ARRANGEMENT OF RESPIRATORY NITRATE REDUCTASE IN THE CYTOPLASMIC MEMBRANE OF *ESCHERICHIA COLI*

Location of β subunit

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1. Introduction

The membrane-bound nitrate reductase (EC 1.7.99.4) from *Escherichia coli* functions as the terminal enzyme of the respiratory chain of the organism, when grown anaerobically in the presence of nitrate as electron acceptor. The transfer of reducing equivalents from reduced ubiquinol, through cytochrome b-556$^{NO_3^-}$ and nitrate reductase, to NO_3^- is accompanied by the net translocation of protons across the cytoplasmic membrane [1].

The topography of the cytochrome b-556$^{NO_3^-}$–nitrate reductase complex has been studied both functionally and structurally. The use of artificial permeant and non-permeant reductants [2] has established that the cytochrome b-556$^{NO_3^-}$ can accept electrons at the periplasmic surface and that at least part of nitrate reductase is able to donate electrons to oxidised dyes at the internal surface of the cytoplasmic membrane. Lactoperoxidase-catalysed radio-iodination [3] has located the cytochrome b-556$^{NO_3^-}$ at the periplasmic face and the α-subunit of nitrate reductase (M_r 150 000) at the cytoplasmic face of the membrane. This location of the α-subunit has been confirmed by transglutaminase-catalysed covalent labelling [4] and by immunofluorescence studies using antibodies specific for the α-subunit [5]. The location of the β-subunit (M_r 59 000) of nitrate reductase has not been established.

Here we have investigated the topography of nitrate reductase using two non-membrane permeant reagents, diazotised [^{125}I]DDISA and [^{35}S]DABS, both of which are capable of modifying the β-subunit in the isolated enzyme. We conclude that the β-subunit is located at the cytoplasmic surface of the membrane.

Abbreviations: [^{125}I]DDISA, diazotised [^{125}I]diiodosulphanilic acid; [^{35}S]DABS, diazobenzene [^{35}S]sulphonate; SDS, sodium dodecyl sulphate

2. Materials and methods

2.1. *Growth of bacteria, preparation of spheroplasts and membrane vesicles*

Escherichia coli (strain EMG 29) was grown anaerobically at 37°C, in the presence of glycerol (0.5%, w/v) and KNO_3 (1%, w/v), in the medium defined [6]. Spheroplasts were prepared from bacteria by a lysozyme/EDTA method [7]. Bacteria were harvested by centrifugation at 7000 × g for 15 min, and the cells were resuspended in 0.75 M sucrose, 20 mM sodium phosphate (pH 7.5) to give A_{600} 10. Lysozyme (2 mg/ml) was added to 100 μg/ml final conc., the mixture was incubated at 4°C for 5 min, then slowly diluted with 0.5 vol. cold 1.5 mM EDTA (pH 7.5). The spheroplasts were used immediately.

Membrane vesicles were prepared from spheroplasts by ultrasonic disruption, followed by centrifugation at 7000 × g for 15 min, all at 4°C. The supernatant so obtained was centrifuged at 250 000 × g for 1 h and the pellet (membrane vesicles) was resuspended in 0.5 M sucrose, 20 mM phosphate, 1 mM EDTA (pH 7.5) (buffer A). The membrane vesicles were stored at −20°C prior to use.

2.2. *Reagent preparation and surface labelling conditions*

[^{125}I]DDISA was synthesised from sulphanilic acid

and K^{125}I following the procedure in [8]. The reagent (spec. act. 100 Ci/mol) was prepared in buffer A and was used immediately after synthesis. [^{35}S]DABS was synthesised from [^{35}S]sulphanilic acid according to [9]. The reagent (spec. act. 4 Ci/mol) was also prepared in buffer A and was used immediately after synthesis.

K^{125}I and [^{35}S]sulphanilic acid were obtained from The Radiochemical Centre, Amersham.

Spheroplasts (30 mg protein) and membrane vesicles (15 mg protein) were mixed and [^{125}I]DDISA was added to 1 mM final conc. After 30 min at 4°C, labelled spheroplasts and membrane vesicles were separated by centrifugation at 2500 × *g* for 15 min. The pellet (spheroplasts) was resuspended in 0.5 M sucrose, 20 mM Tris–HCl (pH 7.5) (buffer B) and recentrifuged 3 times at 5000 × *g* for 10 min. Membrane vesicles were prepared from the washed spheroplasts by ultrasonication as above. This vesicle preparation contained cytoplasmic membranes labelled on their periplasmic surface (subsequently termed P-vesicles).

The supernatant from the 2500 × *g* centrifugation, containing the membrane vesicles labelled directly (vesicles labelled mainly on the cytoplasmic surface, C-vesicles) was recentrifuged at 5000 × *g* for 10 min. Vesicles were washed 3 times by suspension in buffer B and re-sedimentation at 250 000 × *g* for 1 h.

The labelled vesicle preparation (P-vesicles and C-vesicles) were suspended separately to the same protein concentration, dispersed with Triton X-100 (final conc. 2% (w/v)) and centrifuged at 100 000 × *g* for 1 h. Equal volumes of the extracts were used for immunoprecipitation, using antibodies specific for nitrate reductase or (F_1)ATPase, to recover the respective enzymes. The purified *E. coli* (F_1)ATPase used for the production of antibodies was a gift from Dr G. Vogel (Max-Planck-Institut für Biologie, Tübingen). [^{35}S]DABS labelling conditions were identical to those for [^{125}I]DDISA except that spheroplasts (15 mg) and membrane vesicles (8 mg) were used, and [^{35}S]DABS was added to 2 mM final conc. After 15 min at 4°C, the spheroplasts and membrane vesicles were separated and treated as above.

2.3. *Other methods*

Nitrate reductase was purified essentially as in [10]. Immunoprecipitated material was collected, washed [11] and analysed by SDS–polyacrylamide gel electrophoresis [12] in 10% polyacrylamide gels. The distribution of ^{35}S radioactivity in 1 mm slices of polyacrylamide gels was determined; by liquid scintillation counting [13], and ^{125}I was determined by direct counting of the slices in a gamma spectrometer. Protein was assayed by the Lowry method [14].

3. Results

3.1. *Labelling of isolated nitrate reductase*

Earlier studies on the arrangement of the subunits of nitrate reductase with the cytoplasmic membrane of *E. coli* have failed to locate the β-subunit [3,4]. Neither lactoperoxidase-catalysed iodination [15] nor transglutaminase-catalysed covalent modification [4] labels the β-subunit in either the isolated enzyme or in membrane preparations. We investigated the reactivity of two other reagents [^{125}I]DDISA and [^{35}S]-DABS towards isolated nitrate reductase. Both of these reagents have been used to study the topography of membrane proteins in other systems [16,17].

Nitrate reductase was purified as in [10]. Contrary to the findings in [10] but in accordance with those in [18], the isolated enzyme was free of spectroscopically detectable cytochrome. The enzyme was recovered after chemical modification by immunoprecipitation with antibodies specific for nitrate reductase. Treatment of isolated nitrate reductase with [^{125}I]-DDISA resulted in both α- and β-subunits being modified (fig.1a). The ratio of radioactivity found in each subunit was approximately equal to the molar ratio of the subunits. [^{35}S]DABS labelling of isolated nitrate reductase resulted in the labelling of both α- and β-subunits in a similar fashion (fig.1b). Both subunits are, therefore, accessible to both reagents in the isolated enzyme. Therefore [^{125}I]DDISA and [^{35}S]DABS appear to be suitable probes for the study of the location of both the α- and β-subunits of the membrane-bound enzyme.

3.2. *Labelling of membrane-bound nitrate reductase with [^{125}I]DDISA*

Under the labelling conditions normally used for [^{125}I]DDISA modification (low reagent concentrations), only a small fraction (assumed to be representative) of all potentially reactive sites on the membrane surfaces are labelled. Spheroplast and membrane vesicles were mixed prior to exposure to [^{125}I]DDISA and subsequently separated. This ensured that the incorporation of the reagent at the periplasmic and

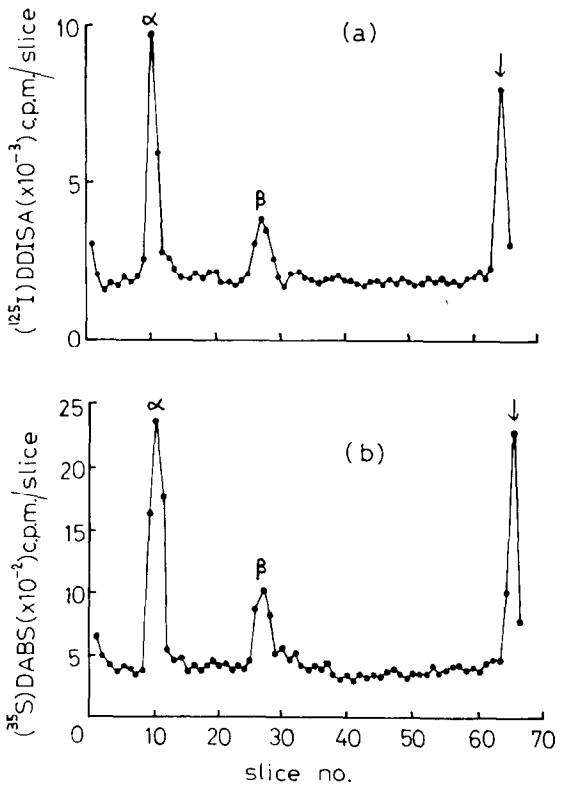

Fig.1. Labelling of isolated nitrate reductase with [^{125}I]DDISA (a) and [^{35}S]DABS (b). Nitrate reductase (30 μg) in 50 mM phosphate buffer (pH 7.5) was mixed with [^{125}I]DDISA (1 mM final conc.) or [^{35}S]DABS (2 mM final conc.) for 10 min at 4°C. 1 ml 50 mM Tris–HCl (pH 7.0) was added, the enzymes were immunoprecipitated with antibodies specific for nitrate reductase, and the immunoprecipitated material was analysed by SDS–polyacrylamide gel electrophoresis. The arrow indicates the position of the dye front in each gel.

cytoplasmic surfaces of the membrane were directly comparable [3]. Control experiments have shown that cross-contamination of labelled vesicles with spheroplasts and vice versa was negligible after their separation by differential centrifugation. The incorporation of label at the cytoplasmic face of the membrane was monitored by the measurement of the extent of modification of (F_1)ATPase in both spheroplasts and membrane vesicles during each experiment. This enzyme is located at the inner face of the cytoplasmic membrane in *E. coli* [19].

The incorporation of [^{125}I]DDISA into membrane vesicles derived from labelled spheroplasts in which the periplasmic face of the membrane is labelled (P-vesicles) and into membrane vesicles directly, in which mainly the cytoplasmic face of the membrane is labelled (C-vesicles), is shown in table 1. The ratio of incorporation into C-vesicles to P-vesicles was about 2:1 indicating that more sites are available to the reagent at the cytoplasmic face of the membrane than at the periplasmic face. The low relative specific radioactivity of the soluble (cytoplasmic) fraction indicated that significant leakage of the reagent into spheroplasts did not occur. From the gel electrophoretic analysis of the immunoprecipitate, it is evident that (F_1)ATPase is not labelled with [^{125}I]DDISA in P-vesicles (fig.2a). However, both subunits I and II of (F_1)ATPase (M_r 56 000 and 52 000, respectively) are labelled in C-vesicles. Since membrane vesicles prepared following ultrasonic disruption in *E. coli* are of predominantly inside-out orientation with respect to the intact organism [20], these results are in accordance with the well-documented location of this enzyme at

Table 1
Incorporation of [^{125}I]DDISA and [^{35}S]DABS into spheroplasts and membrane vesicles

Preparation	[^{125}I]DDISA (cpm . mg protein^{-1})	[^{35}S]DABS (cpm . mg protein^{-1})
P-vesicles	3 099 400	121 444
C-vesicles	5 953 400	280 144
Soluble 'cytoplasmic' fraction	150 000	11 490

Spheroplasts and membrane vesicles were mixed and labelled with [^{125}I]DDISA or [^{35}S]DABS as in section 2. The spheroplasts and membrane vesicles (C-vesicles) were recovered by differential centrifugation. P-vesicles and soluble 'cytoplasmic' fraction were isolated from the surface-labelled spheroplasts following ultrasonic disruption (see section 2)

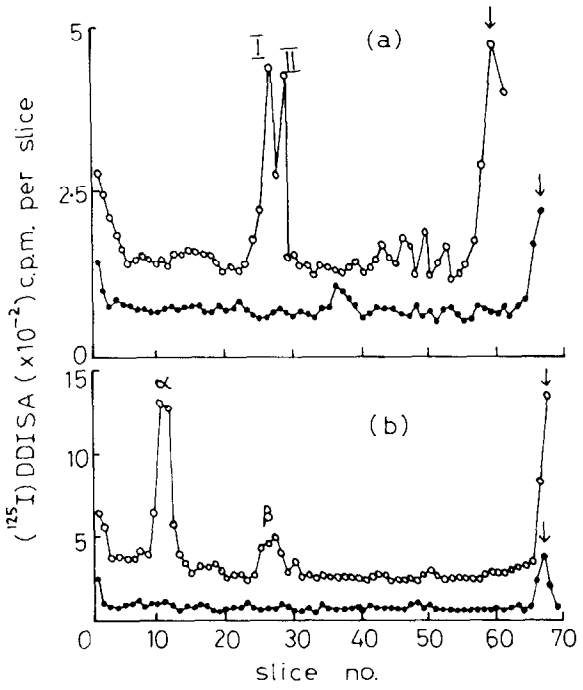

Fig.2. [^{125}I]DDISA labelling of membrane-bound (F_1)ATPase and nitrate reductase. A spheroplast and membrane vesicle mixture was labelled with [^{125}I]DDISA, then each separated as in section 2. Membrane vesicles (P-vesicles) were prepared from the spheroplasts and both vesicle preparations were dispersed separately with Triton X-100. (F_1)ATPase (a) and nitrate reductase (b) were immunoprecipitated, with antibodies specific for (F_1)ATPase and nitrate reductase, respectively. Immunoprecipitated material from P-vesicles (•——•) and from C-vesicles (○——○) was analysed by SDS–polyacrylamide gel electrophoresis. An arrow indicates the position of the dye front in each gel.

the inner face of the cytoplasmic membrane in *E. coli* [19] and support our necessary supposition that [^{125}I]DDISA does not penetrate the cytoplasmic membrane during the course of the experiment. We did not detect radioactivity in the other polypeptides of (F_1)ATPase although any incorporation into the low molecular weight subunits would have been overlooked.

Nitrate reductase, obtained from the same labelled preparations as (F_1)ATPase above, was not labelled in P-vesicles, however, both α- and β-subunits were labelled in C-vesicles (fig.2b). The absence of label incorporated into nitrate reductase in P-vesicles could not be due to lack of reactivity of the DDISA since the enzyme and (F_1)ATPase were modified in C-vesicles which were present in the same reaction mixture. The α- and β-subunits of nitrate reductase are accessible at the cytoplasmic surface of the membrane. The ratio of radioactivity found in the α- and β-subunits is about the same as that observed for the labelled isolated enzyme suggesting that both subunits are equally well exposed in the membrane. Experiments performed with spheroplasts and membrane vesicles labelled with [^{125}I]DDISA independently produced similar results as those found for the mixture (data not shown).

3.3. *Labelling of membrane-bound nitrate reductase with [^{35}S]DABS*

The ratio of the specific radioactivity of [^{35}S]-DABS incorporation into C-labelled and P-labelled

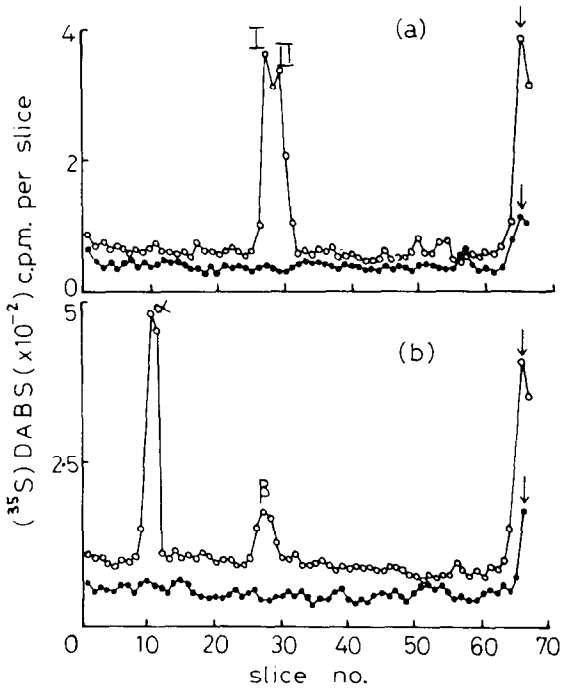

Fig.3. [^{35}S]DABS labelling of membrane-bound (F_1)ATPase and nitrate reductase. A spheroplast and membrane vesicle mixture was labelled with [^{35}S]DABS, then each separated as in section 2. Membrane vesicles (P-vesicles) were prepared from the spheroplasts and both vesicle preparations were dispersed separately with Triton X-100. (F_1)ATPase (a) and nitrate reductase (b) were immunoprecipitated, with antibodies specific for (F_1)ATPase and nitrate reductase, respectively. Immunoprecipitated material from P-vesicles (•——•) and from C-vesicles (○——○) was analysed by SDS–polyacrylamide gel electrophoresis. An arrow indicates the position of the dye front in each gel.

vesicles, as for [^{125}I]DDISA, was also found to be about 2:1 (table 1). The cytoplasmic matrix fraction had a low relative specific radioactivity which indicated that [^{35}S]DABS was essentially non-membrane permeant under these experimental conditions. Subunits I and II of (F_1)ATPase were labelled with [^{35}S]-DABS only when the cytoplasmic face was exposed to the labelling reagent (fig.3a) which, as for the experiments with [^{125}I]DDISA, is in accordance with the location of this enzyme and supports the use of the reagent as a vectorial probe for membrane protein location. The α- and β-subunits of nitrate reductase are labelled with [^{35}S]DABS only when the cytoplasmic face is accessible (fig.3a,b) which agrees with the conclusions drawn from the experiments using the more lipophilic [^{125}I]DDISA.

4. Discussion

This report demonstrates that the β-subunit of nitrate reductase in *E. coli* is located at the internal surface of the cytoplasmic membrane. It also confirms reports, employing direct chemical modification [3,4], that the α-subunit of the enzyme is similarly located at the cytoplasmic face of the membrane. The use of the modification of (F_1)ATPase as an indicator of the accessibility of the cytoplasmic face of the membrane to DDISA and DABS, indicated that these reagents can be reliably used to locate membrane bound proteins in *E. coli*. However, there is a lack of a good marker protein for the periplasmic surface of the membrane so that a similar control for this surface is not available. Further control immunological experiments indicated that membrane vesicles prepared by sonication are of ~70% inside-out orientation with respect to spheroplasts (unpublished).

Chemical modification of *E. coli* spheroplasts by lactoperoxidase-catalysed radio-iodination [3], transglutaminase-catalysed incorporation of dansyl cadaverine [4], [^{125}I]DDISA, [^{35}S]DABS and exposure to antibodies specific for the α-subunit of the enzyme [5], all fail to locate the α-subunit at the periplasmic surface of the membrane. All of these agents interact with the α-subunit in the isolated enzyme. Similarly, the treatment of spheroplasts with antibodies raised to holo-nitrate reductase, which interact specifically with both the α- and β-subunits [5], and these experiments, all fail to locate the β-subunit at the outer surface of the membrane. Therefore, it seems that neither subunit alone nor both subunits as a complex, occupies a transmembranous location in the membrane. However, the cytochrome *b*-556$^{NO_3^-}$–nitrate reductase complex spans the membrane, since the cytochrome *b* has been located at the periplasmic surface [3]. It is not known which of the subunits of nitrate reductase interacts with the cytochrome *b*-556$^{NO_3^-}$ in the membrane nor indeed whether the cytochrome itself spans the membrane.

Spectroscopically detectable cytochrome *b*-556$^{NO_3^-}$, contrary to [21,22], was not found associated with the immunoprecipitates obtained from Triton X-100 dispersed membrane vesicles using antibodies specific for nitrate reductase. No information, therefore, regarding the accessibility of this component to the reagents employed here is available. This discrepancy, which may be due to several factors, is presently under investigation.

It has been proposed that the mechanism of proton translocation in the ubiquinol→nitrate segment of the respiratory chain is a direct and inevitable consequence of the chemistry of the reductant and the transmembranous organisation of the cytochrome *b*-containing nitrate reductase respiratory complex, and is therefore chemiosmotic [23]. Since the protons released at the periplasmic surface come directly from the oxidation of ubiquinol by ferricytochrome *b*-556$^{NO_3^-}$, nitrate reductase serves to conduct electrons to its proton consuming, nitrate-reducing site, which, contrary to [1], has been located at the inner surface [24,25]. It therefore appears, from considerations of the role of the enzyme, unnecessary for part of nitrate reductase to be exposed at the periplasmic surface of the membrane.

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