SYNTHESIS OF 5-DEMETHOXYUBIQUINONE-6 AND UBIQUINONE-6 FROM 3-HEXAPRENYL-4-HYDROXYBENZOATE IN YEAST MITOCHONDRIA

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1. Introduction

Matrix-free mitochondrial membrane preparations of baker's yeast are able to carry out a Mg^{2+}-dependent synthesis of 3-all-*trans*-hexaprenyl-4-hydroxybenzoate, a compound that has been proposed as the first intermediate on the pathway leading from 4-hydroxybenzoate to ubiquinone-6(Q-6) in yeast [1], from 4-hydroxybenzoate, isopentenyl pyrophosphate (IPP) and γ,γ-dimethylallyl pyrophosphate (γ,γ-DMAPP) [Casey, J. and Threlfall, D. R, unpublished]. The membrane preparations were, however, unable to synthesise either any other compounds that might be intermediates on the pathway leading to Q-6 or Q-6. Indirect support for the involvement of 3-hexaprenyl-4-hydroxybenzoate in the biosynthesis of Q-6 has been provided by the isolation from a Q-6-deficient mutant of bakers yeast of 3,4-dihydroxy-5-hexaprenylbenzoate [2], a compound that could be the second intermediate on a pathway having 3-hexaprenyl-4-hydroxybenzoate as the first intermediate. It can of course also be argued that the isolation of this compound provides indirect support for a pathway in which 3,4-dihydroxybenzoate is the first intermediate formed from 4-hydroxybenzoate; c.f. the recent report that 3,4-dihydroxybenzoate can be formed from 4-hydroxybenzoate in rat liver slices and that 3,4-dihydroxy-5-polyprenylbenzoates can be synthesised from 3,4-dihydroxybenzoate and polyprenylpyrophosphates in rat liver mitochondria [3].

In the present paper we report on the ability of mitochondria isolated from yeast protoplasts to synthesise 6-methoxy-2-hexaprenylphenol, 5-demethoxyubiquinone-6 and ubiquinone-6 from either 4-hydroxybenzoate and IPP or 3-hexaprenyl-4-hydroxybenzoate.

2. Materials and methods

2.1. *Materials*

4-Hydroxy[U-^{14}C]benzoate (400 μCi/μmol) was prepared from L-[U-^{14}C]tyrosine (513 μCi/mol) by an enzymatic procedure [Casey, J., unpublished]. Li_3IPP and $(NH_4)_3$-γ,γ-DMAPP, were prepared from the appropriate alcohols [4]. All other reagents were of the best grades available from commercial sources.

2.2. *Isolation of mitochondria*

Saccharomyces carlsbergensis NCYC 74 was grown aerobically at 30°C on 2% (v/v) ethanol, 0.5% (w/v) mycological peptone and 0.3% (w/v) yeast extract in distilled water. The cells were harvested in mid-exponential phase by centrifugation and their protoplasts prepared by a routine procedure [5]. After resuspension in 0.2% (w/v) bovine serum albumin/0.6 M mannitol/0.05 M phosphate buffer, pH 7.4, the protoplasts were ruptured by one passage through a pressure cell (3000 lb/in^2) and the resultant homogenate centrifuged at 1500 × *g* for 10 min followed by 8000 × *g* for 10 min. The 8000 × *g* mitochondrial pellet was washed twice with bovine serum albumin/mannitol/phosphate buffer before final resuspension in a small volume of bovine serum albumin/mannitol/phosphate buffer.

2.3. *Radiochemical assay of biosynthetic activity*

The standard assay mixture was prepared by

adding 0.25 ml of mitochondrial suspension (5–8 mg protein) to 1.15 ml 0.6 M sorbitol/0.05 M phosphate buffer, pH 7.4, which contained 1.1×10^5 dpm (125 pmol) 4-hydroxy [U-^{14}C] benzoate (400 μCi/μmol), 0.5 μmol Li_3IPP, 10 μmol $MgCl_2$, 0.27 μmol *S*-adenosylmethionine (SAM), 0.375 μmol ATP, 0.27 μmol NADPH and 5.4 μmol succinate. The mixture was incubated in a 25 ml conical flask for the appropriate period of time at 30°C with gentle agitation.

At the end of the inocubation period the reaction was stopped by the rapid addition of 5 vol chloroform–methanol (1:2, v/v) and the chloroform-soluble lipids (this extract contained 3-hexaprenyl-4-hydroxybenzoate, 6-methoxy-2-hexaprenylphenol, 5-demethoxyQ-6 and Q-6) extracted and assayed for radioactivity [1]. In some experiments the aq. methanol fraction was adjusted to pH 2 and extracted with diethyl ether (3X) to recover unused 4-hydroxy [U-^{14}C]benzoate and any unprenylated [U-^{14}C]-benzoates produced in the course of the incubation.

2.4. *Identification of ^{14}C-products*

The chloroform-soluble lipids were subjected to thin-layer chromatography (TLC) on silica gel G developed with cyclohexane–diethyl ether (9:2, v/v) (3-hexaprenyl-4-hydroxybenzoate, R_F 0.05; 5-demethoxyQH_2-6, R_F 0.15; 5-demethoxyQ-6, R_F 0.18; QH_2-6, R_F 0.24:Q-6, R_F 0.27 and 6-methoxy-2-hexaprenylphenol, R_F 0.55). The ^{14}C-labelled compounds were located with a Panax TL Chromatogram Scanner, eluted from the gel, assayed for radioactivity content and their identities confirmed by rechromatography in appropriate TLC systems ((3-hexaprenyl-4-hydroxybenzoate; silica gel H developed with acetone–light petroleum (b.p.40–60°C) (3:7, v/v) (R_F 0.33) and then paraffin-impregnated silica gel G developed with aq. 75% (v/v) acetone (R_F 0.44): Q-6, QH_2-6, 5-demethoxyQH_2-6, 5-demethoxyQ-6 and 6-methoxy-2-hexaprenylphenol: paraffin-impregnated silica gel G developed with either aq. 90% (v/v) acetone or aq. 95% (v/v) acetone (see [6] for R_F values)). As final confirmation of their identities, the 5-demethoxy-[^{14}C]Q-6 and [^{14}C]Q-6 were reduced to the corresponding quinols with Zn/HCl and subjected to adsorptive and reversed-phase TLC (see above for R_F values).

The diethyl ether extract of the acidified aq. methanol fraction was examined for the presence of [^{14}C]benzoates by TLC on silica gel H developed with benzene–acetic acid (4:1, v/v) 3,4-dihydroxybenzoate, R_F 0.17; 4-hydroxybenzoate, R_F 0.42; 3-methoxy-4-hydroxybenzoate, R_F 0.57).

3. Results

3.1. *Synthesis of 5-demethoxyQ-6 and Q-6 by yeast mitochondria*

Mitochondria from yeast protoplasts were tested for their ability to form ^{14}C-labelled chloroform- and aq. [^{14}C]methanol-soluble compounds when incubated with 4-hydroxy [U-^{14}C]benzoate, IPP, γ,γ-DMAPP, Mg^{2+} and some of the supplements used in the successful demonstration of Q-9 biosynthesis from 4-hydroxybenzoate in rat liver mitochondria [7]. The results showed that fully supplemented mitochondria incorporated ^{14}C radioactivity into four chloroform-soluble compounds which had TLC properties identical to those of 3-hexaprenyl-4-hydroxybenzoate, 6-methoxy-2-hexaprenylphenol, 5-demethoxyQ-6 and Q-6 (table 1; expt. 1). Mitochondria incubated with 4-hydroxy-[U-^{14}C]benzoate, IPP, γ,γ-DMAPP and Mg^{2+} incorporated radioactivity into 3-hexaprenyl-4-hydroxybenzoate and, to a very limited extent, 5-demethoxyQ-6, whereas mitochondria incubated with 4-hydroxy [U-^{14}C]benzoate and the supplements used to demonstrate Q-9 biosynthesis in rat liver mitochondria formed no ^{14}C-labelled chloroform-soluble compounds (table 1; expt. 1). On examination of the diethyl ether extracts from the aq. methanol washings, it was found that the only radioactive compound they contained was the ^{14}C-labelled substrate. In each incubation the total amount of administered radioactivity was accounted for by the ^{14}C activity recovered in the chloroform-soluble compounds (after correction for loss of $^{14}CO_2$) and the unreacted ^{14}C-labelled substrate.

Subsequent experiments demonstrated that the omission of γ,γ-DMAPP, CoASH and methionine from the incubation mixture had no effect on the types and amounts of ^{14}C-labelled compounds produced, and that to obtain good rates of synthesis of 5-demethoxyQ-6 and Q-6 it is essential to have undamaged mitochondria (table 1; expt. 2).

Table 1
Incorporation of radioactivity from 4-hydroxy[U-^{14}C]benzoate into Q-6 and related compounds in undamaged (expt. 1) and damaged (expt. 2) mitochondria

Incubation conditions	^{14}C Activity (10^{-3} dpm) in $CHCl_3$-soluble compounds[a]			
	3-Hexaprenyl-4-hydroxybenzoate	6-Methoxy-2-hexaprenylphenol	5-Demethoxy-Q-6	Q-6
Expt. 1				
No supplement	0	0	0	0
Complete	41.4	6.3	35.1	11.1
Complete-IPP and γ,γ-DMAPP	0	0	0	0
Mg^{2+}, IPP and γ,γ-DMAPP	75.6	0	1.8	0
Expt. 2				
Standard assay	21.4	2.0	32.5	6.5
– Sorbitol	57.8	0	7.6	0
– Sorbitol + ultrasound	62.7	0	3.4	0

[a] After allowing for the loss of $^{14}CO_2$ in the formation of 6-methoxy-2-hexaprenylphenol, 5-demethoxyQ-6 and Q-6, the administered ^{14}C activity was entirely accounted for by the ^{14}C activity in the $CHCl_3$-soluble compounds + unreacted ^{14}C-labelled substrate

Expt. 1. Mitochondria (6 mg protein) were incubated with 4-hydroxy[U-^{14}C]benzoate and the various combinations of supplements shown in the table for 2 h. The complete system consisted of the standard assay mixture supplemented with 1.0 μmol γ,γ-DMAPP, 0.135 μmol CoASH and 1.35 μmol L-methionine.

Expt. 2. Mitochondria (7.25 mg protein) were (i) incubated under standard assay conditions, (ii) incubated in a sorbitol-deficient incubation mixture and (iii) suspended in a sorbitol-deficient incubation mixture and exposed to ultrasound for 3 s prior to the incubation. The mixtures were all incubated for 2 h

3.2. *Attempt to detect other intermediates*

Attempts were made to cause the accumulation of other possible intermediates on the pathway to Q-6 by incubating the standard assay mixture under anaerobic conditions or by the omission of SAM from the standard assay mixture. Under both sets of conditions the only ^{14}C-labelled compound produced was 3-hexaprenyl-4-hydroxybenzoate. It was also found that the additions of a CoA ester trapping reagent (hydroxylamine) to the standard assay mixture had no effect on the types and amounts of products.

3.3. *Synthesis of 6-methoxy-2-hexaprenylphenol, 5-demethoxyQ-6 and Q-6 from 3-hexaprenyl-4-hydroxybenzoate*

Mitochondria that had been allowed to accumulate 3-hexaprenyl[U-^{14}C]benzoate were incubated with the cofactors needed for the conversion of this ^{14}C-labelled compound into Q-6. The results showed that 3-hexaprenyl-4-hydroxybenzoate can act as a precursor of 6-methoxy-2-hexaprenylphenol, 5-demethoxyQ-6 and Q-6 (fig.1). The relatively poor incorporation of radioactivity into Q-6 was attributed to damage of the mitochondria in the washing procedure which followed the preincubation step.

4. Discussion

The results of this study show that undamaged yeast mitochondria are able to carry out the synthesis of 3-hexaprenyl-4-hydroxybenzoate, 6-methoxy-2-hexaprenylphenol, 5-demethoxyQ-6 and Q-6 starting from 4-hydroxybenzoate and IPP (table 1). The synthesis of the first compound, as in the synthesis of 2-nonaprenyl-4-hydroxybenzoate in rat liver mitochondria [8], is Mg^{2+}-dependent (Casey, J., unpublished) and requires only 4-hydroxybenzoate and IPP, whereas the synthesis of the other compounds needs the addition of supplements similar to those required for the formation of Q-9 from 4-hydroxybenzoate in 'programmed' rat liver mitochondria (mitochondria isolated from homogenates that have been preincubated with MVA to increase the size of

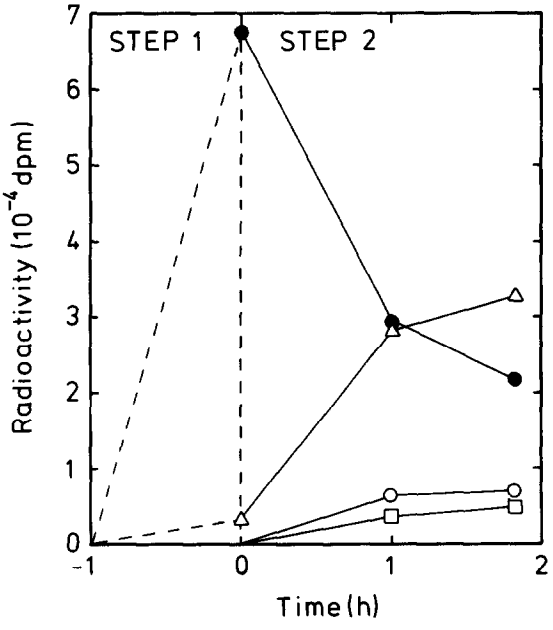

Fig. 1. Synthesis of 6-methoxy-2-hexaprenylphenol (○——○), 5-demethoxyQ-6 (△——△) and Q-6 (□——□) from 4-hexaprenyl-4-hydroxybenzoate (●——●).

Step 1. Mitochondria (21 mg protein) were incubated with 3.3×10^5 dpm (375 pmol) 4-hydroxy[U-^{14}C]benzoate, 1 μmol Li_3IPP, 10 μmol $MgCl_2$, 2 mg bovine serum albumin in 2.7 ml 0.6 M mannitol/0.05 M phosphate buffer, pH 7.4, for 1 h. At the end of this time the volume of the incubation mixture was adjusted to 15 ml by the addition of 0.2% (w/v) bovine serum albumin in ice-cold mannitol/phosphate buffer and the diluted suspension centifruged at 10 000 × *g* for 10 min. The supernatant (this contained all the unreacted 4-hydroxy[U-^{14}C]benzoate) was discarded and the mitochondrial pellet resuspended in 1.5 ml of bovine serum albumin/mannitol/phosphate buffer.

Step 2. One-third of the mitochondrial suspension was analysed for the presence of ^{14}C-labelled $CHCl_3$-soluble compounds. The remaining two-thirds was divided into two equal parts which were then incubated with the standard assay mixture – IPP for 60 min and 110 min, respectively, and then analysed.

the mitochondrial pool of polyprenyl-pyrophosphates) [7].

No evidence was obtained for the formation of:

(i) [^{14}C]Benzoates of the type recently implicated in the biosynthesis of Q-9 in rat liver mitochondria [3].

(ii) Hexaprenyl analogues of benzoyl CoA compounds of the type once considered as intermediates in the biosynthesis of Q-9 in rat tissues [7] e.g., 3-hexaprenyl-4-hydroxybenzoyl CoA.

(iii) Other prenylated compounds such as the 3,4-dihydroxy-5-hexaprenylbenzoate isolated from a Q-deficient yeast mutant [2].

Indirect evidence for the involvement of 3-hexaprenyl-4-hydroxybenzoate in the biosynthesis of 6-methoxy-2-hexaprenylphenol, 5-demethoxyQ-6 and Q-6 was provided by the findings that in those incubations in which as a result of the omission of cofactors other than IPP and Mg^{2+}, anaerobis or damage to mitochondria the incorporation of radioactivity into the last three compounds was reduced there was always a concomittant increase in the amount of radioactivity incorporated into 3-hexaprenyl-4-hydroxybenzoate (table 1). Direct support for a precursor–product relationship between 3-hexaprenyl-4-hydroxybenzoate and 6-methoxy-2-hexaprenylphenol, 5-demethoxyQ-6 and Q-6 was provided by the experiment described in the fig.1. This demonstration is the first to provide good evidence in support of the proposal that 3-polyprenyl-4-hydroxybenzoates can act as precursors of Qs in eukaryotic cells [1,2,8]. In a previous study it was claimed that a pulse-chase experiment with 4-hydroxy-[U-^{14}C]benzoate had established a precursor product relationship between 3-nonaprenyl-4-hydroxybenzoate and Q-9 in rat liver mince [8]. On critical examination of the published data, however, it is apparent that the rate of disappearance of ^{14}C-labelled 3-nonaprenyl-4-hydroxybenzoate was far greater than the rate of appearance of ^{14}C Q-9, i.e., a result that could equally well indicate that there is no precursor–product relationship between these two compounds. If it is accepted that 3,4-dihydroxy-5-hexaprenylbenzoate is an intermediate on the pathway to Q-6 [2], then the results of the present study coupled with the previous demonstration that 5-demethoxyQ-6 is a precursor of Q-6 in yeast [9] provide further support for the pathway that has been put forward for eukaryotic cells [2]: 4-hydroxybenzoate → 3-polyprenyl-4-hydroxybenzoate → 3,4-dihydroxy-5-polyprenylbenzoate → 6-methoxy-2-polyprenylphenol → 5-demethoxyQ-6 → Q-6.

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