The Biosynthesis of Phenylacetic Acids in the Blue-Green Alga *Anacystis nidulans:* Evidence for the Involvement of a Thylakoid-Bound L-Amino Acid Oxidase

W. Löffelhardt

Institut für Allgemeine Biochemie der Universität Wien and Ludwig Boltzmann-Forschungsstelle für Biochemie

(Z. Naturforsch. **32 c**, 345–350 [1977]; received February 14, 1977)

Membrane-Bound Enzymes, *Anacystis nidulans*, Thylakoids, Phenylacetic Acids, L-Amino Acid Oxidase

Phenylacetic acid and *p*-hydroxyphenylacetic acid are formed upon incubation of photosynthetic membranes from the prokaryotic alga *Anacystis nidulans* with L-phenylalanine and L-tyrosine, respectively. The corresponding phenylpyruvic acids act as intermediates as shown by trapping them as the stable oximino acids. The first step in this reaction sequence appears to be catalyzed by a thylakoid-bound L-amino acid oxidase. Already existing evidence concerning phenylacetic acid formation at thylakoid membranes of higher plants via an L-amino acid oxidase and the results obtained with *A. nidulans* give another example of the close analogy in the secondary metabolism of aromatic amino acids between chloroplasts and blue-green algae.

Introduction

Phenylacetic acid and *p*-hydroxyphenylacetic acid are common constituents of higher plants [1, 2]. Recently both acids have been isolated from the brown alga *Undaria pinnatifida* [3]. Furthermore they have been shown to perform auxin-like activities on wheat coleoptiles [3] and on tobacco callus [4]. The growth of the red alga *Porphyra tenera* [5] is stimulated by phenylacetic acid and *p*-hydroxyphenylacetic acid whereas indole-3-acetic acid is inactive. Chloroplasts from higher plants form phenylacetic acid upon incubation with L-phenylalanine [6]. Therefore it seemed interesting to investigate whether a blue-green alga is capable of synthesizing this compound and/or its *p*-hydroxylated analogue. In the case of benzoic acid formation a striking analogy of the pathway in chloroplasts and on thylakoid membranes of *Anacystis nidulans* was previously observed [7].

In this paper the formation of phenylacetic acid and *p*-hydroxyphenylacetic acid from L-phenylalanine and L-tyrosine, respectively, by photosynthetic membranes of *A. nidulans* is reported. Evidence for the occurrence of a thylakoid-bound L-amino acid oxidase in the blue-green alga is discussed.

Requests for reprints should be sent to Dr. W. Löffelhardt, Institut für Allgemeine Biochemie der Universität Wien, Währingerstraße 38, *A-1090 Wien*.

Materials and Methods

Cultivation of algae

Anacystis nidulans (Sammlung von Algenkulturen, Pflanzenphysiologisches Institut, Universität Göttingen, Katalog Nr. L 1402-1) was grown in axenic culture at 37 °C in the medium C of Kratz and Myers [8], containing 1 g/l of sodium bicarbonate under continuous light and aeration (3% CO_2 in air, v/v).

Labelled compounds

L-[U-^{14}C]phenylalanine, L-[4′-^{3}H]phenylalanine, L-[U-^{14}C]tyrosine and L-[3′,5′-^{3}H]tyrosine were purchased from the Radiochemical Centre, Amersham. All compounds were diluted to a specific radioactivity of 10 mCi/mmol and purified by paper chromatography [8] immediately prior to use. L-[methyl-^{14}C]methionine and L-[U-^{14}C]leucine were obtained from the same source and used at a specific radioactivity of 50 mCi/mmol.

[U-^{14}C]phenylpyruvic acid was prepared from L-[U-^{14}C]phenylalanine by the following procedure. A solution (1.0 ml) containing the labelled amino acid (20 μCi) dissolved in 0.1 m Tris buffer pH 7.5, 1.5 units of L-amino acid oxidase (Sigma, type I) and 0.2 ml of catalase (Boehringer, analytical grade) was incubated at 37 °C while oxygen was blown on the surface. After three hours the evolution of ammonia ceased and the reaction mixture was put on top of a 1×10 cm column filled with Dowex 50 W, 100–200 mesh (H^+) and was washed with 150 ml of distilled water. The effluent

was evaporated to dryness and the residue was dissolved in methanol and subjected to paper chromatography in the solvent system A: benzene : acetic acid : water (4/2/1, by volume, upper phase). [U-^{14}C]phenylpyruvic acid was obtained free from phenylacetic acid (R_F-values were 0.66 and 0.89, respectively).

Non-labelled compounds

Oxime of phenylpyruvic acid: 5 g of sodium phenylpyruvate were dissolved in 100 ml of distilled water. After addition of a solution of 1.9 g hydroxylammonium chloride and 3 g sodium carbonate in 20 ml H_2O the mixture was allowed to stand for 12 hours at room temperature. Acidification produced a white precipitate, which was collected and dried. Recrystallization from boiling chloroform yielded 3.8 g of white crystals, m.p. 173 – 174 °C. The oxime of *p*-hydroxyphenylpyruvic acid was prepared in an analogous way. Recrystallization from hot water lead to light tan needles, m.p. 153 – 155 °C.

Sodium phenylpyruvate, *p*-hydroxyphenylpyruvic acid, sodium α-keto-γ-methiolbutyrate and sodium α-keto-isocaproate were purchased from Sigma. All other reagents used were of analytical grade.

Preparation of subcellular fractions from A. nidulans

Homogenates of sonicated cells and thylakoid membranes obtained after ultrasonic rupture or lysozyme treatment of the algal cells were prepared as already described [7].

Sucrose density gradient centrifugation of a homogenate yielded three bands [7]: I (light green, 41% sucrose), II (yellowish, 46% sucrose) and III (dark green, 54% sucrose). The same procedure performed with a thylakoid preparation resulted in the appearance of two bands [7]: IV (green, 54% sucrose) and V (dark green, 58% sucrose). All these bands were assayed for the formation of phenylacetic acids.

Assay conditions and identification of products

Phenylacetic acid formation was measured by the incubation (in a total of 3 ml) of homogenate, a thylakoidal or a supernatant fraction with the corresponding labelled aromatic amino acid in 0.1 M Tris buffer, pH 7.5, at 25 °C. After 30 min carrier was added, the reaction was stopped by acidification and the mixture was extracted with diethyl ether. Labelled phenylacetic acid was identified by twofold paper chromatography followed by preparative gas chromatography as described elsewhere [8]. In the case of *p*-hydroxyphenylacetic acid the last step was replaced by recrystallization to constant specific radioactivity.

L-Amino acid oxidase activity was assayed by adding a solution of 1.9 mg $NH_2OH \cdot HCl$ and 3.1 mg sodium carbonate in 0.2 ml of water to the incubation mixture described above. The oximes were finally characterized by recrystallization to constant specific radioactivity. The R_F-values in solvent system I were: 0.55 (oxime of phenylpyruvic acid), 0.28 (*p*-hydroxyphenylacetic acid) and 0.07 (oxime of *p*-hydroxyphenylpyruvic acid). In solvent system II: *n*-butanol : ammonia : ethanol : benzene (5/3/2/1, by volume) the following R_F-values were observed: 0.65 (phenylacetic acid), 0.65 (oxime of phenylpyruvic acid), 0.38 (*p*-hydroxyphenylacetic acid) and 0.39 (oxime of *p*-hydroxyphenylpyruvic acid).

The keto acids derived from L-methionine and L-leucine are reasonably stable. Therefore the incubation mixture contained the labelled amino acid without added hydroxylamine. α-Ketoisocaproic acid and α-keto-γ-methiolbutyric acid were identified directly by paper chromatography. The R_F-values were 0.63 (I) and 0.61 (II) for the former and 0.22 (I) and 0.52 (II) for the latter compound.

Determination of radioactivity

Radioactive samples were counted in a scintillation spectrometer (Beckman SL 230). Chromatograms were scanned on a chromatogram scanner (4π-geometry, 15% efficiency for ^{14}C).

Other determinations

Chlorophyll a was determined according to Strain *et al.* [9]. The content of phycocyanin, which accounts for about 36% of the soluble proteins of *A. nidulans*, was measured at 620 nm using an extinction coefficient of 7.3 $mg^{-1} \cdot cm^2$ [10]. Polyphenol oxidase activity was assayed at 265 nm using 4-methylcatechol and ascorbic acid as described by Ruis [11].

Sucrose density was determined refractometrically.

Results

Formation of phenylacetic acids

Preliminary experiments should reveal whether homogenates of *A. nidulans* cells procedure significant amounts of phenylacetic acids from aromatic amino acids. Expts 1 and 2, Table I, show that both phenylacetic acid and *p*-hydroxyphenylacetic acid are formed in good yields. In fact they represented

Table I. Conversion of aromatic amino acids into phenylacetic acids by homogenates and subcellular fractions of *A. nidulans*.

Expt. No.	Substrate	[M]	Preparation	Product	nmol × (30 min)$^{-1}$ × (mg Chl a)$^{-1}$
1	L-[4′-^{3}H]phenylalanine	2×10^{-4}	homogenate	phenylacetic acid	1.5
2	L-[3′,5′-^{3}H]tyrosine	7×10^{-5}	homogenate	*p*-hydroxyphenylacetic acid	2.5
3	L-[4′-^{3}H]phenylalanine	2×10^{-4}	thylakoids	phenylacetic acid	0.68
			supernatant (after ultrasonification)	phenylacetic acid	0.11 *
4	[U-^{14}C]phenylalanine	3×10^{-4}	thylakoids	phenylacetic acid	1.08
			supernatant (after lysozyme treatment)	phenylacetic acid	0.02 *
5	L-[3′,5′-^{3}H]tyrosine	8×10^{-5}	thylakoids	*p*-hydroxyphenylacetic acid	0.34
			supernatant (after ultrasonification)	*p*-hydroxyphenylacetic acid	0.06 *
6	L-[U-^{14}C]tyrosine	2×10^{-4}	thylakoids	*p*-hydroxyphenylacetic acid	0.65
			supernatant (after lysozyme treatment)	*p*-hydroxyphenylacetic acid	<0.01 *

* Specific activity is calculated per mg of phycocyanin. The weight ratio chlorophyll a : phycocyanin is about 1 : 18 in *A. nidulans*.

the main products of secondary metabolism of aromatic amino acids in the blue-green alga. The following experiments (Table I) were designed to clarify whether the enzyme activities involved in the formation of phenylacetic acids are bound to photosynthetic membranes as it had been observed in the case of benzoic acid formation in *A. nidulans* [7].

In experiments 3 and 5, where the cells were broken by ultrasonic treatment a substantial part of the total activity was found in the soluble fraction. When cells were opened by a milder method using lysozyme, a considerable increase in the amount of thylakoid-bound activity was observed. Thus it can be concluded that the enzyme activities responsible for the conversion of aromatic amino acids into phenylacetic acids are located at the photosynthetic membranes but are susceptible to solubilization.

For further purification homogenates as well as thylakoid preparations were subjected to sucrose density gradient centrifugation. The results presented in Table II confirm the localization of phenylacetic acid biosynthesis at the photosynthetic membranes. Fraction I, damaged thylakoids, showed only small activity. Formation of phenylacetic acids was not detectable with the yellowish membrane fraction II, possibly containing cell membrane fragments. Fractions III, IV and V represent photosynthetic membranes of increasing intactness. This was reflected by correspondingly higher rates of phenylacetic acid synthesis. Thylakoid preparations IV and V were obtained by milder lysis of the cells [7]. No ^{14}C-label could be found in *p*-hydroxyphenylacetic acid, *i. e.*, hydroxylation does not occur at the level of phenylacetic acid or phenyl-

Table II. Formation of phenylacetic acids upon simultaneous incubation of purified membrane fractions with L-[U-^{14}C]phenylalanine (2.5×10^{-5} M) and L-[3′,5′-^{3}H]tyrosine (2.5×10^{-4} M). Conversion rates are given in nmol × (30 min)$^{-1}$ × (mg Chl a)$^{-1}$. For fractions I to V see under Materials and Methods.

Products	Label	Conversion rates using the following fractions				
		I	II	III	IV	V
phenylacetic acid	[^{14}C] only	0.05	<0.01	0.65	2.1	2.3
p-hydroxyphenylacetic acid	[^{3}H] only	<0.01	<0.01	0.45	0.48	0.71

alanine or of a possible indermediate like phenylpyruvic acid.

Demonstration of the formation of phenylpyruvic acids as intermediates

In higher plants phenylacetic acid is formed from L-phenylalanine via phenylpyruvic acid [1], whereas *p*-hydroxyphenylacetic acid is derived from L-tyrosine, *p*-hydroxyphenylpyruvic acid being the intermediate [12].

Since phenylpyruvic acids are rather unstable and are decomposed upon paper chromatography in alkaline solvent systems, they were converted *in situ* into the stable oximes. These derivatives, obtained by addition of hydroxylamine to the incubation medium, are easily crystallizable and suitable for silylation and gas-liquid chromatography. Expts 1 and 2, Table III, demonstrate that in the presence of hydroxylamine considerable amounts of the oximes of phenylpyruvic acid and *p*-hydroxyphenylpyruvic acid were formed whereas the yields of the corresponding phenylacetic acids were markedly lowered. Oxime formation is comparable to phenylacetic acid formation observed without addition of hydroxylamine. This indicates that the formation of the phenylpyruvic acids is the rate limiting step in the biosynthesis of phenylacetic acids. Once formed the keto acids are rather quickly transformed into the corresponding arylacetic acids unless they are trapped as the oximes. Expt. 3 shows that the conversion of phenylalanine to its keto acid is a process mainly localized on the thylakoid membranes as was expected from the results concerning phenylacetic acid formation. Expt. 4 gives evidence that the solubilization of phenylacetic acid forming enzyme activities by ultrasonic treatment is reflected by the solubilization of phenylpyruvic acid formation by the same procedure. Here specific activities are related to the chlorophyll content of the nascent thylakoids, so short sonication resulted in the appearance of about one quarter of the total activity in the supernatant.

Evidence for a thylakoid-bound L-amino acid oxidase

A transaminase or an L-amino acid oxidase activity could be responsible for the observed conversion of aromatic amino acids into their α-keto acid counterparts on thylakoid membranes of *A. nidulans*. Eventually a side reaction catalyzed by a polyphenol oxidase could be borne in mind. This enzyme has been reported to be a constituent of thylakoid membranes of higher plants [13]. However, with *A. nidulans* no polyphenol oxidase activity could be detected at thylakoids or in the soluble supernatant. Even if one accounts for the lower sensitivity of the optical test used (see methods) compared to the radiochemical enzyme assays described in this paper, this possibility has been ruled out. The results presented in Table IV render also the involvement of an aminotransferase unlikely. Thoroughly washed thylakoids, which should be devoid of transamination cofactors showed about the same specific activity as thylakoids with added α-ketoglutarate and pyridoxal phosphate. However, incubation under an atmosphere of nitrogen effected a remarkable decrease of activity. A similar reduction of the yield was obtained upon addition of

Table III. Products formed from aromatic amino acids by subcellular fractions from lysozyme-treated cells in the presence of hydroxylamine.

Expt. No.	Substrate	Preparation	Product	nmol × (30 min)$^{-1}$ × (mg Chl a)$^{-1}$
1	L-[U-^{14}C]phenylalanine	thylakoids	oxime of phenylpyruvic acid	1.34
			phenylacetic acid	0.11
2	L-[U-^{14}C]tyrosine	thylakoids	oxime of *p*-hydroxyphenylpyruvic acid	0.63
			p-hydroxyphenylacetic acid	0.03
3	L-[U-^{14}C]phenylalanine	thylakoids	oxime of phenylpyruvic acid	0.74
		supernatant	oxime of phenylpyruvic acid	0.02 *
4	L-[U-^{14}C]tyrosine	thylakoids, sonicated for 4 × 15 sec	oxime of *p*-hydroxyphenylpyruvic acid	0.41
		supernatant thereafter	oxime of *p*-hydroxyphenylpyruvic acid	0.14

* per mg phycocyanin.

Table IV. Incubation of thylakoids with L-[U-^{14}C]tyrosine under varying conditions in the presence of hydroxylamine.

Incubation system	Oxime of *p*-hydroxyphenylpyruvic acid formed [nmol × (30 min)$^{-1}$ × (mg Chl a)$^{-1}$]
thylakoids	0.85
thylakoids, 3× washed	0.79
thylakoids + α-ketoglutarate + pyridoxalphosphate *	0.80
thylakoids, under nitrogen	0.06
thylakoids + 10^{-3} M *o*-aminobenzoate	0.08

* In this experiment hydroxylamine was added 10 min after the reaction was started.

the L-amino acid oxidase inhibitor *o*-aminobenzoate. These facts make it likely that the first step in the biosynthesis of phenylacetic acids in *A. nidulans* is catalyzed by a thylakoid-bound L-amino acid oxidase.

Substrate specificity

Thylakoid membranes of *A. nidulans* showed significantly higher conversion rates for L-phenylalanine and L-tyrosine than for L-methionine or L-leucine (Table V). The latter amino acids are good substrates for L-amino acid oxidase of the snake venom type [14] and for the analogous enzyme from rat kidney [15]. However, the membrane-bound enzyme of the blue-green alga appears to be rather specific for aromatic amino acids.

The conversion of phenylpyruvic acid into phenylacetic acid

In order to investigate further the pathway leading from aromatic amino acids to phenylacetic acids phenylpyruvate was incubated with thylakoid membranes. This compound is less unstable than the *p*-hydroxylated analogue and allows better purification by paper chromatography. Table VI shows the results of two experiments, each compared with a blank. Decomposition products accounted for a large part of the radioactivity recovered. Phenylacetic acid formation was in the same order of magnitude for assay and blank. The differences in reisolated phenylpyruvic acid were more pronounced. Upon incubation with active thylakoid preparations less substrate was left than with the blanks.

Table VI. Incubation of *A. nidulans* thylakoids with [U-^{14}C]-phenylpyruvate. 7.2×10^6 dpm were used for Expt. 1 giving a molar concentration of 1.5×10^{-4} whereas Expt. 2 was performed with 4.45×10^5 dpm (1.1×10^{-5} M).

Expt. No.	Incubation system	Phenylpyruvic acid recovered [dpm]	Phenylacetic acid formed [dpm]
1	thylakoids	425000	1753000
	blank (buffer only)	735000	1405000
2	thylakoids	<1000	243000
	blank (autoclaved thylakoids)	47300	204000

These results indicate that at least under the incubation conditions of this study phenylpyruvic acid undergoes a fast nonenzymatic transformation into phenylacetic acid. Membrane-bound enzyme activities may be superimposed but their nature needs further investigation. Blanks obtained with L-phenylalanine or L-tyrosine as substrates showed only very low levels of phenylacetic acids.

Discussion

Phenylacetic acids were found to be major products of aromatic amino acid metabolism in *Anacystis nidulans*. In gram-positive bacteria *p*-hydroxyphenylacetic acid is an intermediate of the homoprotocatechuate pathway for L-tyrosine catabolism [16]. *Achromobacter euridice* degrades L-phenylalanine by inducible enzymes to phenylacetic acid [17]. In both these cases an aminotransferase catalyzes the first step, *i. e.*, the formation of phenylpyruvic acids.

Table V. Incubation of thylakoids with various labelled amino acids.

Substrate	[M]	Product determined	nmol × (30 min)$^{-1}$ × (mg Chl a)$^{-1}$
L-[U-^{14}C]tyrosine	2×10^{-4}	oxime of *p*-hydroxyphenylpyruvic acid	1.64
L-[U-^{14}C]phenylalanine	1×10^{-4}	oxime of phenylpyruvic acid	1.48
L-[methyl-^{14}C]methionine	8×10^{-5}	α-keto-γ-methiolbutyric acid	0.1
L-[U-^{14}C]leucine	8×10^{-5}	α-ketoisocaproic acid	0.14

In *A. nidulans*, however, the homogentisate pathway is operative, where *p*-hydroxyphenylacetic acid is not involved [18]. No hydroxylation at the levels of L-phenylalanine, phenylpyruvic acid or phenylacetic acid could be observed in the blue-green alga. Thus it is here less likely that phenylacetic acids represent members of a pathway for aromatic amino acid catabolism. Yet unpublished results obtained in this laboratory indicate that both acids exert effects on the growth of *A. nidulans*. They possibly take part in the regulation of metabolic processes as has been reported for auxins in higher plants.

In chloroplasts of higher plants phenylacetic acid is formed by thylakoid-bound enzymes [6]. The analogy between these organelles of eukaryotic cells and the blue-green bacterium *A. nidulans* extends further to the mechanism of the first step of the reaction sequence. The formation of phenylpyruvic acid and *p*-hydroxyphenylpyruvic acid at chloroplast thylakoids is catalyzed by a firmly bound L-amino acid oxidase [19]. However, the evaluation of the distribution of this enzyme activity within the cells of higher plants was hampered by contaminating bacteria [20]. With *A. nidulans*, grown axenically, this difficulty was avoided. A similar relationship between chloroplasts of higher plants and of eukaryotic algae and *A. nidulans* concerning the formation of benzoic acids by thylakoid-bound enzyme complexes has recently been reported [7]. Thus, this study adds some new results to the accumulating evidence in favour of the endosymbiotic origin of chloroplasts [21].

This work was supported by the Jubiläumsstiftung der österreichischen Nationalbank. The author thanks professors O. Hoffmann-Ostenhof and H. Ruis for helpful discussions and for reviewing the manuscript.

[1] F. Wightman, Biochem. Soc. Symp. **88,** 247–275 [1973].
[2] G. Billek and H. Kindl, Mh. Chem. **93,** 85–91 [1962].
[3] H. Abe, M. Uchiyama, and R. Sato, Agr. Biol. Chem. **38,** 897–898 [1974].
[4] B. V. Milborrow, J. G. Purse, and F. Wightman, Ann. Bot. **39,** 1143–1146 [1975].
[5] L. Fries and H. Iwasaki, Plant Sci. Lett. **6,** 299–307 [1976].
[6] W. Löffelhardt and H. Kindl, Hoppe-Seyler's Z. Physiol. Chem. **356,** 487–493 [1975].
[7] W. Löffelhardt and H. Kindl, Z. Naturforsch. **31 c,** 693–699 [1976].
[8] W. Löffelhardt, B. Ludwig, and H. Kindl, Hoppe-Seyler's Z. Physiol. Chem. **354,** 1006–1012 [1973].
[9] H. H. Strain, B. T. Cope, and W. A. Svec, Methods Enzymol. **23 A,** 452–476 [1971].
[10] I. W. Craig and N. G. Carr, Biochem. J. **106,** 361–366 [1968].
[11] H. Ruis, Phytochemistry **11,** 53–58 [1972].
[12] H. Kindl, European J. Biochem. **7,** 340–347 [1969].
[13] N. E. Tolbert, Plant Physiol. **51,** 234–244 [1973].
[14] D. Wellner, Methods Enzymol. **17 B,** 597–600 [1971].
[15] M. Nakano and T. S. Danowski, Methods Enzymol. **17 B,** 601–605 [1971].
[16] V. L. Sparnins and P. J. Chapman, J. Bacteriol. **127,** 362–366 [1976].
[17] M. Fujioka, Y. Morino, and H. Wada, Methods Enzymol. **17 A,** 585–596 [1970].
[18] G. R. Whistance and D. R. Threlfall, Biochem. J. **117,** 593–600 [1970].
[19] B. Ludwig, Diplomarbeit, FB Chemie der Univ. Marburg 1973.
[20] B. Ludwig and H. Kindl, private communication.
[21] P. H. Raven, Science **169,** 641–646 [1970].