A CHLOROPLAST-LOCALIZED DIAMINOPIMELATE DECARBOXYLASE IN HIGHER PLANTS

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1. Introduction

Two biosynthetic pathways for lysine have evolved [1]. One proceeds via DAP and is characteristic of bacteria, higher plants, blue-green and green algae, and some fungi. The other route involves α-aminoadipic acid as an intermediate and is restricted to fungi and euglenid types. There have been some indications that this latter pathway can operate under certain conditions in higher plants [2]. It has often been suggested that chloroplasts evolved from symbiotic prokaryotes [3], and it is known that chloroplasts are capable of the biosynthesis of a number of amino acids [4]. The only reports in higher plants on the enzymes unique to lysine biosynthesis are those on the presence of the enzyme dihydrodipicolinate synthase (EC 4.2.1.52) in germinating maize seedling extracts [5], and the partial purification and characterization of DAP decarboxylase from the aquatic monocots *Lemna perpusilla* [6] and *Spirodela oligorhiza* [7]. The intracellular localization and distribution of this enzyme in higher plant tissues is as yet unknown. This report describes a chloroplast-localized DAP decarboxylase in the leaves of *Vicia faba.*

2. Materials and methods

2.1. *Chloroplast isolation*

Whole chloroplasts were isolated from the leaves of 5 to 6 week old *Vicia faba* plants by the use of the extraction and centrifugation procedure of Miflin and Beevers [8]. Prior to use the plants were placed in the dark overnight in order to remove the starch in the chloroplast. The isolation medium was modified in that 2 mM $MgCl_2$ and 25%(w/w) sucrose were used and the buffer concentration was 0.05 M. In addition 25 mM mercaptoethanol was included in the extracting medium. The density gradient employed was as described [8] except for the buffer concentration and the use of 25%(w/w) sucrose as the lowest concentration in the gradient. For larger quantities of chloroplasts the isolation was carried out by the centrifugation protocol of Walker [9].

2.2. *Assay procedures*

DAP decarboxylase activity was measured by the use of single side-arm manometer flasks with a center well. In the main chamber of the flask the reaction mixture consisted of the following: 40 mM potassium phosphate pH 7.0; 25 μM pyridoxal phosphate; 2 mM[1,7-^{14}C]-DL-DAP (ICN) containing 0.2 μC_i, enzyme and distilled water in a final volume of 2 ml. The center well contained 0.2 ml of a 1:2 mixture of ethanolamine and methyl cellosolve. A folded 1.5 cm^2 filter paper was sometimes placed in the center well solution to increase the surface available for CO_2 absorption. The side-arm contained 0.2 ml of 2 M perchloric acid. The vessel was mounted on a manometer and placed in a water bath at 30°C for the desired incubation time, usually 15 or 30 min, with continuous shaking. The reaction was terminated by tipping the perchloric acid into the main compartment and then shaking was continued for a further 60 min. The center well contents were removed and placed in a scintilla-

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Abbreviation: DAP, diaminopimelate.

tion vial. The center well was washed with a further addition of 0.2 ml of the ethanolamine:methyl cellosolve mixture and this added to the vial. Ten ml of a scintillation counting fluid based on that recommended by Hager [10] for $^{14}CO_2$ was added and the activity measured using a Beckman LSC 250 spectrometer. A unit of enzyme activity is defined as that amount of enzyme which will produce one nmole of $^{14}CO_2$ per min.

Triose phosphate isomerase was assayed by the method of Gibbs and Turner [11]. Chlorophyll was determined by the method of Arnon [12]. Sucrose concentrations in the fractions from the gradient were measured by refractometry. Protein concentrations were determined spectrophotometrically or by the Biuret reaction [13].

All chemicals were purchased from commercial sources and were the highest grade available.

3. Results

The intracellular distribution of DAP decarboxylase in a leaf homogenate is shown in fig.1. The enzyme activity is concentrated in the whole chloroplast fraction as indicated by the marker enzyme, triose phosphate isomerase. The major chlorophyll-containing peak represents chloroplasts which have lost their stroma and large chloroplast fragments.

The decarboxylase and isomerase are both removed when the integrity of the chloroplast is lost. The ease with which the decarboxylase can be removed was demonstrated by preparing whole chloroplasts by the method of Walker [9]. The supernatant solution from which whole chloroplasts had been removed was decanted and assayed for the decarboxylase. The whole chloroplast pellet was resuspended in 0.05 M tricine buffer pH 7.5 with a glass homogenizer and then centrifuged for 5 minutes at 12 000 *g*. The supernatant solution which would contain any material solubilized from the chloroplasts was decanted, and the pellet was suspended by means of a glass homogenizer in 0.05 M tricine buffer, pH 7.5. Both these fractions were also assayed for decarboxylase activity and the results are given in table 1. The procedure effectively removes almost completely the activity present in the intact chloroplast. After solubilization the enzyme was precipitated by the addition of $(NH_4)_2SO_4$ to 75% saturation. The precipitate was dissolved in 0.2 M potassium phosphate buffer at pH 7.0 which was 25% (v/v) glycerol and also contained 5 mM dithiothreitol. Under these conditions the enzyme was stable at $-10°C$ and also to freezing and thawing for at least several days.

The properties of the solubilized enzyme were examined to a certain extent. The pH optimum was 7.0 using potassium phosphate as the buffer. The K_M of the enzyme for DAP was determined by the use of

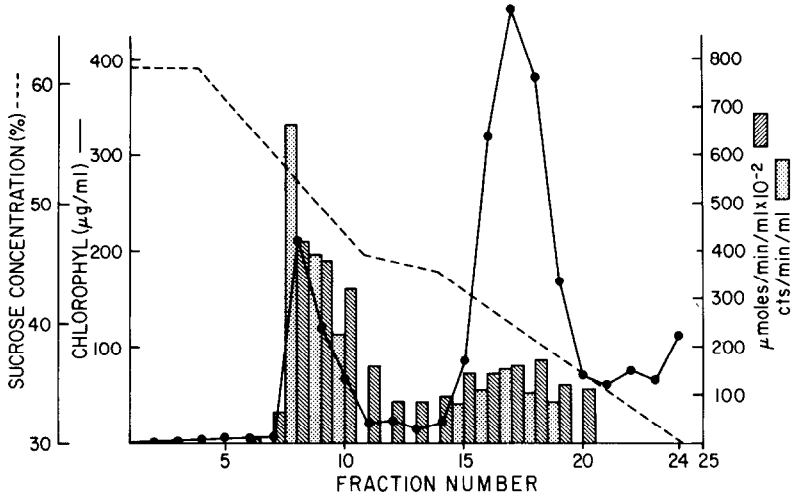

Fig.1. The distribution of DAP decarboxylase and triose phosphate isomerase on a sucrose density gradient of a leaf homogenate from *Vicia faba*. Cross-hatching: Triose phosphate isomerase. Stippling: DAP decarboxylase.

Table 1
Solubilization of DAP decarboxylase from isolated chloroplasts

Enzyme fraction	Specific activity (units/mg)
Extract from ruptured chloroplasts	4.0
Suspension of ruptured chloroplasts	0.1
First supernatant solution devoid of whole chloroplasts	1.7

The assay procedure is described in Materials and Methods. The incubation time was 30 min.

a computer program for a linear regression curve using the Lineweaver–Burk from of the Michaelis–Menten equation. The correlation coefficient was 0.99 and the K_M calculated was 0.3 mM. Under the assay conditions employed the reaction rate was linear with time for at least 60 minutes, and also with the amount of enzyme added.

There was very little increase in the rate of reaction by the addition of pyridoxal phosphate. The largest effect ever observed was a 15% increase but in general the increase was less than 10%. In the presence of 2.5 mM aminooxyacetate or NH_2OH the activity was inhibited over 90%. Addition of either 5 mM L-lysine, D-lysine, DL-α-NH_2-δ-hydroxyvalerate, or DL-α-aminoadipate had no inhibitory effect. EDTA (10 mM) also had no influence on the reaction rate.

The product of the reaction was demonstrated to be lysine by co-chromatography with known markers of L-lysine and DL-DAP using a glass plate coated with cellulose powder and a solvent system of butanol–acetone–diethylamine–water (40:40:8:20), and also by electrophoresis in 0.5 M sodium veronal buffer pH 8.6 at 1000 mV for 25 min. The radioactive areas were visualized by means of a Birchover radiochromatogram spark chamber (Birchover Instruments, Ltd.) and the amino acid markers by spraying with ninhydrin solution. Lysine was the only product formed. In a control reaction mixture using heat-inactivated enzyme no lysine or any other product was produced.

4. Discussion

In considerations of the path of biochemical evolution the chloroplast has frequently been considered to be a descendant of a symbiotic prokaryote [3]. A type of evidence which would be in support of this view would be finding that the chloroplast possessed prokaryotic systems of biosynthesis. Lysine biosynthesis would be one such system [3]. The results presented in this report are consistent with the localization of a DAP decarboxylase in the chloroplast, and thus infers that the chloroplast synthesizes lysine via the DAP pathway which is characteristic of prokaryotes.

The only plant DAP decarboxylases previously reported [6,7] have almost identical properties to the chloroplast enzyme in regard to pH optimum and K_M. These previous studies did not concern themselves with intracellular localization and the homogenization procedures used were not designed to retain chloroplast integrity. Enzymes described previously have all been specific for the meso-form of the substrate. In the present case a mixture of all the isomers was used as the substrate. If only the meso form was utilized then the K_M for the chloroplast enzyme is even lower than the 0.3 mM value obtained.

There was little effect of added pyridoxal phosphate on the reaction rate. However the large inhibition by aminooxyacetate or NH_2OH indicates the necessity of a carbonyl-containing structure for activity. If pyridoxal phosphate is required as a cofactor it is not easily dissociated from the holoenzyme, which is not unusual in enzymes concerned with amino acid metabolism in higher plants [14–16].

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