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ISOLATION OF A SOLUBLE ENZYME COMPLEX COMPRISING THE UBIQUINONE-8 SYNTHESIS APPARATUS FROM THE CYTOPLASMIC MEMBRANE OF ESCHERICHIA COLI

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SUMMARY: A soluble enzyme complex (~ 2×10^6 daltons) was isolated from the cytoplasmic membrane of Escherichia coli. It contains a high amount of 2-octaprenyl phenol, whereas ubiquinone-8, phospholipids and typical enzymes of the cytoplasmic membrane are largely excluded. Upon addition of a cytoplasmic enzyme and substrates, the pool of 2-octaprenyl phenol was quantitatively processed to ubiquinone-8. It is assumed that this complex represents the ubiquinone-8 synthesis apparatus supplied with the substrate 2-octaprenyl phenol.

INTRODUCTION

In E. coli, 2-octaprenyl phenol (2-OPP) is known as an intermediate on the pathway leading to ubiquinone-8 (Q-8)(1). The procession of this precursor requires the introduction of, alternatingly, 3 hydroxy and 3 methyl groups into the aromatic ring. It was shown that the conversion of 2-OPP to Q-8 is absolutely dependent on molecular oxygen (2) which is introduced into the aromatic ring at C-4, C-5, and C-6 (3).

A study of the enzymes involved in this process requires a cell free system with 2-OPP appropriately membrane-fused. Recently, there was success in the production of a cell free system capable of processing accumulated 2-OPP to Q-8 (4). However, only about 15% of the pool of 2-OPP was actually processed to Q-8 whereas the rest was found to be biosynthetically inactive. This fact stimulated experiments to determine the distribution of 2-OPP within the cytoplasmic membrane.

It is reported here that a soluble enzyme complex was isolated from the cytoplasmic membrane containing a high content of 2-OPP but lacking

Abbreviations used: 2-OPP, 2-octaprenyl phenol; Q-8, ubiquinone-8

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Q-8, phospholipids and typical membrane proteins. It is concluded that this complex represents the Q-8 synthesis apparatus with 2-OPP in intimate contact to the enzyme of its procession. A preliminary account of this work was presented (5).

MATERIALS AND METHODS

Reagents: 4-Hydroxy[U-^{14}C]benzoate was prepared by alkali fusion of L-[U-^{14}C]tyrosine (50 µCi; 10mCi/mmol; Amersham-Buchler) (6) and purification was achieved on silica gel G thin-layer sheets (2). Sepharose Cl 6B and 2B were from Pharmacia. The standard buffer was 50mM Tricine (pH 7.5) containing 5mM $MgSO_4$ and 10mM 2-mercaptoethanol.

Growth of cells and labelling of 2-octaprenyl phenol and ubiquinone-8: E.coli K-12 (ATCC 10798) was cultured on the mineral salts medium number 56 (7) containing 50mM glycerol and 50mM fumarate. The medium was supplemented with 0.1% yeast extract and 0.1% bacto tryptone (Difco). Cells were grown at 37°C anaerobically in a completely filled 24 l fermenter (Metrohm-Biolafitte). A 3 l anaerobic inoculum was used and growth was monitored at 578 nm (d=0.5). At A_{578} = 0.5 4-hydroxy[U-^{14}C] benzoate (5.2 µCi) was injected into the fermenter vessel and growth allowed to proceed to A_{578} = 0.8. Cells were harvested by centrifugation, washed in ice cold standard buffer (yield: 62 g wet weight), and finally suspended in this buffer (1 ml/g wet weight).

Determination of 2-octaprenyl phenol and ubiquinone-8: The total amount of 2-OPP or Q-8 was determined from heptane extracts of the cell sonicate. An aliquot of 10 ml was diluted with 4 volumes of 0.2 M acetic acid in methanol and extracted with 2 volumes of n-heptane. The heptane extract was concentrated to 1 ml and phospholipids were precipitated at 0°C by addition of 0.05 ml of 10% $MgCl_2$ in methanol and 20 ml of acetone. The acetone supernatant was applied to a silica gel G thin-layer sheet containing 2% F_{254} fluorescence indicator (Merck) and chromatographed in $CHCl_3$/n-heptane/ethanol (60/40/0.5; v/v/v). 2-OPP (R_F 0.6) was located by Gibbs reagent (8), and Q-8 (R_F 0.4) was detected under uv-light. Compounds were scraped from the plate, eluted with $CHCl_3$ and rechromatographed once. The concentration of the compounds was measured spectrophotometrically ($\varepsilon^{2\text{-OPP}}$=2 700 $M^{-1}cm^{-1}$ at 272 nm in n-heptane; $\varepsilon^{Q\text{-}8}$ = 12 500 $M^{-1}cm^{-1}$ at 275 nm in ethanol), and the radioactivity was counted as described (2).
Small samples of membraneous material (Fig.1 and 2 ; Table 3) were extracted as described above, but heptane extracts were applied directly to the thin-layer sheets together with unlabelled 2-OPP and Q-8 (~ 5 nmol). Zones containing 2-OPP or Q-8 were scraped into scintillation vials and counted for radioactivity. The amount of 2-OPP or Q-8 was calculated from the specific radioactivity of each compound.

Preparation of the cytoplasmic enzyme component: Fractions 75-110 (Fig.1) containing the soluble enzymes were pooled and 50 ml of settled DE-52 (Whatman; equilibrated with 20 mM Tricine pH 7.5) was added and the slurry poured into a column. The column was washed with about 2 column volumes of 20 mM Tricine pH 7.5 containing 0.1 M KCl, followed by a wash with 2 column volumes of the same buffer containing 0.4 M KCl. The eluent of this last wash was concentrated and desalted in UH 100/10 ultrabags (Schleicher & Schüll) (16 ml; 78.6 mg/ml). This preparation was referred to as DE-I enzyme.

Other methods: Total protein was determined by the biuret method, the reagent containing 5% Triton X-100. Protein eluted from the Sepharose columns (Fig.1 and 2) was measured directly at A_{546} after addition of 0.5 ml of H_2O and 0.5 ml of the reagent to a 0.1 ml aliquot of each fraction. Total phosphorus was assayed by the procedure of Bartlett (9), and D-LDH (D-lactate dehydrogenase, EC 1.1.1.28) and NADH oxidase (EC 1.6.3.1) were measured according to (10). Concentrations of cytochrome b_1 were estimated by dithionite-reduced minus oxidized difference spectra at roomtemperature using a millimolar difference coefficient of 17.5 at 560-575 nm.

RESULTS

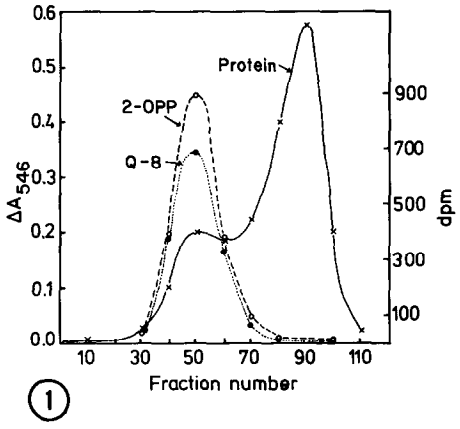
E.coli K-12 was grown anaerobically on a glycerol-fumarate medium and compounds of the Q-8 synthesis pathway were labelled by addition of 4-hydroxy[U-^{14}C]benzoate, the precursor of the quinone ring system (11), to the growing cell suspension (see Methods Section). Analysis of a lipid extract revealed that the label nearly exclusively was introduced into 2-OPP and Q-8. This is consistent with an earlier report showing that high levels of 2-OPP were accumulated under these growth conditions (12).

The cell suspension containing 30-40 nmol of 2-OPP and 80-100 nmol of Q-8 per g wet weight of cells was disrupted by sonic oscillation and centrifuged (4). About 50% of the total amount of 2-OPP was recovered in the 40 000xg supernatant (Table 1) containing all soluble enzymes and a

Table 1

Fractionation of cells containing 2-octaprenyl[U-^{14}C]phenol and [U-^{14}C]ubiquinone-8

Cell fractions	Protein	2-Octaprenyl phenol			Ubiquinone-8		
	mg	dpm	nmol	%	dpm	nmol	%
Sonicate	11 000	234 500	1 540	100	400 500	6 200	100
40 000xg supernatant	4 490	111 200	730	47	89 800	1 390	22
Fraction A	960	89 900	590	38	63 300	980	16
Fraction B_1	202	21 300	140	9	26 500	410	7
Fraction B_2	469	29 700	195	13	5 800	90	1.5
Fraction B_{25}	31	22 800	150	10	520	8	0.13



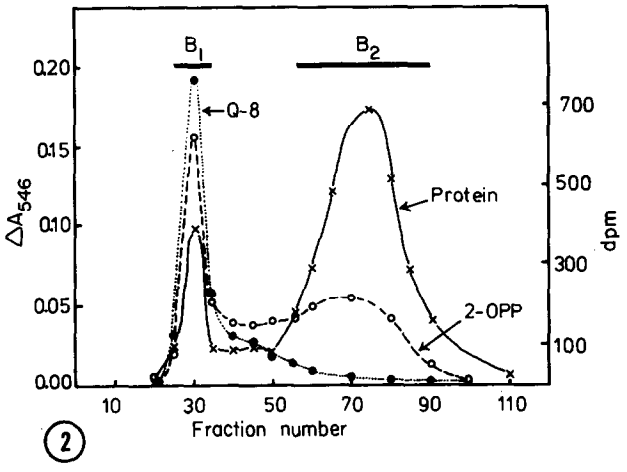


Fig.1. Gel chromatography of the 40 000xg supernatant.

The 40 000xg supernatant (78 ml; 57.6 mg/ml) was chromatographed on a Sepharose Cl 6B column (5x35 cm; standard buffer; 24 ml/h; 6 ml/fraction). Protein was measured by the biuret method and 1 ml aliquots/fraction were extracted by n-heptane and analyzed for 2-OPP and Q-8 (see Methods Section).

Fig.2. Gel chromatography of fraction A.

Fraction A (30 ml; 32 mg/ml) was chromatographed on a Sepharose 2B column (3.5x55 cm; standard buffer; 21 ml/h). Fractions of 6 ml were collected and measured for protein and for the distribution of 2-OPP and Q-8 (see Fig.1).

minor part of membraneous material. The 40 000xg supernatant was chromatographed on a Sepharose Cl 6B column (Fig.1). The soluble enzymes (fractions 75-110) were partially purified on DEAE-cellulose (see Methods Section), and fractions containing Q-8 and 2-OPP were pooled and concentrated by precipitation with 15% (w/v) polyethylene glycol 6000 (SERVA). The precipitate, fraction A, was suspended in standard buffer and subsequently chromatographed on a Sepharose 2B column (Fig.2). Fractions B_1 and B_2 were pooled separately and concentrated by precipitation with polyethylene glycol (B_1: 22.4 mg/ml; B_2: 21.8 mg/ml). Electron microscopy revealed that only fraction B_1 is composed of membrane vesicles.

Fraction B_2 containing a low amount of Q-8 and most of the 2-OPP (Table 1) was finally purified by isopycnic centrifugation. Aliquots of 2ml were layered on 11.5 ml of 25% (w/w) sucrose in standard buffer and centrifuged in a Beckman SW 40 rotor for 16 h at 30 000 rpm . The slight yellow

Table 2

Constituents of fractions B_{25} and B_1

Membrane fraction	Phospholipid	D-LDH	NADH oxidase	Cytochrome b_1
	nmol/mg	μmol/mg.min	μmol/mg.min	μg/mg
Fraction B_{25}	8	0.008	0.012	4
Fraction B_1	170	0.12	0.62	70

band which do not penetrate the sucrose barrier was collected and concentrated with polyethylene glycol (fraction B_{25}; 4.2 mg/ml).

A biochemical analysis of fraction B_{25} and B_1 is presented in Table 1 and 2. Fraction B_{25} contains a high level of 2-OPP (5 nmol/mg) but lacks Q-8 and other typical constituents of the cytoplasmic membrane. By gel chromatography on Sepharose 2B the molecular weight of fraction B_{25} was estimated to $\sim 2 \times 10^6$ daltons. SDS-gel electrophoresis revealed 12 bands ranging from 40 000 to 80 000 daltons (experiment not shown).

Fraction B_{25} was measured for its capability to process the pool of 2-OPP to Q-8 (Table 3). In the presence of the cytoplasmic enzyme component (DE-I enzyme), reduced pyridine nucleotides, S-adenosyl-L-methionine

Table 3

Procession of 2-octaprenyl phenol to ubiquinone-8 in fraction B_{25}

Assay conditions	2-octaprenyl phenol	ubiquinone-8
	nmol/mg	nmol/mg
No additions	5.0	0.25
Complete system	0.0	5.4
Complete system minus dioxygen	4.8	0.4

Aliquots of fraction B_{25} (0.4mg) were mixed with 5.7mg of the DE-I enzyme, 4 μmol of $MgSO_4$, 0.4 μmol of NADH and NADPH. The mixture was filled up to 0.4ml with standard buffer, and the reaction was started with 0.15 μmol of S-adenosyl-L-methionine. The reaction was allowed to proceed at 30°C for 2 h under air or argon (4). The reaction was stopped in acetic acid/methanol and heptane extracts were analyzed for 2-OPP and Q-8 (see Methods Section). 2-OPP or Q-8 were expressed as nmol/mg of fraction B_{25}.

and dioxygen, the pool of 2-OPP was quantitatively and stoichiometrically processed to Q-8.(The membrane vesicles of fraction B_1 were measured also and found to be inactive).

Triton X-100 which was added at a concentration of 50 μM to this system depressed Q-8 synthesis for 30%. At the critical micellar concentration of the detergent (250 μM) the procession of 2-OPP to Q-8 was stopped completely.

DISCUSSION

The availability of an appropriately membrane-fused precursor, a prerequisite for the study of ubiquinone-8 synthesis (2), is conveniently met with E.coli K-12 grown anaerobically on a glycerol-fumarate medium. Under these conditions, 2-OPP the precursor of the aerobic pathway leading to Q-8 is accumulated in the cytoplasmic membrane (0.2-0.4 nmol/mg). Because Q-8 is present in relative high amounts,the existence of an alternative, anaerobic pathway starting also from 2-OPP was postulated (12). It was found here that the procession of 2-OPP in fraction B_{25} is absolutely dependent on molecular oxygen.

A soluble enzyme complex (fraction B_{25}) was isolated from the cytoplasmic membrane which is virtually free of Q-8 but contains a high level of 2-OPP. Typical enzymes of the cytoplasmic membrane and also phospholipids are largely excluded. The very low amount of residual phospholipid (mainly phosphatidylethanolamine) is quite remarkable because the enzyme complex was not solubilized by detergents. This indicates that the complex was broken from the membrane as a distinct and native domain.

The most important was that Q-8 synthesis measured with this enzyme complex was high resulting in the complete procession of the pool of 2-OPP to Q-8. It is assumed that the complex represents the aerobic Q-8 synthesis apparatus with 2-OPP trapped to the enzyme of its procession. The high sensibility against Triton X-100 demonstrated that the complex is stabilized by hydrophobic interactions.

When the complex was isolated from cells which were shaken 4 h under air prior to fractionation, the content of 2-OPP was lowered from 5 to 1.7 nmol/mg but the content of Q-8 did not increase. From this it is clear that in the native membrane de novo synthesized Q-8 was released from the enzyme complex into the membrane phase.

Together with the cytoplasmic enzyme component, presumably a methyltransferase of about 20 000 daltons (unpublished result), the complete aerobic Q-8 synthesis apparatus supplied with its substrate 2-OPP is now available in a soluble form.

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