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SHORT COMMUNICATION

METABOLISM OF AROMATIC AMINO ACIDS IN GLYOXYSOMES

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Abstract—Investigations were carried out on the possible role of glyoxysomes from the endosperm of *Ricinus communis* in the metabolism of aromatic amino acids. L-Phenylalanine was shown to be mainly converted to cinnamic acid by isolated glyoxysomes. Smaller amounts of *p*-coumaric acid and benzoic acid were also detected. *p*-Coumaric acid is formed by hydroxylation from cinnamic acid more actively in proplastids and microsomes than in glyoxysomes. *p*-Coumaric acid was the main product of metabolism of L-tyrosine. A remarkably large amount of *p*-hydroxybenzoic acid was also found indicating a chain shortening process, which *in vivo* has been shown before to be responsible for the formation of hydroxybenzoic acids in higher plants. Formation of *p*-hydroxyphenylacetic acid and homogentisic acid was also detected. The results demonstrate the capability of glyoxysomes to metabolize aromatic amino acids. This finding adds an entirely new aspect to the knowledge on metabolism in glyoxysomes. Proplastids from the same tissue show a different pattern of products.

INTRODUCTION

GLYOXYSOMES are known to be plant cell organelles responsible for a main part of biosynthesis of carbohydrates from fats, namely the β-oxidation of fatty acids and the conversion of acetyl-coenzyme A to succinate via the glyoxylate cycle (for a summary see Ref. 1). They were first detected in the endosperm of germinating castor beans (*Ricinus communis*)[2] and it is now assumed that they generally occur in plant tissues which have to mobilize fat reserves. But these storage cells in seeds only contain up to 60 per cent fat and Stewart and Beevers[3] report also on the occurrence of protein and amino acids in the seeds of *R. communis*. Therefore, it appeared interesting to study the mobilization of the storage protein and the degradation of amino acids in this tissue. It also seemed worthwhile to investigate the question as to whether the very specialized glyoxysomes are responsible for other metabolic pathways apart from the mobilization of fat. This paper demonstrates that glyoxysomes can metabolize and degrade aromatic amino acids.

RESULTS

Metabolism of L-*phenylalanine*

L-(U-^{14}C) Phenylalanine (0·2 μmoles) was incubated with glyoxysomes (2 mg protein) in 400 μl 0·05 M borate (pH 8·8) at 35° for a period of 2 hr. Subsequently, 2 mg of the following compounds were added: cinnamic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, and benzoic acid. The mixture *in toto* was layered on a preparative TLC plate[4] and chromatographed as described in the Experimental. The zones corresponding to the added compound, detected by UV light at 254 nm, were scraped off the plate and the radioactive compounds

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[2] R. W. BREIDENBACH and H. BEEVERS, *Biochem. Biophys. Res. Commun.* **27**, 462 (1967).
[3] C. R. STEWART and H. BEEVERS, *Plant Physiol.* **42**, 1587 (1967).
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eluted and characterized by rechromatography on paper and fractional sublimation. Cinnamic acid, obtained after sublimation at 10^{-1} torr and 140°, contained the preponderant amount of radioactivity metabolized. More than 1 nmole/mg glyoxysomal protein was formed during 1 hr in a series of experiments. Small amounts of radioactivity were detected when benzoic acid or *p*-coumaric acid, respectively, isolated by repeated chromatography and purified by sublimation were recrystallized to constant specific activity after dilution with inactive authentic material. However, the hydroxylation of cinnamic acid to *p*-coumaric acid cannot predominantly be attributed to glyoxysomes, since microsomes as well as proplastids show a considerably higher specific activity for this *p*-hydroxylation as shown below.

4 μmoles (3-^{14}C) cinnamic acid, 1 μmole 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine hydrochloride, 4 μmoles NADPH, and 4 mg of either microsomes, or proplastids, or glyoxysomes, in 0·05 M Tris-HCl buffer, pH 7·5 (total vol. 300 μl) were shaken in an O_2 atmosphere for 1 hr. Working up the reaction mixtures in a similar way as described above gave the following amounts of *p*-coumaric acid: 10 nmoles/mg protein (microsomes), 2 nmoles/mg (proplastids), and 0·1 nmoles/mg (glyoxysomes).

Benzoic acid, a metabolite of L-phenylalanine in glyoxysomes, is subject to *o*-hydroxylation. Using the same conditions as in the experiment with (3-^{14}C) cinnamic acid carboxyl-labelled benzoic acid was converted into salicylic acid. A first crude separation of the radioactive salicylic acid formed from benzoic acid and cofactors was accomplished by TLC and twofold fractional sublimation at 160° and 10^{-1} torr. Further purification achieved by preparative gas chromatography was checked by several-fold recrystallization from water. From various cell fractions the proplastids exhibited the highest transformation (1·5 nmoles/mg protein), whereas less than 0·1 nmoles were formed per mg glyoxysomal protein.

Metabolism of L-*tyrosine*

When 0·2 μmoles L-(U-^{14}C) tyrosine were incubated with 2 mg glyoxysomes in 0·05 M borate (pH 8·8) (total volume 400 μl), *p*-coumaric acid was found to be the main product. During 2 hr more than 80 per cent of the radioactivity applied were metabolized and this fraction contained 60 per cent *p*-coumaric acid. Thorough examination of the zones corresponding to lower R_f values than *p*-coumaric acid on TLC revealed the formation of at least three more radioactive compounds. One of those peaks rechromatographed in solvent system A and B (see Experimental) coincided with *p*-hydroxybenzoic acid. After dilution with 300 mg inactive *p*-hydroxybenzoic acid, the compound was repeatedly recrystallized from water. The specific activities (dpm) after 4 successive recrystallizations were: 24,500, 20,300, 21,100, 22,600. It was calculated that more than 15 per cent of the radioactivity of L-(U-^{14}C) tyrosine was converted into *p*-hyroxybenzoic acid, which is equivalent to 15 nmoles/mg glyoxysomal protein.

Another peak eluted from the original TLC plate was characterized by paper chromatography in solvent system A and by preparative gas chromatography at 120° on Varaport coated with SE-30. These procedures together with recrystallization to constant specific activity demonstrated that at least 8 per cent of the original radioactive material was converted to *p*-hydroxyphenylacetic acid.

Finally the radioactive zone which migrated only 1 cm on the original TLC plate was purified by descending paper chromatography (for 30 hr) in system B, allowing the solvent to run off the end of the paper. Preparative gas chromatography and subsequent paper

chromatography in the system benzene–methanol–acetic acid (45:8:4, by vol.) unequivocally proved the formation of homogentisic acid.

A high percentage of conversion of C_6–C_3 compound into a benzoic acid derivative could be demonstrated by incubation of (3-^{14}C) *p*-coumaric acid (2 μmoles) with ATP (10 μmoles), coenzyme-A (2 μmoles), and 4 mg glyoxysomes at 35° during 2 hr (using 0·05 M Tris-HCl, (pH 7·5); total vol. 400 μl). Separation and isolation of *p*-hydroxybenzoic acid in the manner described above indicated that conversion of 24 nmoles/mg protein has taken place under the conditions employed.

DISCUSSION

The results obtained in experiments *in vitro* with glyoxysomal protein demonstrated the capability of glyoxysomes of metabolizing aromatic amino acids. By control experiments with other cell fractions, which have been described previously,[5] the possibility could be excluded that the activities observed in glyoxysomes are only due to contaminations from those fractions. Recent work in this laboratory[5] unequivocally proved the occurrence of L-phenylalanine ammonia-lyase and L-tyrosine ammonia-lyase in glyoxysomes. For example, in the case of L-tyrosine ammonia-lyase approximately 10 per cent of the total activity of the endosperm tissue was detected in glyoxysomes. But since glyoxysomes are severely damaged during the isolation procedure the activities reported here have to be considered as minimal amounts of conversion. Comparison with some other typical glyoxysomal enzymes would suggest that the actual enzyme activities associated with the intact organelles could be much higher.

The first of the metabolic pathways detected starts with the elimination of ammonia catalyzed by the ammonia lyases mentioned above. This may be followed most likely by a chain shortening reaction converting cinnamic acids into benzoic acid derivatives. This type of reaction (for a summary see Ref. 6) is already known to take place in higher plants since in 1962 Kindl and Billek[7] reported the conversion of ferulic acid to vanillic acid and of cinnamic acid to gentisic acid. It probably involved removal of acetyl-coenzyme-A similar to the β-oxidation of fatty acids.[8] The latter metabolic process has been shown previously in castor bean endosperm to be located virtually exclusively in glyoxysomes.[9] It seems remarkable that an analogous reaction has now been also detected in glyoxysomes. It has also been demonstrated that this degradation of a C_6–C_3 unit to a C_6–C_1 compound represents the physiological pathway leading to *p*-hydroxybenzoic acid[10] the precursor of ubiquinone.

p-Hydroxyphenylacetic acid and homogentisic acid are probably formed via a common intermediate, *p*-hydroxyphenylpyruvic acid, which could be formed from L-tyrosine by an enzyme already known to occur in glyoxysomes, L-amino acid oxidase.[11] So not only a pathway leading to a precursor of ubiquinone but also another one to homogentisic acid, an important precursor of plastoquinone,[12] has been found in glyoxysomes.

The *p*-hydroxylation of cinnamic acid or the *o*-hydroxylation of benzoic acid cannot be

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considered to be typical for glyoxysomal organelles. But specific activities observed for *p*-hydroxylation in microsomes from castor bean endosperm are comparable with values obtained with microsomes from other tissues from dark grown plants.[13,14]

Most of the reactions reported here take place predominantly in glyoxysomes since e.g. proplastids exhibit, as preliminary experiments indicate, a different specificity in the metabolism of various aromatic compounds.

In the last few years a picture has emerged from work on metabolism in glyoxysomes[1] which seemed to show these organelles as a quite highly specialized compartment of the cell. Thus the work we are presenting here adds an entirely new aspect to our knowledge on their metabolic activities.

EXPERIMENTAL

Radioactive compounds. L-(U-^{14}C)Phenylalanine (400 mC/m-mole) and L-(U-^{14}C)tyrosine (400 mC/m-mole) were products of The Radiochemical Centre, Amersham. (3-^{14}C)Cinnamic acid (550 μC/m-mole) and (carboxyl-^{14}C)benzoic acid (500 μC/m-mole) were synthesized by conventional methods.[15] (3-^{14}C)*p*-Coumaric acid (300 μC/m-mole) was prepared from (carbonyl-^{14}C)*p*-hydroxybenzaldehyde.[15,16]

Plants material. R. communis seeds were prepared for germination in the following way: they were surface sterilized by stirring them for 6 min in a 0·05% aqueous $HgCl_2$-solution. Then they were rinsed 4 × with sterile H_2O. They were transferred to a flask containing sterile H_2O and air, filtered through a plug of sterile cotton wool, was bubbled through for 90 min. Seed coats were then cautiously broken on one side to facilitate water uptake. The seeds were then germinated in the dark on sterile moist cellulose at 30° for 5 days.

Preparation of cellular subfractions. Glyoxysomes and proplastids were isolated from the endosperm of germinating castor beans as described by Cooper and Beevers.[17] Stepped or linear sucrose gradients were used. Centrifugations were carried out in a Beckmann SW 25·1 rotor at 25,000 rev/min at 2° for 4 hr. Fractions containing glyoxysomes or proplastids, respectively, were combined, diluted four-fold with grinding medium and the organelles collected by centrifugation. Microsomes were obtained from the supernatant of the crude mitochondrial pellet by a 60 min centrifugation at 100,000 *g*. Purity of glyoxysomes was checked with the aid of marker enzymes as described previously.[5]

Chromatographic procedures. The solvent system benzene–HOAC (4:1, v/v) was used for TLC chromatography on silica gel G. Utilizing preparative TLC plates the R_f values of the compounds investigated were: L-phenylalanine (0·00), L-tyrosine (0·00), homogentisic acid (0·06), *p*-hydroxyphenylacetic acid (0·19), *p*-hydroxybenzoic acid (0·26), *p*-coumaric acid (0·33), salicylic acid (0·65), benzoic acid (0·80), and cinnamic acid (0·85).

Radioactive compounds were further purified by descending paper chromatography using the solvent system n-BuOH–NH_4OH–EtOH–benzene (5:3:2:1, v/v) (=A) and benzene–HOAC–H_2O (4:2:1, v/v, upper phase)[10,18] (=B).

Gas chromatographic analysis was performed with a Varian aerograph 1800 apparatus equipped with a hydrogen flame ionization detection system. The columns used contained silicone nitrile XE-60 or silicone gum SE-30, respectively, on Varaport-30. Utilizing the SE-30 column at 130°, cinnamic acid, salicylic acid, and benzoic acid were effectively separated (relative retention times 1·00, 0·39, and 0·18). Homogentisic acid (as lactone, 5-hydroxy-2-coumarone) could be characterized using a column where silicone nitrile XE-60 served as the stationary phase. At 100° the relative retention time (in relation to cinnamic acid) was 1·0.

Fractional sublimation. A micro apparatus with a temperature gradient as described previously[18] was used. At 10^{-3} torr and 170° cinnamic acid and *p*-coumaric acid could easily be separated. *p*-Hydroxybenzoic acid was sublimed at 190° and 10^{-3} torr.

Determination of radioactivity. This was carried out with a Nuclear Chicago Liquid Scintillation counter (Series 720). The scintillation fluids used and the technique employed have already been described.[19]

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