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Received September 9th, 1966

Biochim. Biophys. Acta, 132 (1967) 197–200

BBA 63229

A constitutive aldolase for 4-hydroxy-2-ketoglutarate in soil bacteria

4-Hydroxy-2-ketoglutarate (HKG), a product of mammalian enzymes[1–3], has also been isolated from higher plants[4,5]. HKG aldolase, which catalyzes the reversible formation of HKG from glyoxylate and pyruvate, has been purified from rat liver[6–8] but has not been described in plants or bacteria; HKG formation in Acetobacter extracts, however, has recently been reported[9] as an enzymic product of glyoxylate and oxaloacetate. This communication reports the partial purification of a constitutive enzyme from soil bacteria which catalyzes reversible cleavage of HKG to glyoxylate and pyruvate. From $NaBH_4$ inactivation studies, the bacterial aldolase may bind both glyoxylate and pyruvate as Schiff base ligands. The enzyme also appears to catalyze a non-stereospecific cleavage of HKG. In these unusual respects it resembles the highly purified mammalian enzyme[8].

DL-HKG was made chemically by the method of RUFFO *et al.*[10], using 10 mmoles each of sodium glyoxylate and oxaloacetic acid. After incubation (3 h, 37°), the reaction mixture was acidified to pH 4.0 (formic acid) and stirred 30 min to remove CO_2 and to complete decomposition of residual oxaloacetate. HKG was isolated by Dowex 1 formate chromatography (2 cm × 40 cm column), essentially as described earlier[2] except that the formic acid gradient was made with a 200-ml mixing chamber (initially 3.8 M formic acid) and a reservoir of 7 M formic acid. Residual glyoxylate appeared at about 100 ml, pyruvate at about 280 ml, and HKG at about 370 ml. HKG-containing eluates were concentrated to dryness several times from added water in a rotary evaporator (40°) to remove formic acid. The yield of DL-HKG averaged 75%, and neutralization required 2.00 to 2.16 equivalents of NaOH, relative to the assay with glutamic dehydrogenase[11] (EC 1.4.1.2). Time of exposure to acid was minimized to avoid decomposition (see below).

Certain properties of HKG were noted in addition to those already described[2,3]. Although neutralized solutions of HKG are stable for at least several months in the cold[11], acid solutions were unstable. In one trial, a solution of HKG initially at 86 mM and pH 2.6 (formic acid eluate) showed first-order decomposition (glutamate dehydrogenase assay) at 37°, with a half-life of about 9.5 days. Appreciable decomposition also occurred in acid solutions stored at −15°. The decomposition products were not identified; however, they appeared to be acid (relatively constant titration value during decomposition), and unreactive with 2,4-dinitrophenylhydrazine. In experiments with [5-^{14}C]HKG, synthesized enzymically[11] from pyruvate and [1-^{14}C]glyoxy-

Abbreviation: HKG, 4-hydroxy-2-ketoglutarate.

late, the decomposition products (2 major peaks were detected by radioactivity eluted from paper chromatograms) moved differently from [^{14}C]malic acid, a plausible decomposition product[2].

An aerobic Gram-negative rod was obtained from enrichment cultures of soil with HKG as carbon source. Cells grew well on 0.2% DL-HKG in liquid media containing mineral salts[12] and 0.1% $(NH_4)_2SO_4$; HKG was added by sterile filtration to the autoclaved medium. Growth and concomitant disappearance of HKG was most rapid at 37° and pH 5–6, of several conditions tested. Resting cells oxidized HKG without lag and at a similar rate after growth on HKG, α-ketoglutarate or L-alanine as carbon source. Stoichiometric experiments with resting cells indicated uptake of approximately 3 μmoles of O_2 per μmole of HKG added, about 85% of theory. To obtain extracts, cells were grown on α-ketoglutarate rather than HKG since the former provided better growth (2–3 g wet cells per l).

Cells were extracted by sonic lysis in water[12]. After low-speed centrifugation of sonicates (25 000 × *g*, 30 min), HKG aldolase activity of the supernatant fluid was assayed in 1 ml volume containing 5 μmoles of DL-HKG and 0.25 M Tris at pH 8.4. After 10 min at 37°, the reaction was stopped with trichloroacetic acid and the pyruvate formed was measured with lactate dehydrogenase (EC 1.1.1.27) and DPNH; this measurement was made in 0.25 M Tris (pH 8.4) under conditions permitting an estimate of pyruvate selectively in the presence of glyoxylate. One unit of enzyme refers to 1 μmole of pyruvate formed per min under the above conditions. Activity was linear with time and enzyme concentration up to about 0.04 units. All the activity of initial sonic extracts was recovered in high-speed supernatants (100 000 × *g*, 90 min), whose specific activity varied between 0.02 and 0.04 unit per mg of protein; this value is at least twice that of crude preparations of rat-liver HKG aldolase[8]. Specific activity was similar in extracts from cells grown on HKG, α-ketoglutarate or alanine, confirming the indication from whole cell studies that HKG enrichment had served to select a strain with a constitutive rather than an inducible HKG aldolase.

Enzyme was purified from α-ketoglutarate-grown cells through several steps

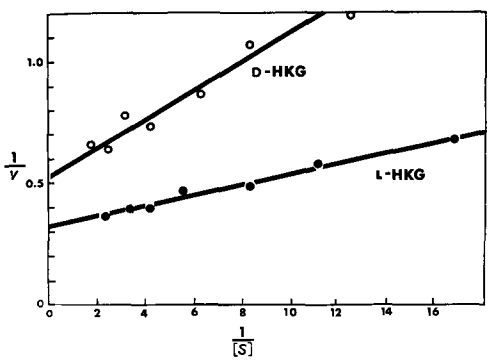

Fig. 1. Reciprocal plots of substrate-activity curves for L-HKG and D-HKG. Enzyme was a Sephadex filtrate and was assayed by glyoxylate formation as noted previously[8]. Ordinate units are reciprocal milli-units of activity, abscissa units are reciprocal millimolar substrate concentrations. From these data, K_m and v_{max} for L-HKG were estimated as $6.8 \cdot 10^{-5}$ M and 0.052 units/mg of protein, respectively; K_m and v_{max} for D-HKG were estimated as $11.1 \cdot 10^{-5}$ M and 0.033 units/mg of protein, respectively.

(precipitation of inert material with 0.1% protamine sulfate, fractionation between 0.35 and 0.5 saturation in $(NH_4)_2SO_4$, and filtration through Sephadex G-200) with approximately 10-fold purification and a 60% yield from initial low-speed supernatants. A further purification of 2–3-fold, but with poor yield, was obtained by elution of enzyme from calcium phosphate gels with 0.5 M Tris or phosphate (pH 8.4). Using the enzyme eluted from calcium phosphate, approximate stoichiometry was demonstrable between HKG disappearance and the formation of pyruvate and glyoxylate, the latter being assayed colorimetrically[2]. Purified enzyme also catalyzed HKG formation from glyoxylate and pyruvate.

Aldolase at the Sephadex-stage cleaved both D- and L-HKG*. The isomers were not markedly different as substrates either with respect to K_m or v_{max} (Fig. 1). Inactivation of the enzyme in the presence of $NaBH_4$ and either glyoxylate or pyruvate was tested by a procedure described for rat-liver HKG aldolase[8], except that only unlabeled substrates were used. Enzyme (0.8 unit, Sephadex filtrate) was treated for 30 min with 5 μmoles of $NaBH_4$ at pH 6.5 following 5-min preincubation at pH 8.4 with 0.5 μmole of pyruvate, 1.5 μmoles of glyoxylate or no substrate. $NaBH_4$-enzyme lost 22%, pyruvate-$NaBH_4$ enzyme lost 50%, and glyoxylate-$NaBH_4$ enzyme lost 55% of the initial activity.

The wide occurrence of HKG and its ready formation from glyoxylate and pyruvate suggest metabolic functions independent of hydroxyproline degradation. Other roles proposed for this compound (and for the aldolase as a possible biosynthetic enzyme) include glutamate synthesis in organisms lacking a citric acid cycle[13], regulation of the citric acid cycle[10,14], and cyclic glyoxylate oxidation[15]. The present report of a constitutive HKG aldolase in bacteria strengthens the impression of a general, although as yet unclear, metabolic function for this compound. Conclusive definition of this enzyme as an aldolase specific for HKG must await further purification and specificity studies, especially since partly purified ketodeoxyglutarate aldolase of *Escherichia coli* catalyzes slow HKG formation[16]. However, the data suggesting that the bacterial enzyme appears to be non-stereospecific for HKG isomers and that glyoxylate, as well as pyruvate, may bind to a lysine of the enzyme support its kinetic homology with mammalian HKG aldolase. Both HKG non-stereospecificity[11,7] and glyoxylate binding[8] have been more clearly established for the mammalian enzyme.

This work was supported by a grant (GM-11105) from the U.S. Public Health Service. We thank Mrs. GLORIA ROSSO for skillfull assistance with certain of the experiments.

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* The isomers of HKG were obtained by reducing DL-HKG to L-*threo*- and L-*erythro*-γ-hydroxyglutamate and separating these by ion-exchange chromatography[11], then converting each amino acid diastereomer to L- or D-HKG with pyridoxal[3]. Validation of this procedure will be presented separately.

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Received September 19th, 1966

Biochim. Biophys. Acta, 132 (1967) 200–203

BBA 63233

A staining procedure for demonstration of multiple forms of aldolase

In animal tissues two isoenzymes of aldolase (ketose-1-phosphate aldehyde-lyase, EC 4.1.2.7 or fructose-1,6-diphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13) have been differentiated. Aldolase A catalyses the reaction fructose 1-phosphate⇌dihydroxyacetone phosphate + glyceraldehyde at about 1/50th of the rate at which it catalyses the reaction fructose 1,6-diphosphate⇌dihydroxyacetone phosphate + glyceraldehyde phosphate. Aldolase B utilizes fructose 1-phosphate (Fru-1-*P*) and fructose 1,6-diphosphate (Fru-1,6-P_2) at about the same rate. Measurement of the ratio of aldolase activities (Fru-1,6-P_2/Fru-1-*P*) in different tissues of many species has shown a variation between 50/1 (muscle) and 1/1 (liver)[1,2].

The simplest method of screening tissue extracts for the presence of multiple molecular forms of any enzyme is electrophoresis, followed by staining. No such method has been available for the detection of aldolase, and after electrophoresis the enzyme could only be identified by elution and subsequent spectrophotometric assay. A method has been developed for the detection and identification of multiple forms of aldolase on starch-gel electrophoresis which utilizes the ready reducibility of alkaline silver nitrate by products of aldolase catalysis.

Human tissues were obtained either at operation, or from autopsy when death had occurred within 12 h. Rat tissues were obtained from white Sprague–Dawley or Chester–Beatty hooded rats. Tissues were homogenized at 0° in equal amounts (w/v) of 0.24 M sucrose containing $2 \cdot 10^{-5}$ M EDTA; for muscle, 2 vol. of sucrose solution were used. A crude supernatant fraction was separated after ultracentrifugation at 80 000 × *g* for 30 min.

Electrophoresis was performed on vertical starch gel. For electrophoresis with citrate–phosphate buffer (pH 7.0) the starch gel was made up in a solution of $1.8 \cdot 10^{-3}$ M citric acid and $13 \cdot 10^{-3}$ M Na_2HPO_4, and the "bridge" solution contained $6 \cdot 10^{-3}$ M