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FORMATION OF α,β-UNSATURATED CARBOXYLIC ACIDS FROM AMINO ACIDS IN PLANT PEROXISOMES

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Abstract—Investigations were carried out on the metabolism of ^{14}C-labelled L-phenylalanine, L-tyrosine, L-histidine, L-tryptophan, and L-aspartic acid in isolated leaf peroxisomes from spinach and sunflowers. All the amino acids were converted to the corresponding α,β-unsaturated carboxylic acids. Cinnamic acid was formed from L-phenylalanine, *p*-coumaric acid from L-tyrosine, and indolyl-3-acrylic acid from L-tryptophan. Urocanic acid and fumaric acid were characterized as metabolic products of L-histidine and L-aspartic acid, respectively. The experiments lead to the suggestion that in higher plants ammonia-lyases may be generally associated with microbodies, and that leaf peroxisomes may have more importance in amino acid metabolism than was previously assumed.

INTRODUCTION

IN HIGHER plants the formation of α,β-unsaturated acids from L-amino acids plays a central role in the metabolism of some of the amino acids. This type of reaction is generally catalyzed by ammonia-lyases. The presence of L-aspartate ammonia-lyase,[1] L-phenylalanine ammonia-lyase,[2] and L-tyrosine ammonia-lyase[3] in higher plants has been reported. The formation of cinnamic acid or of *p*-coumaric acid catalyzed by the two latter enzymes is the starting reaction for the biosynthesis of most plant constituents derived from the shikimic acid pathway. Whereas L-phenylalanine ammonia-lyase has been detected in a large number of vascular plants and in several genera of Basidiomycetes[4] and has been well characterized already[5] less information is available on the two other enzymes or on the presence of additional ammonia-lyases in higher plants.

Nothing was known about the intracellular localization of ammonia-lyases in eucaryotic cells until the recent report from this laboratory of the presence of a number of ammonia-lyases in glyoxysomes from the endosperm of germinating castor beans.[6] Investigations described in this paper show that leaf peroxisomes are also capable of catalyzing the formation of α,β-unsaturated acids from a number of amino acids.

RESULTS

Identification of Incubation Products

Sunflower. 50 μC of either L-(1-^{14}C)phenylalanine, L-(1-^{14}C)tyrosine, L-(U-^{14}C)histidine, L-(U-^{14}C)aspartic acid, or L-(methylene-^{14}C)tryptophan were incubated separately with peroxisomes (for further experimental details see Table 1). After a first crude separation of the components using TLC (see Methods), the zones corresponding to the α,β-unsaturated

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[3] A. C. NEISH, *Phytochem.* **1**, 1 (1961).
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[5] E. A. HAVIR and K. R. HANSON, *Biochem.* **7**, 1896 (1968).
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carboxylic acids were cut out and the compounds eluted with methanol. The eluate representing the crude *p*-coumaric acid (110,000 dis/min) was concentrated and rechromatographed on paper, 90 per cent of the radioactivity being localized in the zone coinciding with *p*-coumaric acid. The compound eluted from the paper was diluted with 200 mg highly purified inactive *p*-coumaric acid and then subjected to fractional sublimation. The sublimate and the crystalline product obtained after recrystallization from water had the same specific activity (480 dis/min). A similar procedure was employed in order to isolate and characterize cinnamic acid as a metabolic product from L-phenylalanine.

TABLE 1. FORMATION OF α,β-UNSATURATED CARBOXYLIC ACIDS FROM THE CORRESPONDING AMINO ACIDS IN PEROXISOMES FROM SUNFLOWERS AND SPINACH

Precursor	Product	Conversion (nmoles/mg protein) Sunflowers	Spinach
L-Phenylalanine	Cinnamic acid	12	1·0
L-Tyrosine	*p*-Coumaric acid	1·4	0·4
L-Aspartic acid	Fumaric acid	4·0	1·2
L-Histidine	Urocanic acid	0·5	1·0
L-Tryptophan	Indolylacrylic acid	1·0	—

For sunflowers: 0·8 μmoles L-(1-^{14}C)phenylalanine or 0·3 μmoles L-(1-^{14}C)-tyrosine or 0·04 μmoles L-(U-^{14}C)histidine or 0·06 μmoles L-(U-^{14}C)aspartic acid or 0·2 μmoles L-(methylene-^{14}C)tryptophan, respectively, (each representing 50 μC) were incubated with 0·5 mg peroxisomal protein in a total volume of 300 μl at 35° for 1 hr.

For spinach: The same concentrations as for sunflower peroxisomes were used; but the volume of incubation mixtures was reduced to 1/3. For isolation and quantitation of products see text.

Crude urocanic acid was purified after the first TLC separation by repeated paper chromatography. Finally, the radioactive material was diluted with 400 mg urocanic acid and recrystallized from water; the specific activity (dis/min/mg) was constant after 5 recrystallizations (number of recrystallization in parenthesis): 840(1)–770(2)–760(3)–760(4)–790(5).

Indolylacrylic acid was separated from the components of the incubation mixture but could only be obtained free of contaminating indolylacetic acid by repeated chromatography on paper in solvent system A (see Experimental). Subsequent chromatography in solvent B showed only one sharp peak. The radioactive material (110,000 dis/min) was eluted from the paper with methanol and 200 mg indolylacrylic acid, purified by recrystallization immediately before use, was added. Avoiding bright light, the indolylacrylic acid was first separated from small amounts of polymers by filtering through silica gel (0·05–0·20 mm); then the compound was recrystallized three times from ethanol. The specific activity remained constant.

Fumaric acid was characterized by rechromatography; malate or acetate was not detectable in the rechromatographed material. Conversions calculated from these data are summarized in Table 1.

Spinach. Since thin layer chromatography provided an effective separation of the α,β-unsaturated carboxylic acids from the corresponding amino acids the conversions summarized below (Table 1) are based on the values determined by counting the product adsorbed on silica gel. The great differences in R_f values and the sharp bands localized by

fluorescence justify this method.[7] Only when L-histidine was used, was it necessary to rechromatograph the urocanic acid formed twice to separate it from small traces of imidazolyl acetic acid.

Distribution of Activities among various Cell Fractions

To make certain that α,β-unsaturated acids are actually formed by peroxisomes and that the observed conversions are not a result of contamination of the isolated peroxisome fraction by other cell organelles, incubations were carried out with a number of other cell fractions. Table 2 summarizes the results of a typical experiment with L-phenylalanine.

TABLE 2. FORMATION OF CINNAMIC ACID FROM L-PHENYLALANINE BY VARIOUS CELL FRACTIONS

Cell fraction	Amount of cinnamic acid formed	
	nmoles	nmoles/mg protein
37,000 *g* Supernatant	620	0·32
Gradient supernatant	9·6	0·74
Crude mitochondria	21·6	0·50
Peroxisomes	1·6	2·60

0·5 μmoles L-(1-^{14}C)phenylalanine were incubated with protein from cell fractions at 35° for 30 min; total vol. 300 μl. Protein was determined according to Lowry *et al.*[8]

As Table 2 shows, much less cinnamic acid was formed in the peroxisome fraction than in the supernatant or mitochondrial fractions. However, one has to remember the fact that peroxisomes are quite labile and are heavily damaged during both disruption of cells and the purification procedure. Some peroxisomal enzymes are more easily solubilized than others,[9] and are then found in the supernatant. Therefore the amount of cinnamic acid formed per mg of protein should give more reliable information as to whether peroxisomes are actually capable of forming the unsaturated carboxylic acids. Since the specific activity of the enzymes is by far the highest in the peroxisomes, at least part of this activity must be compartmented in these organelles. In addition it could be shown that only about 0·02 per cent of the activity of a number of enzymes present in the supernatant were found as contaminating the peroxisome fraction. Therefore the level of supernatant proteins in this organelle fraction is at least 10 times lower than the percentage of ammonia lyases detected in peroxisomes. In agreement with our results, Rocha and Ting[10] have shown recently that leaf peroxisomes isolated by suitable density gradients are of high purity, being less contaminated by other fractions than all the other organelles isolated from spinach leaves. From the relatively high activity and specific activity of the gradient supernatant fraction (Table 2), one can see that a good part of the activity gets lost from the organelles during isolation. The possibility that the observed activities result from contamination by chloroplasts could

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not be ruled out when peroxisomes from the first gradient were utilized. During fractionation chloroplasts and chloroplast fragments are distributed among several fractions, so that it would be difficult to decide which of these 'chloroplast fractions' would be suitable for a quantitative comparison of specific activities with peroxisomes. However, it could be shown that a chloroplast-free peroxisome preparation purified by a second density gradient (as described in the Experimental) had the same activity as the crude preparation.

DISCUSSION

The results described in this paper show that leaf peroxisomes, like glyoxysomes, are capable of converting certain amino acids to the corresponding α,β-unsaturated acids. It can be assumed also that these conversions can take place *in vivo* in peroxisomes. Formation of the unsaturated acids should be a result of the action of a number of specific ammonia-lyases or one relatively unspecific ammonia-lyase localized in peroxisomes; but these enzymes will have to be characterized and compared with similar enzymes from other compartments by future work.

To our knowledge formation of urocanic acid from L-histidine has hitherto been shown only in one higher plant system, glyoxysomes from castor bean endosperm.[6] Indolyl-3-acrylic acid has recently been isolated from plants[11] but this is the first report of its metabolic formation from L-tryptophan in higher plants. Peroxisomes have been demonstrated by Tolbert *et al.*[9,12] to be involved in glycolate metabolism; in connexion with the glycolate pathway they participate in the metabolism of the amino acids glycine and L-serine. But, as shown in this paper, quite different pathways of amino acid metabolism, namely the formation of α,β-unsaturated acids, can also take place in these organelles. Metabolic capacities of this type have never before been demonstrated in peroxisomes from any organism. The fact that these conversions could be shown in two different kinds of plant microbodies which otherwise have entirely different metabolic functions may indicate that at least plant microbodies are generally equipped with this metabolic capacity. Although from the published results no conclusions can be drawn on the quantitative importance of the observed conversions for metabolism *in vivo*, they do show that leaf peroxisomes are not as highly specialized in their metabolism as they appeared to be from previous work.

EXPERIMENTAL

Radioactive precursors. L-(1-^{14}C)phenylalanine (12·5 mC/m-moles), L-(1-^{14}C)tyrosine (33 mC/m-mole), L-(U-^{14}C)histidine (250 mC/m-mole), and L-(U-^{14}C)aspartic acid (160 mC/m-mole) were purchased from NEN-Chemicals, Dreieichenhain. L-(methylene-^{14}C)Tryptophan (50 mC/m-mole) was from The Radiochemical Centre, Amersham.

Isolation of peroxisomes. Peroxisomes were prepared from leaves of spinach (*Spinacia oleracea*) and sunflowers (*Helianthus annuus*). A procedure modified from that of Tolbert *et al.*[9] was used. 100 to 200 g of washed and deribbed leaves were ground in 50 g portions in a Waring blendor for 10 sec in 75 ml of 0·5 M sucrose in 0·02 M glycylglycine or Tricine (pH 7·5). This and all the following procedures were carried out at 2°. After filtration through nylon mesh the extract was centrifuged for 7 min at 1000 *g*, and the pellet discarded. The supernatant was then centrifuged for 20 min at 6000 *g*. In some cases this was followed by a centrifugation of the 6000 *g* supernatant for 20 min at 37,000 *g*, whereby a crude mitochondrial pellet was obtained. The 6000 *g* pellet was suspended in 3 ml of grinding medium and the suspension layered on top of a discontinuous density gradient consisting of 7·5 ml 60% sucrose, 15 ml 50% sucrose, and 5 ml 30% sucrose in 0·02 M glycylglycine or Tricine (pH 7·5). Gradients were centrifuged in a Beckman SW 25·1 ultracentrifuge rotor for 4 hr at 25,000 rev/min. Peroxisomes, which were identified by their catalase content, were collected from the 50–60% interphase. The combined peroxisome fractions were diluted 4-fold with grinding medium and were centrifuged for 20 min at 18,000 rev/min. To obtain peroxisomes entirely free of chloroplasts this peroxisome preparation was further purified over a continuous gradient made from 13 ml 60% and

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13 ml 45% sucrose. After 4 hr of centrifugation at 25,000 rev/min in the SW 25·1 rotor, one green and one colourless band were obtained from the gradients. The colourless peroxisomes were collected as described above. Catalase as marker enzyme was assayed spectrophotometrically at 240 nm.[13]

Isolation procedure. The products of the reactions with various cell fractions were isolated by separating the components of the incubation mixture using preparative TLC on silica gel. The entire reaction mixture was put on the plate (2 mm thick) and subsequently a solution of the expected compounds in HOAc was layered on the start region; the principle has been already described for L-tyrosine and L-phenylalanine ammonia-lyases.[7] When L-phenylalanine, L-tyrosine, or L-tryptophan were the substrates of the reaction the solvent system benzene–HOAc (4:1, v/v) was utilized.[7] In the case of metabolism of L-histidine or L-aspartic acid, respectively, the TLC plates were developed in *n*-BuOH–HOAC–H_2O (4:1:1, v/v). R_f values: fumaric acid 0·85, malic acid 0·70, aspartic acid 0·20; urocanic acid 0·50, imidazole acetic acid 0·46, histamine 0·18, histidine 0·06.

Re-chromatography of the reaction products was performed on paper with two solvent systems. System A: *n*–BuOH–NH_4OH–EtOH–benzene (5:3:2:1, v/v); system B: benzene–HOAc–H_2O (4:2:1, v/v).[14] In system A the following $R_{\text{salicylic acid}}$ values were found: histidine 0·30, imidazole acetic acid 0·43, urocanic acid 0·50, tryptophan 0·66, indolylacetic acid 0·83, indolylacrylic acid 0·90.

Determination of radioactivity. Radioactivity of compounds adsorbed on TLC plates was assayed by suspending the finely powdered silica gel containing the compound in a thixotropic gel; the procedure, as well as the methods used for liquid scintillation counting, were as reported previously.[15] In the cases where preparative TLC plates coated with a fluorescent indicator were used, it was necessary to keep the scintillation vials in the dark before counting until chemiluminescence had decayed completely.

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