separated on 12.5% acrylamide gels as described by Laemmli (19). Samples were prepared for electrophoresis by treatment with 67.5 mM Tris-HCl pH 6.8 containing 3% SDS, 20% glycerol, 5% β-mercaptoethanol, and .03% bromophenol blue. Gels were stained with Coomassie blue, and scanned on a Bio-Image densitometer to quantify relative band intensity.

RESULTS AND DISCUSSION

Purification of A. vinelandii KDPG aldolase. The purification is summarized in Table I. The specific activity of the enzyme is increased 500 fold, with 73% yield through the S-Sepharose step. Figure 1 illustrates the progress of the purification. The purified protein consists of a single subunit of M_r ca. 24,000. Scanning densitometry indicates that the enzyme is more than 99% pure after S-sepharose chromatography. The slight increase in

Table I
Purification of *A. vinelandii* KDPG aldolase

	Volume (ml)	activity (units/ml)	protein (mg/ml)	Specific activity (units/mg protein)	Extent of purification	Yield (%)
Cell extract	76	30.6	36.4	1.2	-	100
Q-Sepharose	63	32.8	3.6	9.1	7.4	89
Acid precipitation	69	33.6	2.45	13.7	11.2	100
S-Sepharose (after concentrating pooled fractions)	2.45	696	1.15	605	492	73
S-200	33.6	19.5	0.031	625	508	28

activity after acid treatment is reproducible. The only other purification to homogeneity for this enzyme, from *Pseudomonas putida*, required seven purification steps, including crystallization (6).

Quaternary structure of KDPG aldolase. Crosslinking studies (Figure 2) show that the M_r 24,000 subunits are converted to a major species with M_r 70,000 by SDS-PAGE. This M_r value is consistent with a trimeric native quaternary structure, as previously determined for *P. putida* KDPG aldolase by X-ray crystallography (10). A higher molecular mass species

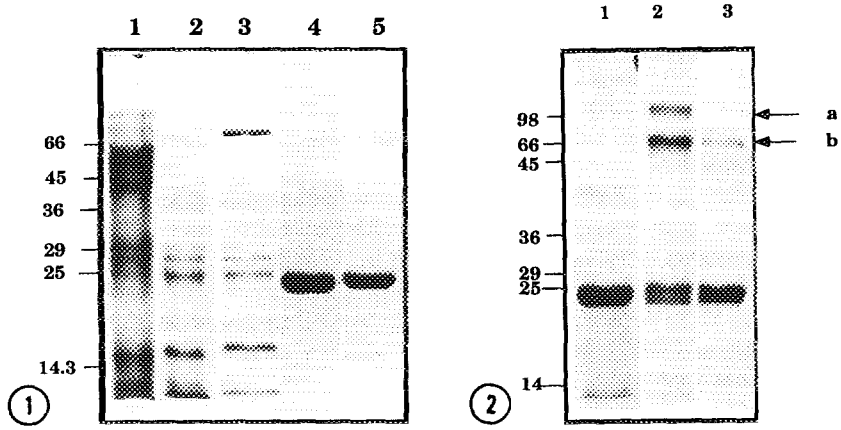

FIG. 1. SDS-PAGE showing the progress of purification of KDPG aldolase. Lane 1, extract prior to Q Sepharose chromatography; lane 2, pooled fractions after Q-Sepharose chromatography; lane 3, material after acid precipitation; lane 4, pooled fractions after S-Sepharose chromatography; lane 5, pooled fractions from S-200 chromatography.

FIG. 2. Examination of the subunit structure of KDPG aldolase by chemical crosslinking. The crosslinking reaction was carried out as described in EXPERIMENTAL PROCEDURES. Lane 1, untreated KDPG aldolase; lane 2, KDPG aldolase crosslinked at pH 8.0 in 10 mM HEPES buffer; lane 3, KDPG aldolase crosslinked at pH 5.5 in 10 mM MES. The position of the tetramer and trimer are indicated by (a) and (b), respectively.

(possibly a tetramer) is present when the crosslinking experiment is carried out at pH 8.0 (Figure 2, lane 2) but not at pH 5.5 (Figure 2, lane 3). This may be a consequence of the higher reactivity of the enzyme to crosslinker at higher pH, so that intermolecular crosslinking becomes significant.

Kinetic properties of A. vinelandii KDPG aldolase. The purified enzyme has a specific activity of 625 units/mg protein (Table I) and a K_m for KDPG of 38 μM. The specific activity is higher, and the K_m value lower than those reported for the *P. putida* (330 units/mg and 73 μM) (6) and *Z. mobilis* (600 units/mg and 250 μM) KDPG aldolases (7). Our lower K_m values may reflect our efforts to minimize the anion concentration in our assay system; NaCl is a competitive inhibitor of *A. vinelandii* KDPG aldolase with a K_i of 25 mM (Figure 3); the effect is due to the anionic moiety (Table II). *A. vinelandii* KDPG aldolase also catalyzes KHG cleavage, albeit much more slowly than KDPG cleavage. While the K_m value for KHG was 39 μM, essentially identical to the value for KDPG, V_{max} for KHG aldolase was 4.8 μmol/min-mg protein, or 140-fold slower. Despite this low rate, the V_{max} value compares favorably with that reported for the KHG aldolase of *E. coli*, 3.4 μmol/min-mg (12). The K_m value we observe is significantly lower than that reported for *E. coli* KHG aldolase, 2.4 mM, and may also reflect differences in ionic strength between assay conditions (see above).

Inhibition of KDPG aldolase activity by KHG. We have tested whether KHG and KDPG share common binding sites on the enzyme; Figure 4 shows that KHG inhibits KDPG

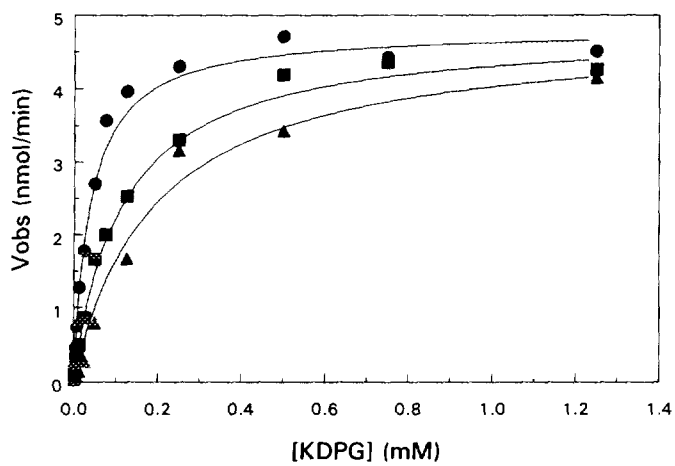

FIG. 3. Effect of salt on KDPG aldolase catalysis. Assays were carried out in the assay mix as described EXPERIMENTAL PROCEDURES with the addition of 0 mM (●), 50 mM (■), or 100 mM (▲) sodium chloride. Lines drawn represent the calculated effect of a competitive inhibitor with K_i = 25 mM on enzyme activity, given a K_m of 38 mM and a V_{max} of 4.8 μmol/min.

Table II
Effect of salts on *A. vinelandii*
KDPG aldolase catalyzed KDPG cleavage

Salt added	% Activity
None	100
Sodium Fluoride	55
Potassium Chloride	33
Sodium Chloride	24
Sodium Bromide	12
Potassium Iodide	7
Sodium Iodide	7

Inhibition was determined with 100 mM of the indicated salt and 50 μM KDPG in the assay mixture described in EXPERIMENTAL PROCEDURES.

aldolase catalyzed KDPG cleavage with an apparent K_i value of 0.17 mM, independent of the concentration of KDPG present. The fact that KHG inhibits KDPG aldolase activity in a concentration range comparable to the measured K_m value for KHG catalysis supports a

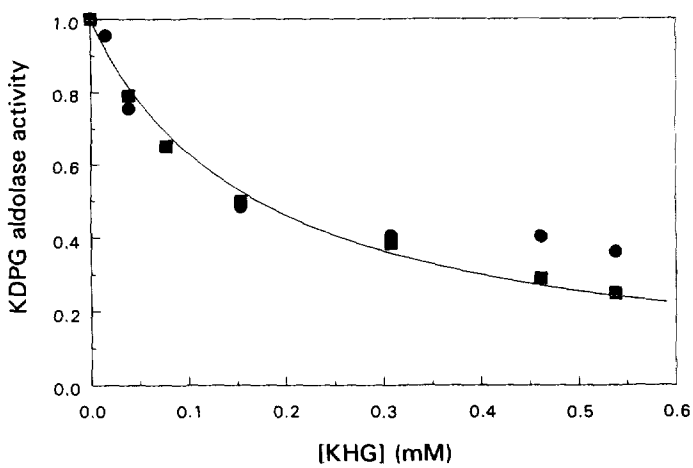

FIG. 4. Effect of KHG on KDPG cleavage catalyzed by KDPG aldolase. Rates of KDPG cleavage were measured in the presence of the indicated concentrations of KHG at KDPG concentrations of 33 μM (●) and 1.42 mM (■). The line drawn represents the calculated effect of a inhibitor with an apparent inhibition constant of 0.17 mM. The two sets of data were normalized to the same rate in the absence of inhibitor. The small amount of KHG cleavage that accompanies a study of this type will contribute less than 1% of the observed rate, even at high KHG concentrations.

model in which KHG and KDPG cleavage occur in the same active site. In the simplest case, it would be expected that alternate substrates sharing the same active site would compete for binding and thus affect V_{max}/K_m but not V_{max} (26). The V_{max} for KDPG is clearly affected by KHG, since even 40-fold increase in KDPG concentration does not materially affect the ability of KHG to inhibit the enzyme. This effect can be explained by postulating that a step occurring at a rate significantly slower than the turnover rate for KDPG occurs between KHG binding and product release. One plausible site for such a slow step would be in steps following formation of the Schiff base intermediate of KHG. *A. vinelandii* KDPG aldolase was 99.9% inactivated by 20 mM pyruvate plus 10 mM sodium borohydride treatment and 90% inhibited by 20 mM 3-bromopyruvate, as expected for a Class I aldolase. A rate of breakdown of the Schiff base intermediate 140-fold slower than the turnover of KDPG would account for the observed difference in turnover number, and would lead to a substantial effect on V_{max} for KDPG. According to this hypothesis KHG acts essentially as a mechanism-based inhibitor of KDPG catalysis. Mechanism-based inhibitors typically affect both V_{max} and V_{max}/K_m values (27), as observed. We can infer further that since enzymatic cleavage of either KHG or KDPG must produce the same Schiff base intermediate (between the enzyme and pyruvate), it is likely that the slow step in the reaction precedes formation of this common intermediate.

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