Polyprenyl Pyrophosphate-*p*-Hydroxybenzoate Polyphenyltransferase Activity in Mitochondria of Broad-Bean Seeds and Yeast

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Cell-free homogenates prepared from broad-bean seeds and yeast cells are capable of synthesizing 4-carboxy-2-polyphenylphenols from *p*-hydroxybenzoate and either isopentenyl pyrophosphate or protein-bound polyphenyl pyrophosphates (produced by incubating a *Micrococcus lysodeikticus* extract with isopentenyl pyrophosphate). The mitochondria contained all the polyphenyl pyrophosphate-*p*-hydroxybenzoate polyphenyltransferase activity; however, unlike the homogenates they could not synthesize a side chain from isopentenyl pyrophosphate and had to be provided with protein-bound polyphenyl pyrophosphates.

It is now well established that *p*-hydroxybenzoate is the specific distal precursor of the *p*-benzoquinone ring of the ubiquinones (see Threlfall & Whistance, 1971). The pathways leading from this acid to the ubiquinones appear to be similar in photosynthetic bacteria, facultative Gram-negative bacteria, some aerobic bacteria and animals, and all have as their first step the formation of 4-carboxy-2-polyphenylphenols (Scheme 1) (see Threlfall & Whistance, 1971; Momose & Rudney, 1972). The enzymic nature of the first step has been studied by Rudney and his co-workers, who first of all showed that chromatophores of *Rhodospirillum rubrum* and homogenates of rat tissues will synthesize 4-carboxy-2-polyphenylphenols and, in the case of the chromatophores, 2-polyphenylphenols (the next intermediates) from *p*-hydroxybenzoate and protein-bound polyphenyl phosphates produced by incubating a polyphenyl pyrophosphate synthetase preparation from *Micrococcus lysodeikticus* with isopentenyl pyrophosphate (Raman *et al.*, 1969; Winrow & Rudney, 1969). They then went on to demonstrate that in animal tissues the inner mitochondrial membrane is capable of the synthesis of 4-carboxy-2-polyphenylphenols from *p*-hydroxybenzoate and isopentenyl pyrophosphate, a finding that showed that this membrane, as well as containing a polyphenylpyrophosphate-*p*-hydroxybenzoate polyphenyltransferase (*p*-hydroxybenzoate polyphenyltransferase), must also contain a system of enzymes for synthesizing polyphenyl pyrophosphates (Momose & Rudney, 1972). It is noteworthy that both the *Rsp. rubrum* and animal polyphenyltransferases show little specificity with regard to the length of the polyphenyl side chain that they transfer to *p*-hydroxybenzoate.

In higher plants, algae and fungi the nature of the pathway is far from resolved. There is some evidence that in the last two groups of organisms the terminal steps are the same as those in bacteria and animals. Thus 5-demethoxyubiquinones have been isolated

from the alga *Euglena gracilis* and a number of fungi, and have been shown to be precursors of ubiquinones in the yeasts *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis* and the mould *Phycomyces blakesleeanus* (Threlfall & Whistance, 1970; Ah Law *et al.*, 1971; Ah Law & Threlfall, 1972). In the case of the fungus *Aspergillus flavus* evidence has been presented that the pathway *p*-hydroxybenzoate → 2-decaprenyl(X-H₂)phenol → 6-methoxy-2-decaprenyl(X-H₂)phenol → 6-methoxy-2-decaprenyl(X-H₂)-1,4-benzoquinone → 5-demethoxyubiquinone-10(X-H₂) → ubiquinone-10(X-H₂) is operative in the biosynthesis of ubiquinone-10(X-H₂) by this organism (Ah Law *et al.*, 1971).

The failure to detect in higher plants, algae and yeast intermediates, other than 5-demethoxyubiquinones, of the type found on the bacterial and animal pathways of ubiquinone biosynthesis indicated that either they are present in amounts too small to be detected by the analytical methods used or they are not involved in ubiquinone biosynthesis in these organisms. In a final effort to find some evidence for the operation of the early steps of the animal- and bacterial-type pathway in these organisms, it was decided to attempt to demonstrate the appropriate enzymic activities in cell-free preparations.

In the present paper we report the demonstration of polyphenyl pyrophosphate-*p*-hydroxybenzoate polyphenyltransferase activity in homogenates and mitochondrial preparations of the yeast *S. carlsbergensis* and seeds of the higher plant *Vicia faba* var. Aquadulce.

Experimental

p-Hydroxy[U-¹⁴C]benzoic acid (7.7 mCi/mmol) and *p*-hydroxy[7-¹⁴C]benzoic acid (6.7 mCi/mmol) were prepared by alkaline fusion of L-[U-¹⁴C]tyrosine hydrochloride and DL-[β-¹⁴C]tyrosine respectively

Scheme 1. Formation of 4-carboxy-2-polyprenylphenols

(Whistance *et al.*, 1967). Triammonium $[1\text{-}^{14}\text{C}]$ isopentenyl pyrophosphate (60mCi/mmol) was purchased from The Radiochemical Centre (Amersham, Bucks., U.K.) and trilithium isopentenyl pyrophosphate was synthesized from isopentenol (Aldrich Chemical Co., Milwaukee, Wis., U.S.A.) by the method of Cornforth & Popják (1969).

S. carlsbergensis Herb I.M.I. 80178 was grown for 24h under the conditions described by Ah Law & Threlfall (1972). After being harvested and washed by resuspension in 0.1 M-potassium phosphate buffer, pH7.1, the cells (from 2 litres of medium) were ground with 5g of neutral alumina (M. Woelm, Eschwege, Germany) for 5min. At the end of this time the suspension was centrifuged at 4000g for 10min to remove alumina, whole cells and cell debris. The resulting supernatant (yeast homogenate) was used either for incubation studies or for the preparation of a particulate fraction (mitochondrial preparation). In the latter case it was centrifuged at 10000g for 15min, after which the mitochondrial pellet was washed and then resuspended in a small volume of buffer.

V. faba var. Aquadulce seeds were soaked overnight in running tap water. The testas were then removed and 20g of tissue was homogenized in 20ml of 50mM-Tris-HCl buffer, pH8.2, containing 0.3M-sucrose and 1mM-EDTA for 30s in an MSE homogenizer. The homogenate was then filtered through two layers of muslin and centrifuged at 2000g for 10min. The supernatant (bean homogenate) was either used for incubation studies or centrifuged at 10000g for 20min, after which the mitochondrial pellet was washed with homogenizing medium and then resuspended in 3ml of 0.1M-potassium phosphate buffer, pH7.1.

Two incubation procedures were used in these studies. In the first the incubation mixture consisted of 5ml of 0.1 M-potassium phosphate buffer, pH7.1, containing 100 μ mol of MgCl_2 , 15 μ mol of cysteine, 10–150mg of homogenate or mitochondrial protein and either 0.37 μ Ci of p -hydroxy $[^{14}\text{C}]$ benzoate and 1.4 μ mol of isopentenyl pyrophosphate or 7.25 μ mol of p -hydroxybenzoic acid and 0.5 μ Ci of $[1\text{-}^{14}\text{C}]$ isopentenyl pyrophosphate. In the second the incubation mixture was the same as the first except that the unlabelled

or the $1\text{-}^{14}\text{C}$ -labelled isopentenyl pyrophosphate was omitted and the mixture was supplemented with 3 ml of an *M. lysodeikticus* extract that had been preincubated with either 11.4 μ mol of isopentenyl pyrophosphate or 0.5 μ Ci of $[1\text{-}^{14}\text{C}]$ isopentenyl pyrophosphate under the conditions described by Raman *et al.* (1969). Irrespective of their natures the mixtures were incubated under O_2 -free N_2 for 2h at 30°C with gentle agitation.

At the end of the incubation periods the mixtures were heated to 100°C to terminate the reactions and to precipitate the protein. The protein was collected by centrifugation and extracted with acetone followed by diethyl ether. The extracts were then combined and evaporated to dryness, and the light-petroleum (b.p. 40–60°C)-soluble fraction was chromatographed on thin layers of silica gel H developed with acetone–light petroleum (b.p. 40–60°C) (3:7, v/v). After development the $[^{14}\text{C}]$ 4-carboxy-2-polyprenylphenols (tri-, R_F 0.4; deca-, R_F 0.6) were eluted with ether, assayed for ^{14}C content and rechromatographed on thin layers of paraffin-impregnated silica gel G developed with either acetone–water–acetic acid (70:30:1, by vol.) (tri-, R_F 0.84; tetra-, R_F 0.72; penta-, R_F 0.57) or acetone–water–acetic acid (80:20:1, by vol.) (hexa-, R_F 0.91; hepta-, R_F 0.77; octa-, R_F 0.65; nona-, R_F 0.52; deca-, R_F 0.39) against hepta-, octa-, nona- and deca-prenyl homologues of $[^{14}\text{C}]$ 4-carboxy-2-polyprenylphenol isolated from *Rsp. rubrum* and 4-carboxy-2-octaprenylphenol from *Escherichia coli*. In some experiments, as an aid to identifying their chain lengths, the 4-carboxy-2-polyprenylphenols were converted into 2-polyprenylphenols by incubating them with cells of *Rsp. rubrum* that had been exposed to ultrasound (Raman *et al.*, 1969). The 2-polyprenylphenols were extracted from the incubation mixtures by the procedure described above for the isolation of 4-carboxy-2-polyprenylphenols and their chain lengths were determined by reversed-phase t.l.c. (Whistance *et al.*, 1969).

Results

On incubation of yeast homogenates with p -hydroxy $[^{14}\text{C}]$ benzoate and either isopentenyl pyrophosphate or *M. lysodeikticus* extract that had been

Table 1. Incorporation of radioactivity from *p*-hydroxy[U - ^{14}C]benzoate into 4-carboxy-2-polyprenylphenols

Cell-free homogenates or mitochondrial fractions were incubated under the conditions described in the text with 0.37 μ Ci of *p*-hydroxy[U - ^{14}C]benzoate and either isopentenyl pyrophosphate or *M. lysodeikticus* extract that had been preincubated with isopentenyl pyrophosphate.

Details of incubation			Incorporation of radioactivity into 4-carboxy-2-polyprenylphenols									
Type of preparation	Protein content (mg)	Source of side chain	(d.p.m.)	(% distribution of radioactivity in homologues)								
Yeast				Di-	Tri-	Tetra-	Penta-	Hexa-	Hepta-	Octa-	Nona-	Deca-
Homogenate	16	None	0	—	—	—	—	—	—	—	—	—
Homogenate	16	Isopentenyl pyrophosphate	25900	2	73	0	0	5	12	8	0	0
Homogenate	24	<i>M. lysodeikticus</i> extract preincubated with isopentenyl pyrophosphate	42500	0	0	0	0	17	11	8	52	12
Mitochondria	16	Isopentenyl pyrophosphate	0	—	—	—	—	—	—	—	—	—
Mitochondria	16	<i>M. lysodeikticus</i> extract preincubated with isopentenyl pyrophosphate	32500	0	0	0	0	17	11	8	52	12
Bean*				Not examined								
Homogenate	90	Isopentenyl pyrophosphate	3200									
Mitochondria	128	<i>M. lysodeikticus</i> extract preincubated with isopentenyl pyrophosphate	60300	0	0	0	0	11	11	21	49	8

* Homogenates without a source of side chain and mitochondria supplemented with isopentenyl pyrophosphate failed to incorporate radioactivity into 4-carboxy-2-polyprenylphenols. Homogenates supplemented with *M. lysodeikticus* extract preincubated with isopentenyl pyrophosphate produced the same mixture of 4-carboxy-2-polyprenylphenols as mitochondria supplemented with that preparation.

preincubated with isopentenyl pyrophosphate radioactivity was incorporated into compounds that had the chromatographic properties expected of 4-carboxy-2-polyprenylphenols. In the incubations with isopentenyl pyrophosphate the tri- (principal component), hexa-, hepta-, octa- and possibly di-prenyl homologues were formed (Table 1). However, when isopentenyl pyrophosphate was replaced by *M. lysodeikticus* extract preincubated with isopentenyl pyrophosphate the hexa- through to deca-prenyl homologues were synthesized and the principal component was now the nonaprenyl homologue (Table 1). The identities of the various phenols were established further by showing that (a) they could be labelled from *p*-hydroxy[7- ^{14}C]benzoate and [1- ^{14}C]isopentenyl pyrophosphate (when incubated with this compound in the absence of *M. lysodeikticus* extract the greater part of the incorporated radioactivity, as expected, was found to be present in squalene) and (b) on incubation with an *Rsp. rubrum* preparation they were converted into compounds having the chromatographic properties of the appropriate 2-polyprenylphenols.

When the yeast homogenate was centrifuged at 11000g for 15 min the *p*-hydroxybenzoate polyprenyltransferase activity was found to be associated with the mitochondrial pellet. The mitochondrial preparation, however, could only synthesize 4-carboxy-2-polyprenylphenols when provided with a polyprenyl pyrophosphate-generating system (*M. lysodeikticus* extract preincubated with isopentenyl pyrophosphate) (Table 1).

At this stage attention was turned to higher plants and algae. Attempts to demonstrate *p*-hydroxybenzoate polyprenyltransferase activity in cell-free preparations of *Eu. gracilis*, mung-bean seedlings and French-bean and spinach leaves were all unsuccessful. However, active preparations were obtained from broad-bean seeds, and similar experiments to those described above for yeast were carried out with this tissue. It was found that, as with yeast, the *p*-hydroxybenzoate polyprenyltransferase activity was associated with the mitochondria. The mitochondrial preparation, when supplemented with *M. lysodeikticus* extract preincubated with isopentenyl pyrophosphate, synthesized the same mixture of 4-carboxy-2-polyprenylphenols as the yeast preparation (Table 1). The homogenate, however, showed very little activity when supplemented with isopentenyl pyrophosphate (Table 1).

In all of the experiments in which *p*-hydroxy[^{14}C]benzoates were used the only radioactive compounds formed were the [^{14}C]4-carboxy-2-polyprenylphenols.

Discussion

Cell-free homogenates prepared from germinating broad-bean seeds and exponentially growing yeast

cells are capable of synthesizing 4-carboxy-2-poly-prenylphenols from *p*-hydroxybenzoate and either isopentenyl pyrophosphate or protein-bound poly-prenyl pyrophosphates (Table 1). In agreement with experiments carried out with cell-free preparations of *Rsp. rubrum* and animal tissues (Raman *et al.*, 1969; Momose & Rudney, 1972), it was found that the composition of the available poly-prenyl pyrophosphates determines which 4-carboxy-2-poly-prenylphenol homologues are synthesized. Thus yeast homogenates, the supernatant fractions of which contain a highly active farnesyl pyrophosphate-synthesizing system, produce mainly 4-carboxy-2-triprenylphenol when supplemented with isopentenyl pyrophosphate and 4-carboxy-2-hexaprenylphenol through to 4-carboxy-2-decaprenylphenol when supplemented with protein-bound 2-hexa- to 2-deca-prenyl pyrophosphates formed by incubating *M. lysodeikticus* extract with isopentenyl pyrophosphate (Table 1). The bean homogenates, although not as active when supplemented with isopentenyl pyrophosphate, gave similar results (Table 1).

The mitochondrial fractions, as is the case with animal mitochondria (Momose & Rudney, 1972), contained the poly-prenyl pyrophosphate-*p*-hydroxybenzoate poly-prenyltransferase activity. In contrast with animal mitochondria, however, which can synthesize 4-carboxy-2-poly-prenylphenols with the same chain lengths as the constitutive ubiquinones from *p*-hydroxybenzoate and isopentenyl pyrophosphate, they could not use isopentenyl pyrophosphate and had to be provided with protein-bound poly-prenyl pyrophosphates. This failure of the mitochondrial fractions to use isopentenyl pyrophosphate is probably due to the loss or inactivation of one of the enzymes required for the synthesis of poly-prenyl pyrophosphates as a result of the relatively crude homogenization procedure used, rather than to the absence of a poly-prenyl pyrophosphate-synthesizing system from plant mitochondria. The ability of the yeast homogenate to make small amounts of 4-carboxy-2-hexaprenylphenol from *p*-hydroxybenzoate and isopentenyl pyrophosphate suggests that in this system the damaged mitochondria are capable of converting some of the dimethylallyl pyrophosphate,

geranyl pyrophosphate or farnesyl pyrophosphate molecules synthesized by the supernatant enzymes into poly-prenyl pyrophosphates with the same chain lengths as the principal constitutive ubiquinone (ubiquinone-6). One obvious advantage to be gained by having a mitochondrial poly-prenyl pyrophosphate synthetase is that it provides a simple and direct method of controlling the lengths of the side chains transferred to *p*-hydroxybenzoate, since the only condition to be fulfilled is that the synthetase should present the *p*-hydroxybenzoate poly-prenyltransferase with a mixture of poly-prenyl pyrophosphates of the same chain lengths as the constitutive ubiquinones.

This study thus provides the first evidence for the operation of the bacterial-type pathways of ubiquinone biosynthesis in higher plants. It also provides the second piece of evidence for the operation of these pathways in yeasts.

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- Ah Law & Threlfall, D. R. (1972) *Phytochemistry* **11**, 481-488
 Ah Law, Threlfall, D. R. & Whistance, G. R. (1971) *Biochem. J.* **123**, 331-339
 Cornforth, R. H. & Popják, G. (1969) *Methods Enzymol.* **15**, 359-390
 Momose, K. & Rudney, H. (1972) *J. Biol. Chem.* **247**, 3930-3940
 Raman, T. S., Rudney, H. & Buzzelli, N. K. (1969) *Arch. Biochem. Biophys.* **130**, 164-174
 Threlfall, D. R. & Whistance, G. R. (1970) *Phytochemistry* **9**, 355-359
 Threlfall, D. R. & Whistance, G. R. (1971) in *Aspects of Terpenoid Chemistry and Biochemistry* (Goodwin, T. W., ed.), pp. 357-404, Academic Press, London and New York
 Whistance, G. R., Threlfall, D. R. & Goodwin, T. W. (1967) *Biochem. J.* **105**, 145-154
 Whistance, G. R., Dillon, J. F. & Threlfall, D. R. (1969) *Biochem. J.* **111**, 461-472
 Winrow, M. J. & Rudney, H. (1969) *Biochem. Biophys. Res. Commun.* **37**, 833-840