THE PURIFICATION OF A RESPIRATORY OXIDASE COMPLEX FROM *ESCHERICHIA COLI*

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1. Introduction

Escherichia coli has the ability to regulate the composition of its respiratory chain according to environmental factors [1,2]. When grown aerobically beyond exponential phase, cells produce two respiratory oxidases, cytochrome *o* and cytochrome *d* [3]. When grown anaerobically with a non-fermentable carbon source (e.g., glycerol) plus fumarate, cells contain fumarate reductase as the major terminal respiratory enzyme. However these cells also contain very high concentrations of the oxidase cytochrome *d*, while no cytochrome *o* is spectroscopically detectable. Cytochromes b_{555}, b_{558} and a_1, are found in addition to cytochrome *d* [2].

Cytochrome *d* is a 1 electron acceptor, but the reduction of O_2 to H_2O requires the transfer of 4 electrons. Known enzymes which also catalyse this oxidase reaction have been shown to contain 4 redox centres: mitochondrial cytochrome *c* oxidase has two haem *a* groups plus 2 redox-active Cu atoms [4] while *Pseudomonas* cytochrome oxidase (nitrite reductase) contains 2 haem *c* plus 2 haem d_1 [5] moieties.

We have solubilized and purified cytochrome *d* to investigate the nature and number of redox centres present and find that cytochromes b_{555} and b_{558} copurify with cytochrome *d*. Evidence from gel electrophoresis suggests that the oxidase is a protein complex containing both cytochromes *b* and *d*.

2. Materials and methods

2.1. *Growth of cells*

Escherichia coli strain EMG-2 (prototroph) was grown in 20 l batches on the mineral salts medium (CR medium) [6] supplemented with vitamin-free casamino acids (0.1%, w/v), glycerol (0.5%, w/v) and fumarate (50 mM). The autoclaved medium was briefly bubbled with nitrogen gas and the culture vessel sealed with a rubber stopper to maintain anaerobiosis. An inoculum of 450 ml was used for each 20 l batch.

2.2. *Harvesting and cell breakage*

Cells were harvested in late exponential phase in an MSE continuous action rotor (43118-503) at 16 000 rev./min with ~300 ml/min flow rate. The pellet was resuspended in CR medium and centrifuged at 10 000 × *g* for 15 min at 4°C. This washing procedure was repeated once. Cells were broken in the French pressure cell and electron transport particles were prepared as in [2].

2.3. *Optical spectroscopy*

Difference spectra were measured with the split-beam spectrophotometer [7] either at room temperature or at 77 K. The molar extinction coefficient of cytochrome *b* in solubilized preparations was determined by comparing reduced minus oxidised difference spectra (room temperature) with alkaline pyridine haemochromogen spectra. Thus $\xi_{560-576}$ was calculated to be 12 mM^{-1}.

2.4. *Polyacrylamide gels*

Electrophoresis under non-dissociating conditions was performed, at pH 8.9, in 7.3% (w/v) polyacrylamide gels as in [8]. Gels were stained for iron as in [9] and for haem as follows: ethanolic *o*-tolidine was acidified with glacial acetic acid to 10% (v/v). Gels were immersed in this reagent 2 min after which the excess tolidine reagent was removed. H_2O_2 3% (v/v) was added to develop the transient blue stain.

2.5. *Assays*

Protein was assayed as in [10] except that the reaction mixture contained sodium dodecylsulphate 0.5% (w/v). Bovine serum albumin (Sigma fraction V) was used as standard.

3. Results

3.1. *Solubilization and assay*

Exposure of membranes to 2% Triton X-100, 50 mM EDTA, 20 mM potassium phosphate (pH 7.2) rendered 60–70% of each of the cytochromes *b*, *a* and *d* soluble. No spectral changes were observed on solubilization (fig.1). In particular it may be noted that no CO-binding *b*-cytochrome (cytochrome *o*) is observable. However in some solubilized samples which had aged and lost oxidase activity, cytochrome *b* was found to bind CO.

The nature of the physiological electron donor to cytochrome oxidase of *E. coli* is unknown. Membranes were found to oxidize ascorbate and other artificial electron donors and the specific ascorbate oxidase activity was not significantly affected by solubilization (~70 ng atom O . min^{-1} . $nmol^{-1}$ cytochrome *b*). However since an assay of activity required the use of significant quantities of material, the cytochrome was routinely assayed spectrophotometrically. Cytochrome *b* was ~0.5 nmol/mg protein in all preparations.

3.2. *Purification*

A column of DEAE-cellulose (DE52, 3.5 × 10.5 cm) was equilibrated with 0.5% Triton X-100, 50 mM EDTA, 20 mM potassium phosphate (pH 7.2) and the solubilized material from ~100 g cell paste was applied directly to the column. Whereas much of the solubilized protein washed straight through, cytochromes *b* and *d* bound firmly and were eluted with a KCl salt gradient (fig.2). The cytochrome elution was observed as a single peak at A_{410}. Spectra indicated the presence of both cytochrome *b* and *d*, and no change in their relative concentrations was detected across the peak fractions.

The peak fractions were pooled, concentrated and chromatographed on a gel filtration column, (Biogel A1.5m, 1.2 × 85 cm). Again the elution patterns of cytochromes *b* and *d* coincided exactly.

3.3. *Gel electrophoresis*

Polyacrylamide gels 7.3% (w/v) run at pH 8.9 were stained for protein, for haem and for iron. A single major band stained for haem, R_F 0.20. This corresponds to the major bands staining for protein and iron (fig.3). The material running close to the top of the gels is probably aggregated oxidase; increased amounts are found on aging of the preparation. The sample indicated in fig.3 may be estimated to be 70% pure. The specific haem content was determined spectrophotometrically to be 4.0 nmol haem *b*/mg pro-

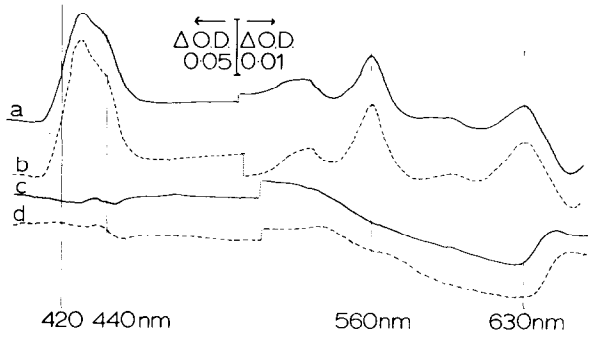

Fig.1. Reduced minus oxidized difference spectra of membranes and of solubilized membranes at room temperature. For spectra (a,b) the sample in the test curette was reduced with a few grains of dithionite, the reference was oxidized with H_2O_2. For spectra (c,d) both samples were reduced and CO was bubbled through the test sample for 1 min before scanning. (a,c) are spectra of membranes; (b,d) of solubilized membranes.

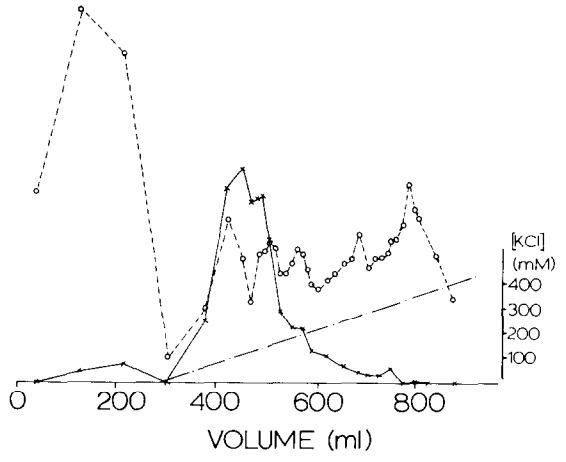

Fig.2. DEAE-cellulose column elution profile. Fractions were assayed for cytochrome (×– –×) by measuring A_{410}, and for protein (○– –○) as in section 2. A linear salt gradient (– . –) was used to elute.

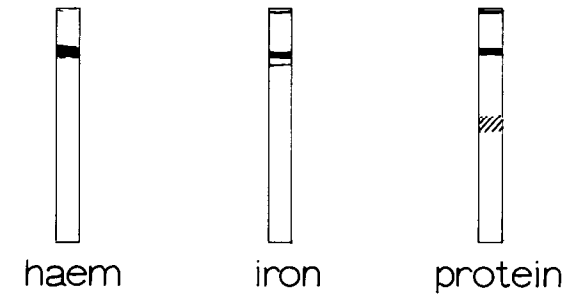

Fig.3. Polyacrylamide gel electrophoresis of purified oxidase. Non-dissociating gels (pH 8.9) were loaded with oxidase and run in parallel. Gels were stained for haem, iron and protein. The origin is at the top and the bromophenol blue front at the bottom.

tein. Two species of cytochrome *b* are resolved spectrally at 77 K (fig.4) in membranes and in purified oxidase. The minimum molecular weight is thus ~350 000. By comparison with the behaviour of *E. coli* nitrate reductase on the gel filtration column, the molecular weight was estimated to be ~400 000.

3.4. *Quantitation of the cytochromes*

For cytochrome *b* in the membranes and solubilized preparations described above the $\xi_{560-576}$ in reduced minus oxidized difference spectra is 12 mM^{-1}. Unfortunately the extinction coefficient for cytochrome *d* cannot be determined as no standard for the quantitation of haem *d* is known. Thus the stoichiometry of cytochromes *b* and *d* in the oxidase cannot readily be determined. The ratio of the α-band signals $\Delta A_{560-575} : \Delta A_{630-610}$ is 2.25 in reduced minus oxidized spectra. Thus if the stoichiometry of haem *b* : haem *d* is 1:1, then $\xi_{630-610}$ = 5.3 mM^{-1} (12 mM^{-1}/2.25) and if it is 2:1.5 then $\xi_{630-610}$ = 10.7 mM^{-1} (2 × 12 mM^{-1}/2.25).

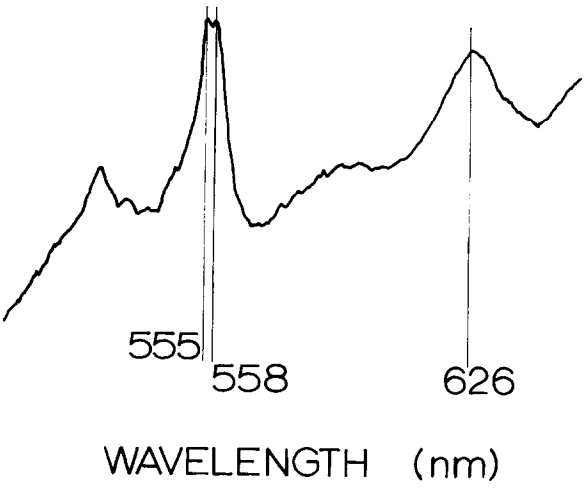

Fig.4. Low temperature (77 K) reduced minus oxidized difference spectrum of purified *E. coli* oxidase. The test sample was reduced with a few grains of dithionite, the reference was oxidized with H_2O_2.

4. Discussion

The above results indicate that the haem *d*-containing cytochrome oxidase from *E. coli* is a large protein containing cytochromes *b* and *d*. Two different cytochromes *b* and a single cytochrome *d* are resolved spectrally and potentiometrically in membranes used for this study [2]. Cytochrome b_{558} and b_{555} have mid-point potentials of +250 mV and +140 mV, respectively at pH 7. Cytochrome *d* has a mid-point at +280 mV. The stoichiometry of these cytochromes in the oxidase complex cannot readily be determined but assuming equimolar amounts of the two *b* cytochromes (as seems likely from spectra) and equimolar *b* and *d* cytochromes, a complex containing 4 heme moieties may be envisaged. Such a stoichiometry would imply $\xi_{630-610}$ = 5.3 mM^{-1}, which is plausible. $\xi_{630-615}$ of *Pseudomonas* cytochrome *cd* was estimated to be 8.5 mM^{-1} [11]. Thus *E. coli* cytochrome oxidase is suggested to contain 4 heme redox centres, but the presence of >4 centres cannot be excluded.

Acknowledgements

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