COMMUNICATION

Oxidation of 2-Keto-4-hydroxyglutarate by Pig Heart and *Escherichia coli* α-Ketoglutarate Dehydrogenase Complex

Enzyme preparations from pig heart and *Escherichia coli* have been found to catalyze a NAD^+- and CoASH-dependent oxidation of 2-keto-4-hydroxyglutarate. Several independent lines of evidence indicate that 2-keto-4-hydroxyglutarate is a substrate for the well-known α-ketoglutarate dehydrogenase complex of the citric acid cycle. The evidence includes (a) a constant ratio of specific activity values for the two substrates throughout purification, (b) identical elution profiles from a $Ca_3(PO_4)_2$ gel–cellulose column, (c) the same sucrose density sedimentation patterns, (d) similar responses in controlled heat inactivation studies, and (e) identical pH–activity curves.

L-Hydroxyproline, a major constituent of collagen, is degraded by mammals with formation of the following intermediates in sequence: Δ^1-pyrroline-3-hydroxy-5-carboxylate, erythro-4-hydroxy-L-glutamate, and 2-keto-4-hydroxyglutarate (1, 2). The latter intermediate, KHG,[1] subsequently undergoes an aldol cleavage yielding pyruvate plus glyoxylate (3), a reaction catalyzed by KHG-aldolase. Methods for preparing KHG-aldolase in highly purified or homogeneous form from extracts of bovine liver (4) and *Escherichia coli* K-12 (5) have been developed. Modes of KHG catabolism, other than aldolytic cleavage, have been suggested. In a brief report, Payes and Laties (6) reported the presence of dehydrogenase activity that catalyzed the conversion of KHG to malate; such activity was detected in mitochondrial preparations from sweet potato and rat liver as well as in acetone powder extracts of *Chlorella*. These investigators found that the level of this dehydrogenase activity decreased with α-KG as substrate but increased with KHG when sweet potato mitochondria were frozen and thawed, the pH–activity maxima were significantly different for the two substrates (i.e., pH 6.5 vs 7.1 for α-KG and KHG, respectively), and the activity in acetone powder extracts of *Chlorella* was five times greater with KHG than with α-KG as substrate. It was concluded that the dehydrogenase activity observed toward KHG is not identical with the α-KG dehydrogenase complex known to function in the citric acid cycle.

We recently obtained evidence that the CoASH-dependent oxidation of KHG by NAD^+ is, indeed, catalyzed by the α-KG dehydrogenase complex.

Materials and methods. DL-KHG was synthesized by nonenzymatic transamination of either threo- or erythro-4-hydroxy-DL-glutamic acid (3). Protein was estimated by the method of Lowry *et al.* (7). KHG-Aldolase activity was determined by measuring the formation of glyoxylate from DL-KHG (8); one unit of enzyme activity is defined as the amount that liberates 1.0 μmol of glyoxylate in 20 min at 37°C. Rat liver mitochondria were prepared by the method of Schneider (9). The procedure of Payes and Laties (6) was followed for polarographic measurement of O_2 uptake as a measure of KHG-dehydrogenase activity. The α-KG dehydrogenase complex was purified from pig heart according to the method of Sanadi *et al.* (10) as modified by Massey (11); specific activity $\cong 20$ to 30. Homogeneous preparations of *E. coli* α-KG dehydrogenase complex were generous gifts from Dr. Lester J. Reed; specific activity $\cong 100$. Absorbance measurements at a fixed wavelength were made with a Gilford Model 2000 spectrophotometer.

The CoASH-dependent oxidation of α-KG or KHG by NAD^+ was followed at 340 nm and 25°C. For the pig heart dehydrogenase complex, the reaction mixtures (1.0 ml) contained (in micromoles): 60, potassium phosphate buffer, pH 7.4; 3, cysteine·HCl; 0.05, CoASH; 0.333, NAD^+; 0.2 to 2.0, α-KG or KHG. For the dehydrogenase complex from *E. coli*, the reaction mixtures (1.0 ml) contained (in micromoles): 50, potassium phosphate buffer, pH 8.0; 3, cysteine·HCl; 1, $MgCl_2$; 0.06, CoASH; 2, NAD^+; 0.2, thiamine pyrophosphate; 0.2 to 2.0, α-KG or KHG. In both cases, the reaction was initiated by adding enzyme; one unit of enzyme activity is defined as the amount that causes an absorbance change of 1.0 per minute. Specific activity is expressed as units per milligram of protein.

Ferricyanide-linked dehydrogenase activity can also be measured. For this purpose, the mixtures (3.0 ml)

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[1] Abbreviations used: KHG, 2-keto-4-hydroxyglutarate; α-KG, 2-ketoglutarate; CoASH, reduced coenzyme A.

contained 150 μmol of potassium phosphate buffer, pH 7.0, 2 μmol of potassium ferricyanide, 2 mg of bovine serum albumin, and 2 to 10 μmol of either α-KG or KHG. Addition of enzyme initiated the reaction which was followed spectrophotometrically at 410 nm and 25°C.

For sucrose gradient centrifugation, gradients (10–40%) were prepared in 0.01 M potassium phosphate buffer, pH 8.0, according to the method of Martin and Ames (12). Sedimentation was performed with an SW-50.1 rotor in a Beckman Model L-350 ultracentrifuge at 40,000 rpm and 4°C for 3.5 h. After centrifugation, each tube was pierced at the bottom and 0.1-ml fractions were collected; these were checked for activity by the NAD$^+$- and CoASH-dependent dehydrogenase assay.

Results and discussion. KHG-Aldolase activity in rat liver mitochondria. When rat liver mitochondrial preparations were tested for their ability to catalyze the aldol cleavage of KHG, significant KHG-aldolase activity was observed (approximate specific activity = 0.16). Since this aldolase catalyzes the formation of equimolar amounts of glyoxylate and pyruvate from KHG [KHG $\rightleftharpoons$ glyoxylate + pyruvate] (3) and since the pyruvate formed in this manner can be oxidized by the pyruvate dehydrogenase system of mitochondria, oxygen will be consumed when KHG is incubated with mitochondrial preparations even though no KHG dehydrogenase activity may be present. Simple measurement of O_2 consumption, therefore, as followed polarographically by Payes and Laties (6) and also ourselves when KHG is added to mitochondria, does not provide a valid measure of dehydrogenase activity toward KHG.

Oxidation of KHG by α-ketoglutarate dehydrogenase complex from pig heart. We purified the α-KG dehydrogenase complex from pig heart and tested all fractions for activity with both α-KG and KHG. The ratio of specific activities for these two substrates was found to remain essentially constant (α-KG/KHG = 16–17) throughout all steps of purification including (a) freezing and thawing of the homogenate, (b) ammonium sulfate fractionation, (c) adsorption and elution from a column of calcium phosphate gel-cellulose, and (d) precipitation of activity from the column eluate with ammonium sulfate. Additionally, as shown in Fig. 1, dehydrogenase activity with α-KG or KHG as substrate has the same elution profile from a calcium phosphate gel-cellulose column and the ratios of specific activities throughout the enzymatically active peak are also quite constant.

The ratio of specific activities toward α-KG and KHG remains unchanged when purified preparations of the pig heart dehydrogenase complex are subjected to controlled heat denaturation (Table I). Very similar results were obtained when we examined the effect that varying pH has on dehydrogenase activity;
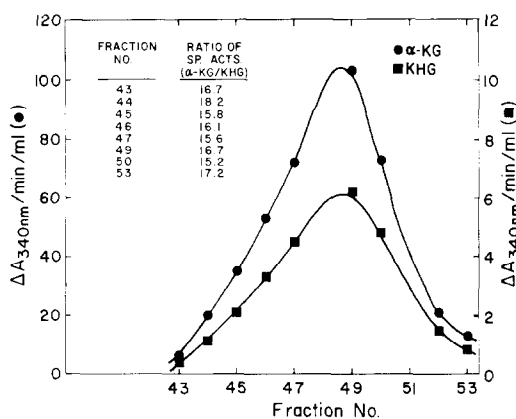

FIG. 1. Elution profile of pig heart dehydrogenase activity from a $Ca_3(PO_4)_2$ gel–cellulose column, using α-KG and KHG as substrates. Fractions were tested by the NAD$^+$- and CoASH-dependent assay.

pH–activity profiles for dehydrogenase activity with α-KG and KHG are identical with a maximum at pH 6.65. Furthermore, when the dehydrogenase complex purified from pig heart was frozen and thawed five times over a period of 3 days, no effect on dehydrogenase activity toward α-KG or KHG was observed.

Oxidation of KHG by α-ketoglutarate dehydrogenase from E. coli. Homogeneous preparations of the α-KG

TABLE I

ACTIVITY WITH α-KG AND KHG DURING HEAT DENATURATION OF PIG HEART α-KG DEHYDROGENASE COMPLEX[a]

Time (min)	Specific activity ($\Delta A_{340\,nm}$/min/mg)		Ratio of specific activities (α-KG/KHG)
	α-KG	KHG	
0	34.4	2.02	17.0
5	25.4	1.59	16.0
10	17.1	1.00	17.1
15	17.3	1.02	17.0
20	13.6	0.72	18.9
25	12.1	0.71	17.0
30	9.5	0.59	16.1
35	8.1	0.40	20.3

[a] Purified dehydrogenase preparations were diluted 10-fold with 0.02 M potassium phosphate buffer, pH 7.4, and incubated at 32°C. Aliquots were removed at 5-min intervals and the rate of α-KG and KHG oxidation was determined by the NAD$^+$- and CoASH-dependent assay.
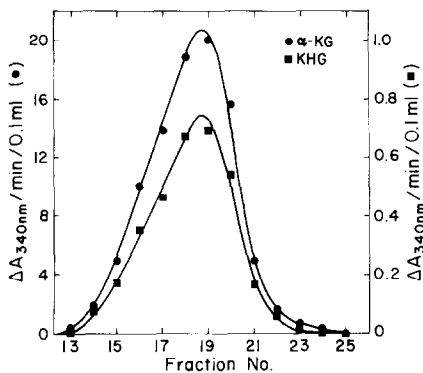
FIG. 2. Sucrose density gradient sedimentation profile of *E. coli* α-KG dehydrogenase complex. Fractions were tested with α-KG and KHG as substrate in the NAD$^+$- and CoASH-dependent assay.

dehydrogenase complex from *E. coli* also catalyze the CoASH-dependent oxidation of either α-KG or KHG by NAD$^+$. The ratio of specific activity (α-KG/KHG), in this instance, is approximately 30. When such preparations are subjected to sucrose density gradient centrifugation, identical sedimentation profiles are obtained when activity is measured with either α-KG or KHG (Fig. 2). With these two substrates, the pH–activity profiles (optimum pH = 8.0) are also the same.

Although the rate of reaction is less with the hydroxylated derivative, these studies with the α-ketoglutarate dehydrogenase complex purified from pig heart and in homogeneous form from *E. coli* indicate that the same complex catalyzes the oxidation of both α-KG and KHG by NAD$^+$. Special dehydrogenase activity for KHG is not required. When ferricyanide-linked dehydrogenase incubation mixtures are examined, malate is readily determined to be the product of KHG oxidation. Furthermore, the expected stoichiometric relationship holds for the dehydrogenase-catalyzed oxidation of KHG; the conversion of 1 μmol of KHG to 1 μmol of malate is associated with the utilization of 2 oxidizing equivalents.

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SUBHASH C. GUPTA
EUGENE E. DEKKER

Department of Biological Chemistry
The University of Michigan
Ann Arbor, Michigan 48109
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