NEW PATHWAY FOR δ-AMINOLEVULINIC ACID BIOSYNTHESIS: FORMATION FROM α-KETOGLUTARIC ACID BY TWO PARTIALLY PURIFIED PLANT ENZYMES[1]

James B. Lohr and Herbert C. Friedmann

Department of Biochemistry, The University of Chicago, Chicago, Illinois 60637

Received February 5,1976

Summary. Two enzymes which catalyze the formation of δ-aminolevulinic acid in two steps from α-ketoglutaric acid have been partially purified from Zea mays leaf extracts. The enzymes catalyze the following reactions: (1) a novel NADH-dependent reduction of the 1-carboxyl group of α-ketoglutarate, yielding 4,5-dioxovaleric acid, followed by (2) a transamination of this product with L-alanine to yield δ-aminolevulinate. The dehydrogenase cannot be demonstrated in crude extracts since it is masked by glutamic dehydrogenase. This pathway, in which the 5-carbon skeleton of α-ketoglutarate is utilized intact for δ-aminolevulinate formation, differs radically from the classical δ-aminolevulinate synthase reaction between glycine and succinyl-CoA.

ALA[2] is the biological pyrrole precursor for such substances as porphyrins and corrins. The classical pathway for ALA formation, demonstrated in mammalian tissues and in bacteria (1,2), involves condensation between glycine and succinyl-CoA, a reaction catalyzed by ALA synthase. This enzyme does not appear to occur in plants. Isotopic in vivo evidence indicates that in higher plants including cucumber (3,4), bean (4), barley (4,5), oat (6), and maize (7) another pathway occurs in which a 5-carbon precursor is converted to ALA without carbon-carbon cleavage. These studies employed various ^{14}C-labeled substances as ALA precursors. Numerous pathways have been postulated (4,5,7,8) in many of which DVA participates. The variety of

[1]Supported by Grant AM-09134 from the National Institutes of Health, United States Public Health Service.

[2]Abbreviations: ALA, δ-aminolevulinic acid; DVA, 4,5-dioxovaleric acid; α-KG, α-ketoglutaric acid.

proposed pathways was necessitated by the lack of direct enzymatic evidence. The participation of DVA, the aldehyde analogue of ALA (8), was surmised because of observations of ALA transaminase activity in various sources (reviewed in 9,10), but strangely enough not in higher plants. We now present the first demonstration of an enzyme, partially purified from extracts of Zea mays leaves, which reduces α-KG to DVA, utilizing NADH as reductant. Furthermore, a transaminase which catalyzes the conversion of DVA to ALA is present in such extracts as well.

Materials and Methods

Chemicals were obtained from the following sources: [U-^{14}C]α-KG and [4-^{14}C]ALA--New England Nuclear; L-[U-^{14}C]glutamic acid--Amersham/Searle; pyruvic acid, NADH, NAD^+, glutamic dehydrogenase, L-alanine, α-KG--Sigma; Dowex 50W-X2, 200-400 mesh, analytical grade--Bio-Rad; pyridoxal phosphate--Calbiochem; DE52--Whatman. Pellicon PSAC membranes were from Millipore, and glass beads, 0.2 mm diameter, from Minnesota Mining and Manufacturing.

Electrophoretic separation and identification of ^{14}C-labeled ALA, glutamic acid, DVA, and α-KG was performed on 22 cm long Whatman No. 1 paper strips at pH 3 (1 N acetic acid) under an applied gradient of 23 volts/cm. Points of application and times for separation were varied depending upon electrophoretic mobilities. The uncorrected mobilities for ALA and for glutamate applied 5 cm from the anode end were 9.05×10^{-5} and 3.7×10^{-5} $cm^2\ volt^{-1}\ sec^{-1}$, respectively, toward the cathode, while the uncorrected mobilities for DVA and for α-KG applied 7 cm from the cathode end were 0.74×10^{-5} and 1.8×10^{-5}, respectively, toward the anode.

Sterile maize seedlings (type D5) were grown for 2 weeks in the light in sterilized moist vermiculite. Approximately 50 g of green leaves were ground in a mortar with glass beads at 5°. The supernatant solution resulting from centrifugation for 10 min in the cold at 48,000 x g served as enzyme source.

[4-^{14}C]DVA was prepared as follows: 1 ml of the crude corn leaf extract

was exhaustively dialyzed against distilled water in the cold. The dialyzed solution was incubated for 4 hr in a total volume of 2 ml containing 1 M sodium phosphate buffer, pH 6.6; 0.3 M sodium pyruvate; 5.8×10^{-5} M [4-^{14}C]ALA; approximately 10^{-5} M pyridoxal phosphate. The mixture was then placed in boiling water for 5 min and centrifuged for 10 min at 48,000 x g. The supernatant solution was applied to a Dowex 50W column, 0.5 x 6 cm, pH 3. The column was treated with 0.1 M Tris-HCl buffer, pH 8.5. The first 6 ml of eluate were discarded, and the following 4 ml were collected.

Partial purification of the dehydrogenase and the transaminase: The protein fraction salted out from the crude enzyme solution by ammonium sulfate between 50 and 70 per cent w/v was collected, dissolved in a small volume of water, and dialyzed overnight in the cold against three changes of water. The resulting clear solution was applied to a DE52 column, 1.5 x 19 cm, which had been equilibrated against 0.1 M Tris-HCl buffer, pH 8.0. The column was treated with 400 ml of a linear gradient of 0 to 1 M KCl in this buffer. Various fractions were concentrated 20-fold by ultrafiltration through a PSAC membrane. Dehydrogenase activity was detected in a small peak eluting between about 200-210 ml of the gradient, while transaminase was eluted in a large brown fraction between about 220-240 ml of the gradient.

Composition of ALA transaminase assay mixture: (1) For the formation of DVA from ALA: 0.25 M sodium phosphate, pH 6.8; 6×10^{-5} M [4-^{14}C]ALA (appr. 10,000 cpm); 0.15 M sodium pyruvate; 10^{-5} M pyridoxal phosphate. (2) For the formation of ALA from DVA: 0.25 M sodium phosphate, pH 6.8; 5 µl [4-^{14}C]DVA solution (appr. 10,000 cpm); 0.15 M alanine; 10^{-5} M pyridoxal phosphate. In all cases 5 µl of enzyme solution was used. Total assay volume in each system was 12 µl.

Composition of DVA dehydrogenase assay mixture: (1) For the formation of DVA from α-KG: 0.15 M sodium phosphate, pH 6.8; 6×10^{-6} M [U-^{14}C]α-KG (appr. 10,000 cpm); 10^{-5} M NADH. (2) For the formation of α-KG from DVA: 0.075 M Tris-HCl buffer, pH 7.5; 5 µl [4-^{14}C]DVA solution (appr. 10,000 cpm); 10^{-5} M NAD^+. (3) For the conversion of DVA via α-KG to glutamate: To the

second mixture, 1.5 units of glutamic dehydrogenase and NADH to 10^{-5} M were added. The assay volume was 15 µl for the first two mixtures, 18 µl for the third.

All mixtures were incubated for 2 hr at 30° before application to paper strips for electrophoresis.

To follow the conversion of α-KG to ALA, a mixture of the two partially purified enzymes was incubated either in 20 µl total volume containing 0.15 M sodium phosphate, pH 6.8; 6×10^{-6} M [U-^{14}C]α-KG (appr. 10,000 cpm); 0.15 M alanine; 10^{-5} M NADH; 10^{-5} M pyridoxal phosphate, or in 125 µl total volume containing 0.15 M sodium phosphate, pH 6.8; 0.3 M α-KG; 0.3 M alanine; 10^{-5} M NADH; 10^{-5} M pyridoxal phosphate. In the first case [^{14}C]ALA formed was detected electrophoretically as described above, in the second ALA formed was assayed colorimetrically (11).

Results

ALA transaminase: Upon incubation of the crude dialyzed corn leaf extract with [4-^{14}C]ALA, the formation of an acidic labeled substance (mobility 0.74×10^{-5} towards anode as indicated above), dependent upon the addition both of pyruvate and of pyridoxal phosphate, was observed. This substance was tentatively identified as [4-^{14}C]DVA. When this product, isolated by chromatography on Dowex 50W, was incubated with an aliquot of the protein eluate from the DE52 column as indicated above, a radioactive material with electrophoretic mobility corresponding to ALA was found, whose formation was dependent upon the addition of both alanine and pyridoxal phosphate. This was clearly the reverse of the above reaction.

DVA dehydrogenase: When [4-^{14}C]DVA was incubated with an aliquot of the appropriate concentrated DE52 eluate, a labeled material with the electrophoretic mobility of α-KG was formed, provided NAD^+ had been added. The identification of the product as α-KG was confirmed by the fact that upon the addition of glutamic dehydrogenase and NADH a labeled substance with the electrophoretic

mobility of glutamic acid appeared instead. Conversely, when [U-^{14}C]α-KG was incubated with the same enzyme fraction, a labeled material electrophoretically identical to DVA was formed, provided NADH had been added.

α-KG to ALA: A radioactive material with the electrophoretic mobility of ALA was formed when [U-^{14}C]α-KG was incubated with the above two enzyme fractions. The formation of [^{14}C]ALA was strictly dependent upon the presence of both enzymes, NADH, alanine, and pyridoxal phosphate. A positive Ehrlich test was obtained after unlabeled α-KG was incubated under similar conditions. This again required the presence of both enzymes.

Discussion

In this paper the direct formation of ALA from the ubiquitous α-KG by two enzymatic steps, a reduction followed by a transamination, has been shown with an *in vitro* system. This demonstration almost certainly eliminates the other suggested pathways from a 5-carbon precursor (4,5,8).

The other photosynthetic organisms in which the conversion of DVA to ALA by transamination has previously been shown are *Rhodopseudomonas spheroides* (12) and *Chlorella vulgaris* (13). The present paper presents evidence for the occurrence of such a transaminase in a higher plant, and shows that DVA is derived from α-KG.

The reduction of α-KG to DVA cannot be demonstrated in crude extracts because of the predominance of glutamic dehydrogenase.

The evolutionary significance of the existence of at least two different pathways for ALA synthesis, depending upon the organism, is unknown. If one accepts the view (14) that in plants the light-controlled step in chlorophyll synthesis is in fact the formation of ALA, then the evolution of a plant pathway for ALA synthesis with special features is not surprising.

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