UBIQUINONE SYNTHESIS IN VITRO STARTING FROM 2-OCTAPRENYL PHENOL

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1. Introduction

The membrane-bound synthesis of ubiquinone-8 (Q-8) in *Escherichia coli* is in principle deduced from the structures of various precursors accumulated by mutant strains, each blocked in a reaction of the pathway [1]. But only some few enzymes of the pathway are described preliminary [2] and no information is available how the 3 oxygen functions are introduced into the aromatic nucleus.

It was shown [3] that Q-8 synthesis in vivo is absolutely dependent on molecular oxygen. That molecular oxygen indeed is a substrate was confirmed by experiments with $^{18}O_2$ (H.E.K., in preparation). This can be evaluated as a possible involvement of a cytochrome *P*-450 enzyme, but a closer study requires the purified components of the pathway. However, until now there was no success on the production of a cell-free preparation from *E. coli* or other bacteria capable of complete ubiquinone synthesis.

It is reported here that the integrity of the Q-8 synthesis in vitro depends critically on the growth phase of the disrupted cells. Intact synthesis occurs with crude enzyme preparations from early logarithmic-phase cells. Furthermore, membrane-integral external monooxygenases are part of the enzyme system.

2. Materials and methods

2.1. *Reagents*

4-Hydroxy[U-^{14}C]benzoic acid was prepared as in [3] and *S*-adenosyl-L-[*methyl*-^{14}C]methionine according to [4]. L-[*Methyl*-^{14}C]methionine (56 mCi/mmol) and *S*-adenosyl-L-[*methyl*-^{3}H]-methionine (10 Ci/mmol) were from Amersham Buchler. The standard buffer was 50 mM Tricine (pH 7.5) containing 5 mM $MgSO_4$ and 10 mM β-mercaptoethanol.

Abbreviations Q-8, ubiquinone-8, 2-OPP, 2-octaprenyl phenol

2.2. *Growth of cells and crude enzyme preparation*

E coli K 12 strain 2847 (*aroB*$^-$) was cultured as in [3]. Growth was monitored at 578 nm (d = 0.5 cm) and allowed to reach 0.45 units. Centrifugation yields about 1 g wet wt/l. Washed cells were suspended in the standard buffer (1 ml/g wet wt) and charged with 2-OPP [3] using unlabelled 4-hydroxybenzoic acid (0.1 mM) in the experiments of fig.1., table 2, and table 3, and 4-hydroxy[U-^{14}C]benzoic acid (90 nmol, 1 μCi) in the experiment of table 1 (2-OPP was isolated as in [3] and measured at 272 nm in *n*-heptane using a molar extinction coefficient of 7950 $M^{-1}cm^{-1}$). Disruption of the cells was performed under air with a Branson Sonifier at maximal output (5 × 10 s, 0–8°C). DNase (50 μg/ml) was added and the suspension centrifuged at 40 000 × g for 20 min. The slightly turbid supernatant (52–56 mg/ml) containing membrane vesicles and all soluble enzymes was used directly or it was stored frozen at −70°C without loss of activity for weeks.

Protein was determined by the biuret method the reagent containing 5% Triton X-100.

2.3. *Enzyme assay*

Aliquots of the crude enzyme preparation (20–25 mg) were made 10 mM in KCN and D,L-dithiothreitol. *S*-Adenosyl-L-[*methyl*-^{14}C]methionine

was added (330 000 dpm; 75 nmol) and the mixture was shaken under air at 30°C for 1 h. The reaction was stopped, 10 nmol Q-8 carrier added, and the mixture was extracted by *n*-heptane [3]. Extracts were applied to silica gel G thin-layer sheets containing 2% F_{254} fluorescence indicator (Merck) and chromatographed in $CHCl_3$/*n*-heptane (60/40; v/v). Q-8 bands (R_F = 0.3) were scraped into scintillation vials and counted for radioactivity.

2.4. *High-pressure liquid chromatography*

Q-8 bands were scraped from thin-layer plates and eluted with ethanol. The extracts were analyzed on a Waters high-pressure liquid chromatograph at 274 nm by injection onto a μPorasil column (4 mm i.d. X 30 cm) which was run with 1.25% dioxane in *n*-heptane [5] at 1.5 ml/min (800 p.s.i). Fractions (0.5 ml) were collected and 50 μl counted for radioactivity. The retention time of Q-8 is 17.5 min.

3. Results and discussion

Crude enzyme preparations from ultrasonically disrupted *E. coli* K12 (*aroB*$^-$) incorporate the ^{14}C-labelled methyl group of *S*-adenosyl-L-methionine into compounds that can be extracted into the heptane phase. About 30% of the label was found in the phospholipid fraction (cyclopropane fatty acid formation [6]) whereas the main compound migrated like Q-8 upon thin-layer chromatography. Evidence that this compound in fact is Q-8 was given by high-pressure liquid chromatography where the radioactivity eluted together with the Q-8 carrier.

Interestingly, this Q-8 synthesis in vitro depends critically on the growth phase of the harvested cells (fig.1.). In preparations from young cells ~0.2 nmol label/mg protein were found in Q-8. Those from late-logarithmic or stationary phase cells were nearly inactive. Effects of the harvest time on full recovery of 2-OPP in *E. coli* [7] or polyprenyl phenol in *Rhodospirillum rubrum* [8] were also observed earlier. Because Q-8 synthesis in vivo is dependent on the motility of its precursors [3], these effects may be due to the lowered membraned fluidity caused by the dramatic increase in phospholipid methylation during the transition from exponential to stationary growth [6]. Possibly, ultrasonic treatment of this

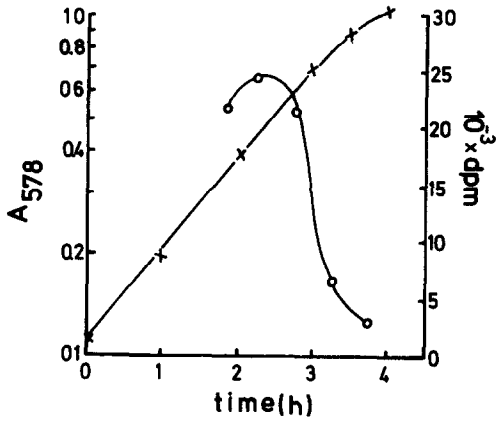

Fig.1. Q-8 synthesis in vitro depending on the growth phase of harvested cells. At various times of growth cells were centrifuged and aliquots (25 mg) of crude enzyme preparations were assayed for Q-8 synthesis as in section 2. (X) Turbidity; (o) radioactivity

cytoplasmic membrane which is less fluid and more rigid may lead to irreversible disorganization of structural and functional components of the pathway.

Cells harvested early in the logarithmic phase dispose of a pool of 15–20 nmol 2-OPP/g wet wt, but the cytoplasmic membrane can be charged with additional 5 nmol 2-OPP by incubation under argon with 4-hydroxybenzoic acid. A double label experiment was performed with a crude enzyme preparation from cells charged with 2-octaprenyl[U-^{14}C]-phenol. Addition of *S*-adenosyl-L-[*methyl*-^3H]-methionine should label de novo synthesized Q-8 by ^{14}C and ^3H in a ratio of 1 3, provided that the complete pathway from 2-OPP to Q-8 is intact.

It is shown in table 1 that in the presence of air >15% (2–3 nmol) of the ^{14}C-labelled 2-OPP pool was converted to Q-8 and ~9 nmol [^3H]methyl were incorporated. Thus, the integrity of the complete pathway in vitro is evident though the amount of processed 2-octaprenyl[U-^{14}C]phenol was relatively low It was found that under the described conditions a fraction of the membrane vesicles containing 2-octaprenyl[U-^{14}C]phenol is biosynthetically inactive (unpublished result). Also, there might be a negative effect of KCN which is required to block the respiratory chain and to inhibit the *S*-adenosyl-L-methionine decarboxylase [4].

Addition of ATP (1 mM) or NADH (1 mM) to

Table 1
Q-8 synthesis by crude enzyme preparations from cells charged with 2-octaprenyl[U-^{14}C]phenol

Compound	2-Octaprenyl phenol		Ubiquinone-8	
Gas phase	Argon	Air	Argon	Air
^{3}H incorp. (dpm)	0	0	0	140 000
^{14}C incorp (dpm)	12 500	9800	0	2300

A crude enzyme preparation (4 ml, 54 mg/ml) containing 2-octaprenyl[U-^{14}C]phenol (28 nmol, 26 000 dpm) was made anaerobic by repeated evacuation and flushing with argon at 0°C. KCN (40 μmol), DL-dithiothreitol (40 μmol) and *S*-adenosyl-L-[*methyl*-^{3}H]-methionine (1.02 μmol, 15.12 × 10^{6} dpm) were added and evacuation and flushing repeated Then 2 ml mixture were shaken under air and the rest was kept under argon (30°C; 1 h). 2-OPP and Q-8 were isolated upon thin-layer chromatography of heptane extracts (2-OPP was located by Gibbs reagent [9]) and counted for radioactivity contributed by ^{3}H and ^{14}C

crude enzyme extracts could not stimulate the incorporation of ^{14}C-labelled methyl into 2-OPP. This situation was changed by gel filtration of the 40 000 × *g* supernatant previous to the enzyme assay (table 2). Now, Q-8 synthesis was exclusively restored by NAD(P)H and dioxygen. Radioactive intermediates were not accumulated under anaerobic conditions. This striking requirement for reduced pyridine nucleotides and dioxygen argues in favor of the involvement of external monooxygenases catalyzing the 3 hydroxylations necessary to process 2-OPP.

Upon fractionation of the 40 000 × *g* supernatant into membrane vesicles and soluble proteins it is shown (table 3) that both fractions are required for Q-8 synthesis. To be sure that the membrane vesicles

Table 3
Reconstitution of Q-8 synthesis from fractions of the 40 000 × *g* supernatant

Enzyme fraction	Ubiquinone-8 synthesis (dpm)
40 000 × *g* supernatant (27.5 mg)	21 500
Membrane vesicles (2.1 mg)	60
Soluble proteins (2.5 mg)	150
Membrane vesicles (2.1 mg) + soluble proteins (2.5 mg)	4200

A 40 000 × *g* supernatant (20 ml) was separated into membrane vesicles and soluble proteins by column chromatography on Sepharose CL 6B (4 × 30 cm, standard buffer) Q-8 synthesis was measured with the fractions as indicated, the assay mixture (see section 2) containing additionally 1 mM NADH

Table 2
Reduced pyridine nucleotides and dioxygen as substrates of Q-8 synthesis

Crude enzyme preparation	Gas phase	Additions	% Formation of [*methyl*-^{14}C]Q-8
Untreated	Air		100
Gel filtrated	Air		2
		1 mM NADH	108
		1 mM NADHP	110
		1 mM $NADP^{+}$	4
	Argon	1 mM NADH	6

A crude enzyme preparation was gel filtrated on Sephadex G-25 and aliquots of the eluted protein assayed for Q-8 synthesis under air or argon with the additions as indicated

are not only the source of the membrane-bound 2-OPP they were treated with trypsin prior to the enzyme assay. A 1 min treatment with 2% (w/w) trypsin reduced Q-8 synthesis by ~50% (data not shown). This indicates that Q-8 synthesis starting from 2-OPP comprises soluble as well as membrane-integral enzymes. Because the transmethylase catalyzing the last step in Q-8 synthesis is a soluble enzyme [2] it can be speculated that the membrane-integral enzymes are external monooxygenases.

References

[1] Young, I G., Stroobant, P., MacDonald, C. G. and Gibson, F. (1973) J. Bacteriol. 114, 42–52.
[2] Leppik, R. A., Young, I. G. and Gibson, F (1976) Biochim. Biophys. Acta 436, 800–810.
[3] Knoell, H. E , Kraft, R and Knappe, J (1979) Eur J Biochem. in press
[4] Tabor, H and Tabor, C. W. (1971) Methods Enzymol. 17 (B), 393–397.
[5] Lichtenthaler, H. K. and Prenzel, U. (1977) J. Chromatog. 135, 493–498
[6] Cronan, J E. (1968) J. Bacteriol. 95, 2054–2061.
[7] El Hachimi, Z , Samuel, O. and Azerad, R. (1974) Biochimie 56, 1239–1247.
[8] Raman, T S , Rudney, H. and Buzzelli, N. K. (1969) Arch. Biochem. Biophys. 130, 164–174.
[9] Gibbs, H. D. (1927) J. Biol. Chem. 72, 649–664.