FORMATION OF α-KETOGLUTARATE SEMIALDEHYDE AN INTRAMOLECULAR DISMUTATION REACTION[1]

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The metabolism of L-arabinose by Ps. saccharophila (ATCC 9114) leads to the formation of L-2-keto-3-deoxyarabonate (L-KDA)(Weimberg and Doudoroff, 1955). Subsequently, it was shown that extracts of Ps. saccharophila converted L-KDA to α-ketoglutarate (αKG) in the presence of DPN or TPN (Weimberg, 1959). We have demonstrated that α-ketoglutarate semialdehyde (KGSA) is an intermediate in this conversion of L-KDA to αKG:

$$\underset{(KDA)}{CH_2OH\text{-}CHOH\text{-}CH_2\text{-}CO\text{-}COO^-} \longrightarrow \underset{(KGSA)}{CHO\text{-}CH_2\text{-}CH_2\text{-}CO\text{-}COO^-} \xrightarrow{TPN} \underset{(\alpha KG)}{COO^-\text{-}CH_2\text{-}CH_2\text{-}CO\text{-}COO^-}$$

KGSA has been previously reported as an intermediate in the degradation of hydroxyproline to αKG (Singh and Adams, 1964); and it occurs as an intermediate in D-glucarate metabolism (Dagley and Trudgill, 1964).

The formation of KGSA from L-KDA represents a new intramolecular dismutation reaction. Previously recognized dismutations, which seem to proceed by carbanion mechanisms, involve the elimination of H_2O α,β to a carboxylate group (Meloche and Wood, 1964); whereas the formation of KGSA involves an elimination of H_2O β,γ to a carbonyl group. The enzyme which carries out this reaction has been purified 100-150 fold; it will be called 2-keto-3-deoxy-L-arabonate dehydrase (KDA-dehydrase). KGSA has been characterized by its

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chemical oxidation to αKG by $KMnO_4$, by its enzymic oxidation to α KG by a partially purified aldehyde oxidase and TPN, and by oxidative decarboxylation to succinic semialdehyde. The disemicarbazone and the bis-2,4-dinitrophenylhydrazone (DNPH) derivatives of KGSA have been prepared and characterized.

When crude extracts (Weimberg, 1959) of Ps. saccharophila were incubated with L-arabonate in the absence of TPN, it was noted that the periodate sensitive substrate disappeared with the concurrent accumulation of a stable product, which could be demonstrated by paper chromatography. This new product was a periodate insensitive α-keto acid. The enzyme which catalyzed the formation of this α-keto acid from L-KDA was purified as described later. With the purified enzyme and chemically synthesized KDA it was possible to accumulate and isolate quantities of the α-keto acid which were sufficient for chemical characterization. This α-keto acid, which was isolated from chromatography on Dowex 1-X8 formate, appeared chemically and chromatographically homogeneous. Chromatography on paper, pyridine:n-butanol:H_2O (6:4:3) and n-propanol:0.2 N NH_4OH (3:1), and thin layer chromatography, Silica Gel-G (Brinkman), methanol:n-butanol:HOAc (6:2:2), revealed only a single component.

The isolated α-keto acid was identified as α-ketoglutarate semialdehyde by the following procedures. By oxidative decarboxylation of 260 μmoles of KGSA with a 25-fold molar excess of H_2O_2 at pH 7 and 25°, succinic semialdehyde was formed in > 95% yield. The DNPH of succinic semialdehyde was isolated, mp 199° (lit. 201° (Kondo and Suzuki, 1936) and 197-203° (Clauson-Kaas and Elming, 1952)). Elemental analysis showed 19.98% N; calculated 19.95% N. Hydrogenation of this derivative for 4 hrs. at 50° under 40 lb/in^2 led to the formation of a single ninhydrin positive material which migrated identically with authentic γ-amino butyric acid during paper chromatography in three solvent systems, methanol:n-butanol:benzene:H_2O (2:1:1:1), isopropanol:pyridine:H_2O (1:1:1), and phenol:NH_3 (200:1).

The isolated KGSA was oxidized to αKG by two means. First, 1.75 μmoles

TABLE 1

Purification of 2-Keto-3-Deoxy-L-Arabonate Dehydrase

Procedure	Units*	Spec. Act.	Yield
1. Sonicate	160	0.10	100
2. Streptomycin sulfate	157	0.07	98
3. 0-47% $(NH_4)_2SO_4$	152	0.13	95
4. $CaPO_4$-cellulose column	103	3.0	82
5. DEAE column	38	10.0-14	24

*Unit = μ mole/min.

1-Cultures of Ps. saccharophila are grown at 30° with vigorous aeration and pH control (6.4-6.9) in 20 liter carboys containing an inorganic medium (Doudoroff, 1940) with 0.25% L-arabinose as the sole carbon source. Harvested cells are washed once with 0.033 M potassium phosphate buffer, pH 6.8 and frozen until used. Forty ml 0.1 M phosphate buffer, pH 7.5 are added to 40 g frozen cells and the suspension is sonicated 3 minutes with an MSE, 500 watt sonicator. 2-To the sonicate at pH 7.0, after sufficient buffer is added to make the protein concentration 30 mg/ml, is added 0.1 volume of 40% streptomycin sulfate (Lilly). 3-The $(NH_4)_2SO_4$ precipitation is carried out at pH 7.5. 4-The $CaPO_4$-cellulose column (4.0 x 15 cm) is prepared by previous procedures (Massey, 1960) using cellulose powder (Whatman, standard grade) and $CaPO_4$ (Swingle and Tiselius, 1951). The protein is eluted with a discontinuous gradient of potassium phosphate buffers, pH 7.5:0.002 M, 0.006 M, and 0.012 M. The enzyme is eluted with the last buffer. 5-The enzyme from 4 is put onto a DEAE column (2.2 x 20 cm) pre-equilibrated against 0.012 M phosphate buffer, pH 7.5; it is eluted with a discontinuous gradient of phosphate buffers, pH 7.5:0.012 M, 0.04 M, and 0.07 M. The enzyme is eluted with the last buffer.

KGSA and 9.0 μmoles TPN in the presence of a 13-fold purified aldehyde oxidase from Ps. oleovorans[3] (Baptist, Gholson, and Coon, 1963) was converted to 1.65 μmoles αKG and 1.78 μmoles TPNH. Secondly, 120 μmoles KGSA were oxidized at pH 7.5 and 0° with a 6-fold molar excess of $KMnO_4$. αKG was recovered in 74%

[3]Dr. M. J. Coon kindly furnished a culture of this bacteria.

[4]We wish to thank Professor Adams for a gift of authentic KGSA·DNPH.

yield. In both cases the αKG produced was identified by its reaction with TPNH, NH_4^+, and glutamic dehydrogenase (Boehringer) to produce TPN and glutamate. The αKG was identified further by paper chromatography in two solvents, H_2O saturated n-butanol:HCOOH (95:5) and n-butanol:n-propanol:H_2O (10:7:3).

KGSA was also isolated as the DNPH. This derivative was not entirely satisfactory. Although the infrared spectrum of our compound correlated in all absorption regions with the infrared spectrum obtained with authentic KGSA-DNPH[4], our derivative, however, did not give reproducible elemental analyses, even between similar preparations. Satisfactory results were obtained with the disemicarbazone derivative. This material was isolated as the monohydrate, mp 173-175°, 65% yield after recrystallization. Elementary analysis 32.1% C, 5.7% H, and 32.0% N; calculated 32.1% C, 5.3% H, and 32.1% N for $C_7H_{12}N_6O_5 \cdot H_2O$.

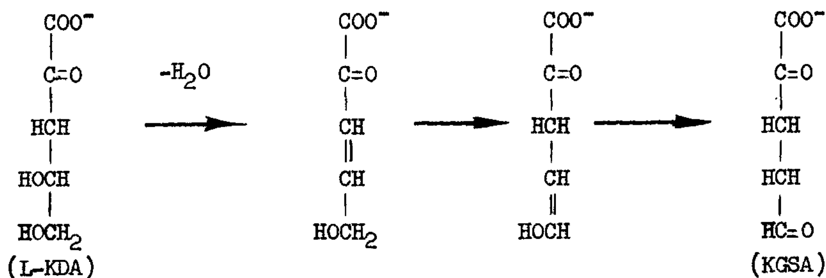
The purification of 2-keto-3-deoxy-L-arabonate dehydrase is summarized in Table I.

The purified KDA dehydrase converts enzymically prepared L-KDA (Weimberg, 1959) into KGSA stoichiometrically. Beginning with chemically synthesized DL-KDA, one-half of this substrate is converted stoichiometrically to KGSA; the D isomer is not utilized. From an incubation mixture, which contains 1.98 mmoles DL-KDA and 25 units of KDA-dehydrase in 80 ml 0.1 M phosphate buffer, pH 7.0, 0.90 mmoles of KGSA and 1.17 mmoles KDA were isolated by chromatographing the reaction mixture on Dowex 1-X8 formate.

The properties of the highly purified KDA-dehydrase have been partially investigated. The dehydrase has optimal activity at pH 7.2 at 30°. There is no evidence for the reversibility of the reaction it catalyzes, nor is there any evidence that KGSA is inhibitory. The activity is not diminished by the following chelating agents or thiol reagents: EDTA, 2,2'-bipyridine, o-phenanthroline, iodoacetate, or N-ethyl maleimide (each at 10^{-3} M). p-HMB inhibits about 50% at 5×10^{-4} M and 15% at 5×10^{-5} M. This enzyme contains no significant amount of B_{12}-type compounds; less than 10μμg/ml (protein

0.02 mg/ml) as assayed with L. leichmannii.[5]

The structures of the substrate and the product of this enzymic reaction suggest the following possible mechanism:

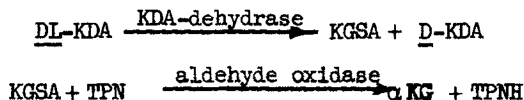


We are currently investigating the possibility of such a mechanism with the use of appropriately labeled isotopic intermediates.

EXPERIMENTAL

The substrate, DL-KDA, is prepared in a non-enzymic condensation of 12 mmoles oxaloacetic acid (Nutritional Biochemicals) with 8 mmoles glycolaldehyde (Sigma) at pH 7 in 0.05 M phosphate buffer. This reaction is analogous to the condensation of oxaloacetic acid and methylglyoxal (Henze, 1930). DL-KDA is first purified by elution from Dowex 1-X8 chloride with 0.05 M HCl. Then it is chromatographed on Dowex-1-X8 formate, using a convex gradient elution, 0.1 M - 0.5 M formic acid, pH 3.0 (pH adjusted with pyridine). KDA and KGSA are purified and separated completely by this latter column.

KDA-dehydrase, L-KDA, and KGSA each may be assayed in the coupled system shown below when the appropriate reagent is made limiting:



In the presence of excess substrate, the assay for KDA-dehydrase is linear over the range 0.3 - 2.0 mμmoles/min.

Carbonyl compounds are assayed with DNPH (Bohme and Winkler, 1954); α-keto acids, with semicarbazide (MacGee and Doudoroff. 1954). KDA may be

[5] B_{12} assays were performed by Dr. R. Kisliuk, Tufts University, Boston.

assayed after periodate oxidation by the colorimetric assay for β-formylpyruvate (Weissbach and Hurwitz, 1959). Protein is determined by the method of Lowry (Lowry, et al., 1951) with crystalline bovine serum albumin standards.

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