# A TRANSMEMBRANE LOCATION FOR THE PROTON-TRANSLOCATING REDUCED UBIQUINONE→ NITRATE REDUCTASE SEGMENT OF THE RESPIRATORY CHAIN OF *ESCHERICHIA COLI*

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## 1. Introduction

Membrane-bound nitrate reductase [EC 1.7.99.4] of *Escherichia coli* is the terminal enzyme in an energy-conserving respiratory pathway. Two components of this pathway which are induced by nitrate are, a *b*-type cytochrome (cyt $b_{556}^{NO_3^-}$) and nitrate reductase itself [1]. The transfer of one pair of reducing equivalents from $UQH_2^*$, through cyt $b_{556}^{NO_3^-}$ to nitrate reductase and $NO_3^-$ is accompanied by the outward translocation of two protons across the cytoplasmic membrane [23]. Mitchell's proposals for the mechanism of respiratory-driven proton translocation involve a trans-membrane orientation of the oxido-reduction components involved [4]. In this paper we present the results of and experimental test of his proposal in respect of components of the $UQH_2$-$NO_3^-$ reductase segment (here termed the ntirate respiration complex) of the anaerobic respiratory chain of *E. coli*. We have measured the accessibility to lactoperoxidase-catalysed radio-iodination [5] of the immunologically defined nitrate respiration complex [6] in situ in the cytoplasmic membrane. Two types of preparation have been labelled: spheroplasts, in which only the periplasmic surface of the cytoplasmic membrane is exposed to lactoperoxidase, and ultrasonically prepared membrane vesicles which are predominantly inverted [7]. Our experimental design ensures that data from spheroplast and vesicle labellings are directly comparable. We report that the nitrate respiration complex has a transmembrane orientation.

* *Abbreviations*: $UQH_2$ -reduced ubiquinone; EDTA - ethylene diamine tetraacetate; EGTA - ethyleneglycol bis-(aminoethyl)-tetraacetate; ONPG - *o*-nitrophenyl-β-[D]-galactoside; SDS-sodium dodecyl sulphate; TCA - trichloroacetic acid.

## 2. Materials and methods

### 2.1. *Organism*

*Escherichia coli* strain A1002 (K12 Ymel, ato⁻, fadR$^c$, ilv⁻, lacI⁻, metE⁻, rha⁻), a gift from Dr H. U. Schairer, Max Planck Institute fur Biologie, 73-Tubingen, West Germany, was used throughout. Cells were grown anaerobically in a medium containing 10 g tryptone, 5 g yeast extract (both from Oxoid Ltd.) 7.5 g KCl, 12 g $K_2HPO_4$, 3 g $KH_2PO_4$, 5 g glucose and 10 g $NaNO_3$ per litre. Harvesting, at mid-exponential phase was by centrifugation at room temperature.

### 2.2. *Preparations*

Spheroplasts were prepared at room temperature by the lysozyme/EDTA method of Birdsell and Cota-Robles [8] and re-suspended in 0.5 M sucrose, 50 mM Tris–HCl, 2 mM $MgCl_2$, 1 mM EGTA pH 7.5 (buffer A). They were used immediately following preparation. Vesicles were prepared from spheroplasts by ultrasonic disruption in 50 mM Tris–HCl, 1 mM $MgCl_2$ pH 7.5 (buffer B) followed by differential centrifugation, all at 4°C. Vesicles were twice washed by re-suspension in buffer B and resedimentation. Finally they were resuspended in buffer A and stored at −20°C until used.



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2.3. *Surface labelling*

Spheroplasts (0.2–0.8 mg protein) and vesicles (2–8 mg protein) were suspended together, at room temperature, to a final vol of 0.44 ml in buffer A containing 20 μg lactoperoxidase and 3 nmol $K^{125}I$ (spec. act. 140 mCi/μmol: The Radiochemical Centre, Amersham, U.K.). Five additions, each of 0.2 μmol $H_2O_2$ were made at intervals of two minutes. Iodinated spheroplasts and vesicles were separated, after measuring the intergrity of the spheroplasts in the incubation mix (see below), by differential centrifugation and each washed three times by re-suspension in buffer A + 2 mM KI and resedimentation. A membrane vesicle fraction was then prepared in buffer B + 2 mM KI from the iodinated protoplasts exactly as above.

2.4. *Other methods*

The integrity of spheroplasts was determined from the latency of ONPG hydrolysis. For immunoprecipitation of the nitrate respiration complex, vesicles were first dispersed in 10 mM potassium phosphate buffer, 2% Triton X-100 pH 7.8 and centrifuged for $6 \times 10^6$ g.min to remove insoluble outer membrane material [9]; sufficient anti-nitrate reductase serum was then added to the supernatant to precipitate all the nitrate reductase present. Immuno-precipitates were collected and washed as described by Werner [10].

Sample disaggregation, electrophoresis in SDS–7.5% polyacrylamide gels and gel counting procedures were as described elsewhere [11]. Anti-nitrate reductase serum was raised in rabbits and standardized by titration against the nitrate reductase activity of an ultrasonic vesicle preparation. TCA-insoluble radioactivity was estimated by collection and counting of TCA precipitates on filter discs (Whatman GF-A) according to standard procedures. Protein was determined by the Lowry method [12].

## 3. Results and discussion

Labelling with lactoperoxidase/$^{125}I$ is usually carried out under conditions that do not lead to saturation of all available sites. This, coupled with the endogenous catalase activity of many biological preparations, complicates the quantitative comparison of separately iodinated preparations. Such complication was particularly apparent in the iodination of *E. coli* spheroplasts (high catalase activity) and ultrasonic vesicle preparations (low catalase activity). We overcame this difficulty by iodinating, in a single incubation, a mixture of spheroplasts and membrane vesicles. Thus we obtained identical conditions of iodination for both preparations, which were subsequently separated by differen-

Table 1
Iodination of vesicles and spheroplasts

| Fractions recovered after iodination | $^{125}I$-spheroplast fractionation products | Protein (mg) | T.C.A.-insoluble $^{125}I$ (c.p.m./mg protein × $10^{-4}$) |
|---|---|---|---|
| Spheroplasts[a] | | 16.9 | 160.9 |
| | Ultrasonicated spheroplasts | 16.7 | 169.0 |
| | $12 \times 10^4$ *g*.min pellet[b] | 1.8 | 474.1 |
| | $10 \times 10^6$ *g*.min supernatant | 9.5 | 38.4 |
| | P-labelled vesicles ($10 \times 10^6$ *g*.min pellet) | 4.1 | 333.3 |
| C-labelled vesicles | | 0.8 | 1757.5 |

Spheroplasts and C-labelled vesicles were separated from 3 parallel iodinations as described in Materials and methods. The spheroplasts were ultrasonicated (in buffer B + 2 mM KI) and centrifuged for $12 \times 10^4$ *g*.min. The supernatant was recentrifuged for $10 \times 10^6$ *g*.min. The resulting pellet (P-labelled vesicles) was twice washed in buffer B + 2 mM KI.

[a] Control experiments showed a 1.2% contamination of spheroplasts (and hence, of P-labelled vesicles) with C-labelled vesicles after their separation following labelling.

[b] The $12 \times 10^4$ *g*.min pellet is enriched with outer membrane which becomes labelled to a high specific activity.

tial centrifugation. A membrane vesicle preparation was then made from the iodinated protoplasts. This vesicle preparation (subsequently termed P-labelled vesicles) contained cytoplasmic membranes labelled on their periplasmic surface. The other vesicle preparation (subsequently termed C-labelled vesicles), iodinated as such, contained cytoplasmic membranes labelled predominantly on their cytoplasmic surface.

Table 1 summarizes the distribution of radioactivity in fractions obtained from a labelling experiment as described above. The low incorporation of $^{125}I$ into the $10 \times 10^6 g$·min supernatant (soluble cell contents) relative to the membrane fractions supports our necessary supposition that lactoperoxidase does not penetrate the cytoplasmic membrane.

The subunit composition of the nitrate respiration complex is shown in fig.1. Subunits $\alpha$ and $\beta$ (mol. wt 150 000 and 66 000) are from nitrate reductase itself, and $\gamma$ (mol. wt 20 000) is the haem-bearing polypeptide of cytochrome $b_{556}^{NO_3^-}$ [1,6,13]. The extent to which these subunits are labelled from the periplasmic or the cytoplasmic surface of the cytoplasmic membrane is shown in fig.2 (A and B respectively).

From the gel analysis in fig.2B it is evident that, in C-labelled vesicles, the $\alpha$ and $\beta$ subunits are both labelled and are, therefore, exposed on the cytoplasmic membrane. The amount of label in each subunit is roughly in proportion to their molecular weights. A proportional labelling of the $\gamma$ subunit would be scarcely detectable in this gel and in fact no peak of radioactivity can be seen in the position of the $\gamma$ subunit. In P-labelled vesicles, the $\gamma$ subunit is the most heavily labelled (see fig.2A). Label incorporated into the $\alpha$ subunit in P-labelled vesicles is less than 4%, per mg membrane protein, of that in C-labelled vesicles. This value corresponds to the measured protoplast breakage after iodination; consequently we can attribute this incorporation to labelling from the cytoplasmic aspect of the cytoplasmic membrane. We conclude, therefore, that the $\alpha$ and $\beta$ subunits are not iodinated in intact spheroplasts whereas the $\gamma$ subunit is. The magnitude of background radioactivity in the gel in fig.1B obliges us to leave open the question of the exposure of cytochrome $b_{556}^{NO_3^-}$ on the cytoplasmic surface of the cytoplasmic membrane. These conclusions are supported by the results of preliminary duplicate experiments using [$^{35}S$]diazobenzene sulphonate in place of $^{125}I$/lactoperoxidase, by experiments using permeant and non-permeant reductants of nitrate reductase [14] and antibody agglutination studies (unpublished).

The nitrate respiration complex therefore spans the cytoplasmic membrane of *E. coli*. Elsewhere [15] we relate these findings to the apparent location of the nitrate site [16] and to the possible mechanism of the

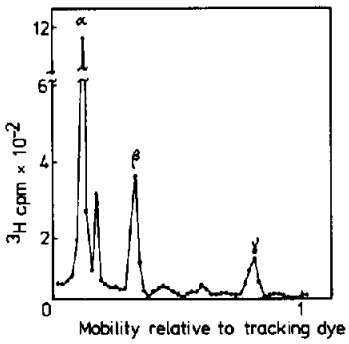


Fig.1. The subunit composition of the nitrate respiration complex. Material cross reacting with anti-nitrate reductase serum was collected from membrane vesicles prepared from *E. coli* grown in the presence of [$^3H$]valine. The immune precipitate was analyzed by SDS–polyacrylamide gel electrophoresis.

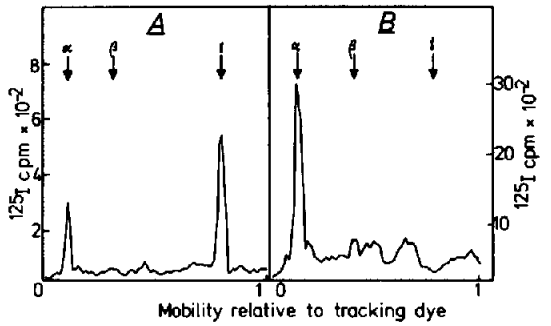


Fig.2. Surface accessibility of subunits of the nitrate respiration complex in situ in the cytoplasmic membrane. The nitrate respiration complex was immuno-precipitated from 1.4 mg (protein) P-labelled vesicles (A) and 0.25 mg (protein) C-labelled vesicles (B). Immuno-precipitates were fractionated by SDS–polyacrylamide gel electrophoresis and the distribution of $^{125}I$ in the gels was measured. The positions of the $\alpha$, $\beta$ and $\gamma$ subunits, determined in gels run in parallel with those shown, are marked with arrows. The iodinated components at positions other than those arrowed (particularly in gel B) are strongly iodinated outer membrane components sometimes present as trace contaminants of the immunoprecipitates.

proton translocation observed to be associated with the flow of reducing equivalents through this oxido-reduction carrier complex.

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