Functional size measurements of the ubiquinol oxidase activity of the cytochrome *o* terminal oxidase complex of *Escherichia coli*

Huw D. WILLIAMS,*† Julia A. M. HUBBARD,†§ Jonathan H. A. NUGENT† and Robert K. POOLE*

*Microbial Physiology Research Group, Biosphere Sciences Division, King's College London, Campden Hill Road, London W8 7AH, and †Department of Biology, University College London, Gower Street, London, WC1E 6BT, U.K.

The functional molecular mass for ubiquinol-1 oxidation by the *Escherichia coli* terminal oxidase, cytochrome *o*, was determined by radiation-inactivation analysis of membranes from a cytochrome *d*-deficient mutant. The functional molecular mass for ubiquinol-1 oxidase activity was found to be 38.3 ± 2.65 kDa.

INTRODUCTION

The aerobic respiratory chain of *Escherichia coli* contains two terminal oxidases. When grown under conditions of high aeration, the terminal oxidase cytochrome *o* is synthesized, while under oxygen-limited conditions the synthesis of a second terminal oxidase is induced, namely cytochrome *d* (Ingledeu & Poole, 1984).

Each of these enzymes catalyses the two-electron oxidation of ubiquinol-8 and the four-electron reduction of oxygen to water. The cytochrome *o* or *bo* complex contains copper and 2 mol of *b*-type haem, one cytochrome *b* and copper forming a binuclear centre analogous to that in *aa*₃-type cytochrome *c* oxidases (Salerno *et al.*, 1990). The purified cytochrome *bo* complex has been reported to contain 2–4 polypeptide subunits (Matsushita *et al.*, 1983, 1986; Kita *et al.*, 1984). A two-subunit enzyme was first reported with apparent molecular mass of 55 kDa and 33 kDa (Kita *et al.*, 1984). Matsushita *et al.* (1983, 1986), however, found four subunits (66, 35, 22 and 17 kDa), and this has been confirmed in more recent preparations (Georgiou *et al.*, 1988).

The structural genes for cytochrome *o* have been cloned, and DNA sequence analysis of the *cyo* operon has shown it to contain five open reading frames, (*cyoA*, *B*, *C*, *D* and *E*) (Chepuri *et al.*, 1990); interestingly the sequences of three of these, *cyoA*, *cyoB* and *cyoC*, show significant sequence similarity with subunits II, I and III respectively of the eukaryotic and prokaryotic *aa*₃-type cytochrome *c* oxidases. The deduced molecular masses of the gene products are 35, 75, 23, 12 and 32 kDa for *cyoA*, *B*, *C*, *D* and *E* respectively (Chepuri *et al.*, 1990). As yet, *cyoD* and *cyoE* have not been demonstrated to be related to any components of the purified enzyme. It is not clear how many subunits are required for cytochrome *bo* to function as a ubiquinol oxidase.

Radiation-inactivation analysis is a technique that has proved particularly useful for molecular mass determinations of membrane-bound proteins as it allows measurement of the functional size of an enzyme (Harmon *et al.*, 1985; Kempner & Schlegel, 1979). Samples are irradiated with high-energy ionizing radiation and then assayed for enzyme activity. When a biological material is irradiated, exposure to ionizing radiation leads to a loss of function due to destruction of critical structures: the larger the structure, and the more likely it is to be hit. Because the energy deposition is so large (~66 eV per primary ionization), function is completely destroyed by a single hit and there are no partially destroyed targets. Single-hit target theory predicts an

exponential decay of activity with increasing dose of radiation and the rate of exponential decay is directly proportional to the mass of the enzyme (Kempner & Schlegel, 1979). The slope of this plot can be related to the molecular mass by calibration using enzymes of known molecular mass (Nugent, 1986). The inactivation observed is independent of other components in the sample and so the protein does not need to be pure and measurements can be made *in situ*.

In the present study, we have used radiation-inactivation analysis to determine a functional molecular mass for ubiquinol oxidation by the cytochrome *bo* terminal oxidase in a cytochrome *d*-deficient mutant of *E. coli*.

MATERIALS AND METHODS

Strain, growth conditions and membrane preparation

E. coli AN2343 is a cytochrome *d*-deficient strain which has a mutation in the *cydD* locus (Poole *et al.*, 1989), with the result that it is unable to synthesize a functional cytochrome *d*. *E. coli* AN2343 was grown aerobically in succinate minimal medium (Poole *et al.*, 1989) supplemented with casamino acids, containing (per litre): Na₂HPO₄·12H₂O, 10.96 g; KH₂PO₄, 2.7 g; (NH₄)₂SO₄, 1 g; sodium succinate, 5 g; trace-elements solution, 10 ml. The trace elements solution contained (per litre): EDTA disodium salt, 5 g; FeCl₃·6H₂O, 0.5 g; ZnO, 0.05 g; CuCl₂·2H₂O, 0.01 g; CoCl₂·6H₂O, 0.01 g; H₃BO₃, 0.01 g. After autoclaving, the medium was supplemented with 1 ml of 1 M-MgCl₂ and with 5 ml of 10% casamino acids (Difco East Molesey, Sussex, U.K.). Starter cultures (300 ml), inoculated from rich agar plates, were grown in shake flasks overnight to provide an inoculum for 10 litres of medium in a 12 litre Biostat V fermenter (B. Brown Medical Ltd., Aylesbury, Bucks., U.K.), sparged with air at 10 litres/min and stirred vigorously. The culture was harvested after about 400 min, when the *A*₆₀₀ was 0.7–0.8. Cells were harvested using an Alpha-Laval Camberley, Surrey, U.K. continuous-action centrifuge. The cells were resuspended in 50 mM of Tris-HCl (pH 7.4)/2 mM-MgCl₂/1 mM-EGTA and membranes prepared after ultrasonic disruption of cells as described previously (Poole & Haddock, 1974).

Enzyme assays

Malate dehydrogenase (porcine heart cytoplasmic, EC 1.1.1.37) was assayed as the rate of oxidation of NADH monitored at 340 nm using freshly made oxaloacetate solution as substrate.

§ Present address: Celltech Limited, Bath Road, Slough, Berks., U.K.

† Present address and address for correspondence: Department of Biology, Imperial College of Science, Technology and Medicine, Prince Consort Road, London SW7 2BB, U.K.

Fig. 1. Irradiation of ubiquinol-1 oxidase activity in membranes prepared from *E. coli* AN2343

Frozen samples were irradiated at -196°C and ubiquinol-1 oxidase and malate dehydrogenase assays were performed on irradiated samples at room temperature as described in the Materials and methods section. (a) Ubiquinol-1 oxidase activity; (b) malate dehydrogenase activity.

Oxidation of ubiquinol-1 was measured at room temperature by following the absorbance at 275 nm in a spectrophotometer as described by Matsushita *et al.* (1986). Ubiquinone-1 was reduced by adding small amounts of solid sodium borohydride. Ubiquinone was provided by Hoffman-LaRoche (Basle, Switzerland).

Spectrophotometry

Reduced (sodium dithionite) minus oxidized (ammonium persulphate) difference spectra were recorded at room temperature in a Johnson Foundation DBS-3 dual-wavelength scanning spectrophotometer (Williams & Poole, 1987). CO-difference spectra were obtained after bubbling of dithionite-reduced samples with CO in a cuvette for 5 min.

Sample preparation for radiation-inactivation

Membrane samples (~ 0.5 g) were resuspended in about 5 ml of 50 mM-Tris/HCl (pH 7.4)/2 mM-MgCl₂/1 mM-EGTA/BSA (1 mg/ml)/15% glycerol containing 1–2 μg of the standard, malate dehydrogenase (70 kDa)/ml. The sample was flushed with N₂ gas for 60 min and then 0.3 ml portions were placed in 0.3 cm-diameter silica tubes. Samples were then frozen and stored in liquid N₂ or solid-CO₂ pellets.

Irradiation procedure

Irradiation of frozen samples was carried out at -196°C in liquid N₂ using a 16 MeV electron beam (MEL SL75/20 linear accelerator; Addenbrookes Hospital, Cambridge, U.K.) at a dose rate of 2 Mrad/min as described previously (Nugent & Atkinson, 1984). After irradiation, samples were allowed to

warm to -25°C to allow recombination of any free radicals produced by irradiation. The samples were then thawed at 4°C , aliquots taken and the samples were stored at -196°C until assayed.

RESULTS

Spectrophotometric analysis of membranes of the cytochrome *d*-deficient mutant AN2343 confirmed the absence of spectral signals due to the cytochrome *d* oxidase (Poole *et al.*, 1989).

The absence of cytochrome *d* means that the ubiquinol-1 oxidase activity of membranes of AN2343 can be assumed to be due solely to cytochrome *o*. The ubiquinol-1 oxidase activity of AN2343 membranes was inactivated exponentially with doses of 16 MeV electron beam radiation as predicted by single-hit target theory (Fig. 1a). By using malate dehydrogenase as an internal standard for both the radiation dose and temperature effects (Fig. 1b), this dose-response curve could be used to determine the target size of the ubiquinol-1 oxidase activity of cytochrome *o*. Since the target size for malate dehydrogenase is 70 kDa (Nugent & Bendall, 1987), the target size for cytochrome *o*-dependent ubiquinol oxidase activity was calculated to be 38.8 kDa. Three independent irradiations of the cytochrome *o* ubiquinol oxidase activity in frozen membranes gave molecular masses in the range 35.5–40.7 kDa, with an average value of 38.3 ± 2.65 kDa (mean $\pm$ s.d.).

DISCUSSION

Radiation-inactivation analysis permits the determination of the molecular mass of a membrane-associated enzyme activity *in situ*. We have used this technique to determine the target size for ubiquinol oxidase activity in membranes from a cytochrome *d*-deficient mutant of *E. coli* in which ubiquinol-1 oxidation resulted solely from the activity of cytochrome *o*.

A functional molecular mass determined by radiation-inactivation analysis may correspond to the molecular masses of two or more component subunits, or in some instances to an isolated domain of a larger polypeptide (Solomonson *et al.*, 1986, 1987). For activity associated with diffusible components, such as mobile electron-transfer carriers (Sone & Kosako, 1986), the functional molecular mass would be expected to correspond to the molecular mass of the largest component. The target size for radiation-inactivation of the ubiquinol-1 oxidase activity of cytochrome *o* was found in the present study to be 38.3 ± 2.65 kDa. This is considerably less than the molecular mass of a heterodimer composed of two subunits of molecular mass 55 and 33 kDa (= 88 kDa; Kita *et al.*, 1984) or of a heterotetramer composed of four subunits of molecular mass 55, 33, 18 and 11 kDa (= 117 kDa; Georgiou *et al.*, 1988).

Kempner & Schlegel (1979) in comparing the target molecular masses from radiation-inactivation analysis of a large number of proteins with the 'accepted' value for the molecule or subunit found the average 'error' for all the data to be 14%. Therefore, taking into account both the error associated with radiation-inactivation analysis and the often-observed anomalous behaviour of membrane proteins on SDS/PAGE (Gennis, 1989), the value of 38.3 kDa obtained in the present study could correspond to the 33–35 kDa polypeptide purified as subunit II of cytochrome *o* (Matsushita *et al.*, 1983; Kita *et al.*, 1984; Georgiou *et al.*, 1988). An alternative possibility is that the functional molecular mass represents a heterodimer of subunits III (18 kDa) and IV (11 kDa) or a separate domain of subunit I (55–66 kDa). However, this result is somewhat surprising and not straightforward to explain, considering our current knowledge of this enzyme. The oxidation of ubiquinol by cytochrome

o is intrinsically linked to electron flow to oxygen and oxygen reduction to water as described by the overall reaction (Matsushita *et al.*, 1986):

Therefore, oxidation of ubiquinol is functionally linked to oxygen reduction. The site of oxygen binding and reduction is a CO-binding cytochrome *b* (*o*) (Castor & Chance, 1959; Poole *et al.*, 1979; B. Bolgiano, I. Salmon, W. J. Ingledew & R. K. Poole, unpublished work). The current evidence indicates that cytochrome *b*₅₅₈ (*o*) is associated with subunit I (75 kDa), the product of *cyoB*, as probably are the second cytochrome *b* and the copper (Nakamura *et al.*, 1990). Therefore we would have expected a minimum target size, by radiation-inactivation analysis, of 75 kDa (the predicted size from the DNA sequence of *cyoB*), whereas, the current data, on first inspection, suggest that a 38 kDa polypeptide is the minimum molecular mass required for cytochrome *o* catalysis.

One possible explanation is that a 38 kDa polypeptide, possibly corresponding to subunit II, which does not show significant sequence identity with any of the subunits of *aa₃*-type cytochrome *c* oxidases (Chepuri *et al.*, 1990), is involved in binding and oxidation of ubiquinol and that this is rate-limiting for cytochrome *o* catalysis. However, this would only be a valid argument if the 38 kDa polypeptide could communicate with more than one site of oxygen reduction, and there is no evidence for this at present.

The data presented here indicate that a 38 kDa polypeptide, which may be equivalent to subunit II of the cytochrome *o* complex, is essential for cytochrome *o* catalysis and suggests that it may be important in ubiquinol binding. In this context it is noteworthy that subunit II of this ubiquinol oxidase has a molecular mass of 35 kDa and, unlike other subunits in the complex, does not show significant sequence similarities with components of the *aa₃*-type cytochrome *c* oxidases.

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