CONVERSION OF 4-HYDROXYPHENYLPYRUVIC ACID INTO HOMOGENTISIC ACID AT THE THYLAKOID MEMBRANE OF *LEMNA GIBBA*

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1. Introduction

Homogentisic acid (2,5-dihydroxyphenylacetic acid) is known as an intermediate of the catabolism of phenylalanine and tyrosine. In liver [1] or bacteria [2], the formation of homogentisic acid from 4-hydroxyphenylpyruvic acid is catalyzed by a dioxygenase which is, according its mode of action, rather a hydroxylase. The enzyme from heterotrophic plant cell cultures probably has a similar physiological function [3]. In autotrophic cells, however, another isoenzyme should also be present which provides homogentisic acid as precursor for plastoquinone biosynthesis. We report here the occurrence of a homogentisic acid-forming enzyme system in thylakoid membrane preparations from sterile cultures of *Lemna gibba.*

2. Materials and methods

2.1. *Preparation of cell fractions*

Sterile cultures of *L. gibba* G1 were grown under a light–dark regime as in [4]. Cells were harvested and homogenized in a medium containing 12% sucrose (w/w) and 50 mM Hepes–NaOH (pH 7.5). Intact chloroplasts were purified by centrifugation (8000 × *g*, 15 min) in a gradient consisting of 12.5–50% Percoll (Pharmacia, Sweden) in 0.25 M sorbitol. Thylakoidal membranes obtained by hypotonic shock of chloroplasts were subjected to density gradient centrifugation in sucrose gradients ranging from 15–50% sucrose (w/w).

2.2. *Enzyme assay and identification of the product*

4-[U-^{14}C]Hydroxyphenylpyruvic acid (5 μCi/μmol) was prepared from L-[U-^{14}C]tyrosine (The Radiochemical Centre, Amersham) using L-amino acid oxidase [5] and purified by paper chromatography in the solvent system benzene : acetic acid : water, 4:2:1 (v/v/v). A suitable amount of membrane suspension (equivalent to 1 mg chlorophyll) was incubated with 0.5 μmol 4-[U-^{14}C]hydroxyphenylpyruvic acid, 100 μmol ascorbate, 1000 units liver catalase, and 100 μmol Hepes–NaOH (pH 7.2) in 2.0 ml total vol. The mixture was shaken for 30 min and the reaction terminated by addition of carrier homogentisic acid and 4-hydroxyphenylpyruvate acid (5 μmol each) and 10 μmol NH_2OH. Residual labelled 4-hydroxyphenylpyruvate was thus converted into the stable oxime. After 20 min at room temperature the mixture was acidified and extracted 5 times with diethyl ether, the ethereal solution concentrated and subjected to thin-layer chromatography in the solvent system benzene : isoamyl alcohol : acetic acid, 40:20:1.5 (v/v/v). The oxime of 4-hydroxyphenylpyruvic acid (R_F 0.08) was well separated from homogentisic acid; its zone at R_F 0.54 was eluted. One half of the product was diluted with 100 mg inactive homogentisic acid and recrystallized from ethyl acetate–chloroform. The other half of the product was methylated with CH_2N_2, and the derivatives purified by thin-layer chromatography in the system benzene : ethyl acetate, 4:1 (v/v).

The radioactivity determined by a chromatogram scanner was located at R_F 0.35 (methyl 2,5-dihydroxyphenylacetate), R_F 0.55 (methyl 2-hydroxy-5-

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methoxyphenylacetate) and R_F 0.65 (methyl 2,5-dimethoxyphenylacetate). Development and drying of paper and thin-layer chromatograms was performed in an atmosphere of nitrogen.

3. Results

3.1. *Conversion of 4-hydroxyphenylpyruvate into homogentisic acid in chloroplasts*

Chloroplasts from sterile cultures of *L. gibba* were used to demonstrate the hydroxylation and oxidation of 4-hydroxyphenylpyruvic acid leading to 2,5-dihydroxyphenylacetic acid.

Table 1 shows that chloroplasts possess the highest capacity of homogentisic acid formation. This was corroborated in another experiment when chloroplasts were prepared by centrifugation in a Percoll gradient and controlled microscopically. Thylakoids obtained from whole chloroplasts exhibited, on a chlorophyll basis, identical values of conversion. Hence, the data given for the other pellets (P10 and P100, in table 1) reflect that both fractions were contaminated with thylakoidal membranes. On the basis of chlorophyll, the enzyme activities do not differ significantly between the fractions outlined in table 1.

Although residual catalase activity was detected in the chloroplast pellet (table 1) determination of catalase activities in the other fractions, and the calculation of oxygenase activity versus catalase activity, prove that the two enzymes are not housed in the same organelle.

3.2. *Factors influencing the oxygenase reaction*

The conversion of 4-hydroxyphenylpyruvic acid into dihydroxyphenylacetic acid formally requires two oxidation steps, a hydroxylation reaction at the ring and an oxidative decarboxylation. A mono-oxy-

Table 2
Factors affecting 4-hydroxyphenylpyruvate dioxygenase

Additions	Conversion
None	3.4
50 μM Fe^{2+}	5.0
5 mM Ascorbate	6.7
50 mM Ascorbate	7.9
50 mM Ascorbate + catalase (1000 units)	10.4
50 mM Ascorbate + 50 μM Fe^{2+} + catalase (1000 units)	11.6

Bovine liver catalase was preincubated at 70°C for 10 min in order to inactivate contaminating 4-hydroxyphenylpyruvate dioxygenase. Thylakoids purified by sucrose density gradient centrifugation were assayed in the presence of reducing agents and other factors. Conversion rates are given in nmol × $[30\ min]^{-1}$ × $[mg\ chl.]^{-1}$

Table 1
Distribution of 4-hydroxyphenylpyruvate dioxygenase within cell fractions

Preparation	Oxygenase		Catalase Total act. (μmol/min)	Oxygenase/ catalase
	Total act. (units)	Spec. act. (units/mg prot.)		
Chloroplasts	16.2	2.54	24	0.67
P10	8.0	1.16	276	0.03
P100	5.6	1.47	160	0.03
S100	10.1	0.83	763	0.01

20 g of a sterile culture of *Lemna gibba* were homogenized and fractionated under sterile conditions. The following fractions were prepared by differential centrifugation: chloroplasts (1000 × *g*, 2 min), a pellet consisting primarily of mitochondria, peroxisomes and thylakoids (P10; 10 000 × *g*, 20 min), a microsomal fraction (P100; 100 000 × *g*, 1 h), and a supernatant (S100). 1 unit is equivalent to the formation of 1 nmol homogentisic acid during 30 min

genase which is responsible for the side chain migration induced by hydroxylation [6] is assumed to need reducing agents in order to furnish the oxygen species required for hydroxylation. Table 2 shows that ascorbate is a suitable reductant.

We found that the presence of 0.1% H_2O_2 leads to a pronounced inhibition of homogentisic acid formation. Addition of catalase increased the enzyme activity (table 2). Maximum activity was obtained by addition of Fe^{2+} and of high concentrations of ascorbate and catalase as has been reported for the enzyme from *Pseudomonas* sp. [2].

4. Discussion

The results presented complement, in the case of *L. gibba*, our knowledge concerning the conversion of L-tyrosine into prenylquinones, e.g., plastoquinone. The enzymes of homogentisic acid formation, i.e., an L-amino acid oxidase [7] and the 4-hydroxyphenylpyruvate dioxygenase, as well as the enzyme required for conversion of homogentisic acid into polyprenyltoluquinols [8] are all arranged at the thylakoid membranes.

Isolated chloroplasts from spinach leaves possess the capacity for formation of plastoquinone [9], but leaf peroxisomes seem to be responsible for the homogentisic acid production [10]. However in leaf cells from *L. gibba* the cooperation of different compartments for the sequence tyrosine → plastoquinone is not required. An assembly of membrane-bound enzymes in the chloroplasts is channeling L-tyrosine into the pathway of plastoquinone biosynthesis. Chloroplasts contain considerable amounts of ascorbate [11] which might well serve as the in vivo reductant needed for the formation of homogentisate. The soluble 4-hydroxyphenylpyruvate dioxygenase activities described in table 1 probably are localized in the cytoplasm where the degradation pathway of aromatic amino acids may take place.

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