p-HYDROXYBENZOATE SYNTHASE: A COMPLEX ASSOCIATED WITH MITOCHONDRIAL MEMBRANES OF ROOTS OF *CUCUMIS SATIVUS*

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1. Introduction

The enzymes concerned with *p*-hydroxybenzoic acid biosynthesis have long been of special interest because of the role of *p*-hydroxybenzoic acid in the formation of ubiquinone [1] and many other compounds. In vivo-experiments with various plants, cf. [2] have shown that *p*-coumaric acid is the immediate precursor of *p*-hydroxybenzoic acid. In addition, we have been able to demonstrate in vitro the conversion of *p*-coumaric acid to *p*-hydroxybenzoic acid utilizing glyoxysomes [3] and glyoxysomal membranes [4]. Though interesting in its own right, the association of the *p*-hydroxybenzoate-forming enzyme(s) with glyoxysomes apparently restricts this crucial pathway to a certain metabolic situation, i.e. the germination of certain seeds. A more generally occurring compartment may easier be correlated to a pathway leading to the ubiquitous ubiquinone.

This paper presents a *p*-hydroxybenzoic acid-forming system which not only exhibits reasonably high conversion rates; but it also seems to be more representative with respect to the physiological role than any other benzoic acid-synthesizing in vitro system from higher plants, so far known.

2. Materials and methods

2.1. *Materials*

[3′, 5′-^{3}H] *p*-Coumaric acid was prepared from [3′, 5′-^{3}H] tyrosine using a tyrosine-ammonia lyase preparation from *Avena sativa* [5]. [3-^{14}C] Ferulic acid was synthesized from [7-^{14}C] vanillin and malonic acid [6]. [1-^{14}C] Palmitic acid was a product of Radiochemical Centre, Amersham, and [1-^{14}C] palmityl-CoA was purchased from NEN Chemicals, Dreieichenhain.

Seeds of *Cucumis sativus* were surface-sterilized and germinated at 20°C for 5 days.

2.2. *Isolation of organelles*

Several treatments were tried to break up the root cells. Finally the following method was adopted: 30 g of moist roots were chilled, cut with scissors and then ground in a cooled mortar for 20 min in an equal vol of grinding medium containing 17% (w/w) sucrose [7] The mixture was then squeezed through three layers of Miracloth and centrifuged – at 4°C, as were all other operations – at 1000 *g* for 10 min. The supernatant was centrifuged at 15 000 *g* for 30 min to give a mitochondrial, plastids and microbody pellet and this supernatant was in turn centrifuged at 150 000 *g* for 60 min to give a microsomal fraction and the final supernatant. All pellets were resuspended in 17% sucrose, 10 mM Tris–HCl (pH 7.2) and assayed simultaneously at the end of the centrifugation.

When the 15 000 *g* pellet was subjected to an isopycnic gradientcentrifugation a sucrose gradient ranging from 25–55% (w/w) was used. The tubes were centrifuged in a Spinco SW 27 rotor at 27 000 rev/min for 6 h.

In most other cases, a sedimentation velocity centrifugation was performed, where 32 ml of a linear sucrose gradient (20–45%, w/w) and a 4 ml-cushion (60%) was employed. The SW 27 rotor was accellerated in a Beckman L5-65 centrifuge to 25 000 rev/min using the highest accelleration rate. After 25 min the run was stopped. The gradients were fractionated into samples of 1.2 ml on an ISCO gradient fractionator.

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2.3. *Assay of* p-*hydroxybenzoate synthase*

The standard assay mixture contained in a total vol of 1.5 ml: 60 nmol (40 μM) [3′, 5′-^{3}H]*p*-coumaric acid (85 μCi/μmol), 1.0 ml membrane preparation (100 μg protein) and 0.5 ml 0.1 M Tris–HCl buffer (pH 7.5). After 10 min incubation time 0.5 mg inactive *p*-hydroxybenzoic acid was added and the mixture repeatedly extracted with ether. The ether solution was concentrated and the phenolic acids separated by paper chromatography, using the system *n*-butanol–conc. ammonia–ethanol–benzene (5:3:2:1, v/v) [8]. The zone corresponding to *p*-hydroxybenzoic acid was eluted and after dilution with 100 mg *p*-hydroxybenzoic acid as carrier, a recrystallization to constant specific acitivity was performed. Samples of 1 to 3 mg of recrystallized product were disolved in 0.3 ml acetone p.a., mixed with a toluene scintillation cocktail and counted in a scintillation spectrometer. Results are expressed as V_{max} corresponding to a K_M = 175 μM (cf. [3,4]); therefore, the conversion rates obtained with 40 μM substrate were multiplied by 5.

2.4. *Other assays*

Sucrose concentrations of the fractions were determined by refractometry. Cytochrome *c* oxidase [9], glucose 6-phosphate dehydrogenase [10], glucose 6-phosphatase [11] and malate dehydrogenase [12] were assayed according to documented proedures. The assays of fumarase, catalase, citrate synthase and triosephosphate isomerase were performed as summarized elsewhere [7]. Protein was measured at 280/260 nm [13].

3. Results

3.1. p-*Hydroxybenzoate synthase activity in crude fractions*

Comparing - with respect to their capability of converting *p*-coumaric acid to *p*-hydroxybenzoic acid – homogenates from roots of *C. sativus* with roots or leaves of other plants (*Triticum vulgare, Astilbe chinensis*) a clear preference was given to the cucumber system. As shown in table 1, conversion rates up to 10 nmol per g fresh weight in 10 min were found. The highest total or specific activities were attributable to the organelle fractions containing mitochondria and plastids.

3.2. *Separation of components of the 15 000* g *pellet*

An isopycnic gradient centrifugation of the 15 000 *g* pellet yielded one broad protein band between densities 1.17 – 1.19 g/cm^3. This fraction contained more than 80% of the *p*-hydroxybenzoate synthase applied to the gradient. Attempts aimed at a better separation of the organelles led to centrifugation methods based on differences in mass of mitochondria, microbodies, plastids and membranes released from the apparently inevitable breakage of organelles during isolation. It was found that at least 10 min, but rather 25 min, are necessary to give a reasonably good separation of the subcellular fractions. Fig.1 shows the distribution of various enzymatic activities on the sucrose gradient. Only the four fractions rich in protein were tested for *p*-hydroxybenzoate synthase. Besides some activity in fractions which contain impure and partially damaged

Table 1
p-Hydroxybenzoic acid formation (nmoles in 10 min) using homogenates or fractions, respectively, from three plants (10 g fresh weight each)

	Cucumis sativus roots	*Triticum sativum* roots	*Triticum sativum* leaves	*Astilbe chinensis* leaves
Crude homogenate	98 (0.020)	19 (0.011)	14 (0.008)	42 (0.012)
15 000 *g* pellet	75 (0.170)	10 (0.120)	n.d.[a]	26 (0.100)
150 000 *g* pellet	7 (0.055)	1 (0.030)	n.d.	3 (0.050)
Supernatant	21 (0.002)	2 (0.001)	n.d.	5 (0.002)

In parenthesis are the specific activities in nmoles formed/min/mg protein.
[a] n.d. = not determined.

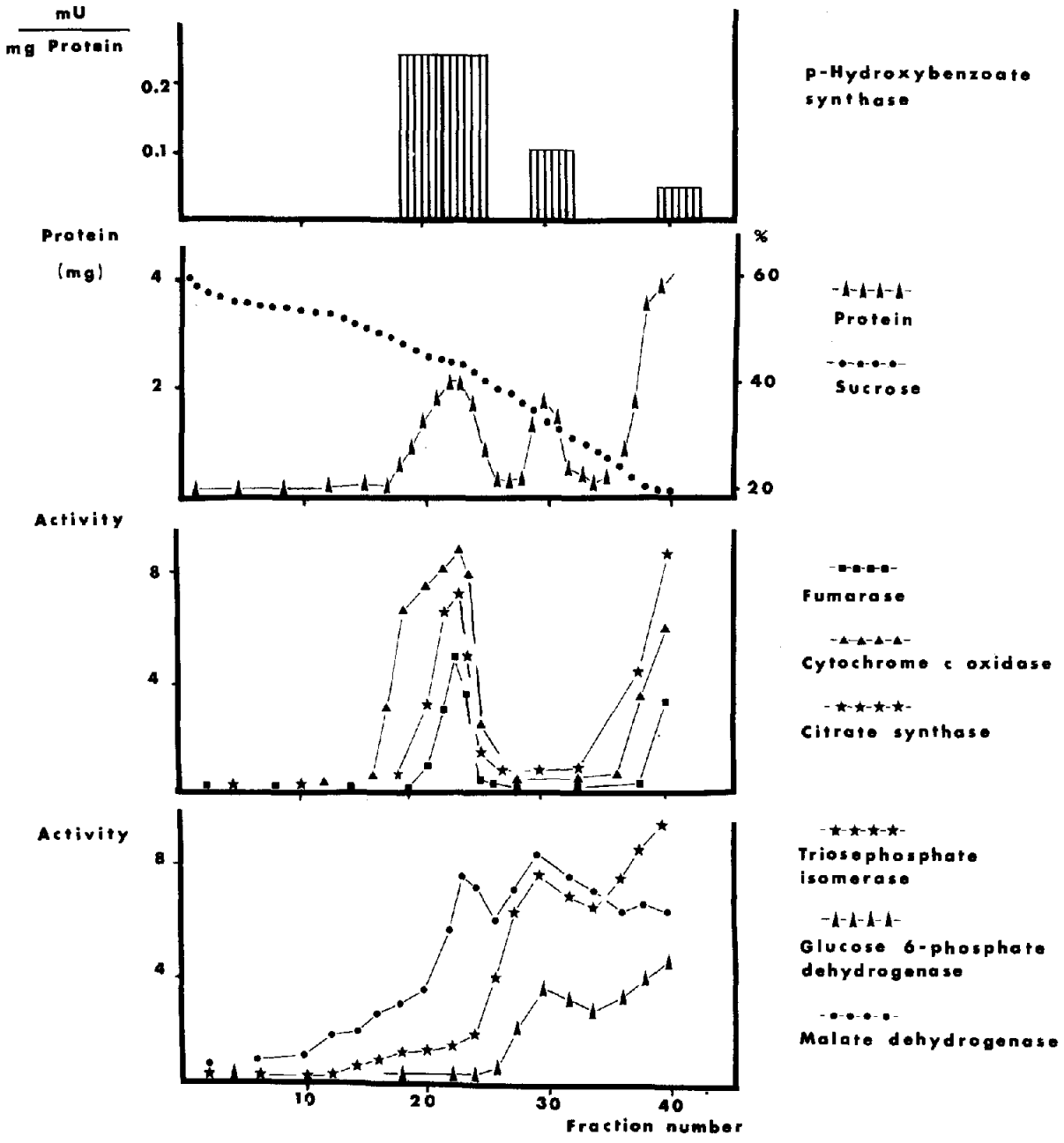

Fig.1. Enzyme profiles after a 25-min centrifugation of a 15 000 *g* pellet. One arbitrary unit of enzyme activity represents the following values for the various enzymes: citrate synthase: 0.5 U/ml; cytochrome oxidase: 0.25 U/ml; fumarase: 3 mU/ml; triose phosphate isomerase: 0.1 U/ml; malate dehydrogenase: 0.1 U/ml; glucose 6-phosphate dehydrogenase: 3 mU/ml. The activity of catalase and glucose 6-phosphatase did not show any significant peak in the gradient.

Table 2
Comparison of *p*-hydroxybenzoate synthase and fatty acid β-oxidation in mitochondrial fractions. 175 μM [3′, 5′-^{3}H] *p*-coumaric acid, 20 μM [1-^{14}C] palmitic acid, or 20 μM [1-^{14}C] palmitoyl-CoA, respectively, were incubated with aliquots of the mitochondrial subfractions for 10 min. Total activities and specific activities are given for these conditions

	p-Hydroxybenzoate synthase		β-Oxidation using palmitic acid		β-Oxidation using palmitoyl-CoA	
	nmol	nmol/mg	nmol	nmol/mg	nmol	nmol/mg
Matrix	1.85	0.37	0.30	0.06	2.00	0.40
Membranes	5.00	1.25	0.04	0.01	0.16	0.04

plastids (fr. no. 29–32) or in the gradient supernatant (fr. no. 30–42), most activity towards *p*-coumaric acid degradation was contributed by two fractions (fr. no. 18–21 and 22–25) at about 41% sucrose. It is concluded that mitochondria are the cellular sites where *p*-hydroxybenzoic acid synthesis takes place.

3.3. *Location of p-hydroxybenzoate synthase within mitochondria*

When a mitochondrial preparation (4 ml, 1.5 mg/ml) was treated 4 times 30 sec with an Ultraturax blendor (Janke und Kunkel, Staufen) and then centrifuged at 50 000 *g* for 30 min, the activity in the supernatant can be compared with the activity at the membrane. On the basis of specific and total activity (table 2) the *p*-hydroxybenzoate synthase activity is assigned to the mitochondrial membrane. In contrast, the β-oxidation of [1-^{14}C] palmitoyl-CoA was most active in the supernatant representing the mitochondrial matrix. A significant CoA-dependence was shown only in the case of fatty acid degradation.

3.4. *Some features of the p-hydroxybenzoate synthase*

Using a suspension of mitochondria in 0.1 M Tris–HCl buffer (pH 7.5) as enzyme preparation, the *p*-hydroxybenzoate synthase exhibited the following features: K_M for *p*-coumaric acid: 175 μM; V_{max}: 240 pmol/mg protein/min; pH optimum: 7.2; no activation by additional ATP, Coenzyme A, ATP + Coenzyme A, or NAD^+ (table 3). The activity towards other cinnamic acids was (with *p*-coumaric acid as 100%): ferulic acid (62%), cinnamic acid (36%).

Attempts to demonstrate the existance of intermediates in the assay solution were unsuccessful. Upon paper chromatography of the assay mixture or of the ether extract of the acidified solution, no other radioactive compounds could be detected except *p*-coumaric acid and *p*-hydroxybenzoic acid.

4. Discussion

A significant activity of *p*-hydroxybenzoate synthase activity was found in roots of cucumber seedlings and assigned to mitochondria. Mitochondrial preparations from cucumber behave similar to organelles from other roots [14,15] when subjected to isopycnic density centrifugation or sedimentation velocity centrifugation. Deviating from the results obtained by Miflin [15] is the sedimentation rate of plastids which appears to be slower in the case of cucumber organelles.

Table 3
Effects of various coenzymes on the *p*-hydroxybenzoate synthase of mitochondria. Gradient-purified mitochondria (0.5 mg protein) were incubated under standard assay conditions for 10 min; the additions were 0.5 mM each

Additions	Activity nmol	% of control
–	1.20	100
ATP	1.15	96
Coenzyme A	1.08	90
ATP + Coenzyme A	1.10	92
NAD^+	1.10	92
$NADP^+$	1.22	103
ATP + Coenzyme A + $NADP^+$	1.18	98

In the case of glyoxysomes – the other hitherto described in vitro-system for the synthesis of *p*-hydroxybenzoic acid – the *p*-hydroxybenzoate synthase is associated with the organelle's membrane as are the enzymes of fatty acid β-oxidation. Therefore, a clear-cut decision whether a separate system working independently from β-oxidation exists for *p*-coumarate degradation was not made in glyoxysomes. Here with mitochondria, two criteria can be applied which clearly distinguish between fatty acid β-oxidation and *p*-hydroxybenzoic acid formation: fatty acid oxidation proceeds via CoA esters and is located in the matrix, while the *p*-hydroxybenzoate synthase is not activated by exogeneously supplied CoA and is associated with the membrane.

The inability to obtain any evidence for intermediates leads to the suggestion that the conversion of *p*-coumaric acid to *p*-hydroxybenzoic acid is catalyzed by an enzyme complex arranged at the mitochondrial membrane, Such membrane-bound multi-enzyme complexes are quite common in the metabolism of aromatic amino acids in higher plants [16,17]. Although it is very likely in other cases [3,18] that acetate or acetyl CoA is the other product of the reaction in question, the lack of effect of coenzyme A on benzoic acid formation on thylakoids [17] or on *p*-hydroxybenzoic acid formation at the mitochondrial membrane allows other interpretations, too. The reaction sequence does not need to proceed via CoA esters. A considerable amount of work is required, therefore, on the mechanism of the multi-step reaction.

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