Resolution of the Nitrate Reductase Complex from the Membrane of *Escherichia coli*

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Received for publication 28 August 1972

A procedure has been developed whereby the components of the nitrate reductase complex from the membrane of *Escherichia coli* can be extracted by means of a nonionic detergent and separated from each other as discrete elements in an active form. It is not yet clear whether these are the dissociated enzymatic elements or whether they are fragments containing other membrane proteins. In any case, they appeared nearly lipid-free and still active.

The manner in which proteins are functionally integrated into biological membranes remains an unanswered question. To attack this problem, procedures are required whereby proteins may be solubilized from membranes in an intact form which might allow the reconstitution of the original membrane. To approach this latter requirement, it seemed important to utilize a membrane system in which the restoration of function could indicate the regaining of structure. For this purpose, we chose to study the nitrate reductase complex present in *Escherichia coli* membranes. This complex is composed of formate dehydrogenase, two cytochrome b₁ molecules, and the nitrate reductase proper (5, 6). It has been shown to be membrane-bound (1, 2, 7) and to be induced when bacteria are grown under anaerobic conditions in the presence of nitrate (5, 7).

The present results indicate that, by the use of a nonionic detergent, BRIJ-36 T, the formate dehydrogenase and nitrate reductase may be separated from each other and from the majority of the membrane proteins and lipids. Preliminary findings obtained with ⁵⁹Fe also give an indication of the elution profile of the cell cytochromes and other iron-containing components.

**MATERIALS AND METHODS**

The strain used was *E. coli* HfrH obtained from J. A. De Moss, University of California, San Diego. Growth conditions and spectrophotometric determinations of nitrate reductase and formate dehydrogenase were performed as previously described (6). Membrane fractions were prepared by the method of Morris and De Moss (4) slightly modified: bacteria were grown anaerobically with nitrate, centrifuged, washed, and resuspended in 0.01 M tris(hydroxy-methyl)aminomethane Tris buffer, pH 7.6, containing 0.01 M MgSO₄, 0.05 M KCl, 20% sucrose, and 1 mg of lysozyme per ml. They were frozen and thawed several times. Ten volumes of deoxyribonuclease solution containing 5 µg per ml were added until the viscosity decreased to a minimum. Whole cells were eliminated by centrifugation at 2,000 rpm (960 x g). The cell membrane fraction was sedimented at 20,000 rpm for 30 min, washed twice by centrifugation with 0.02 M Tris buffer containing 1 mM ethylenediaminetetraacetate (EDTA), and resuspended in the same buffer. Equal volumes of cell membranes and 10 mM BRIJ-36 T in 0.02 M Tris buffer, pH 7.5, containing 1 mM EDTA were then mixed. The mixture was kept at 0°C for 1 or 2 h and subjected to chromatography on agarose gel columns (Sepharose 4B from Pharmacia, Upsala, Sweden). Two different columns were used throughout. One, measuring 2.0 by 50 cm was jacketed and cooled with water maintained at 10°C to protect the formate dehydrogenase. The other (2.5 by 80 cm) was run at room temperature and was mainly used for the identification of the other membrane components.

Spectra of cytochromes were measured at room temperature with a Cary 15 recording spectrophotometer. Protein was measured by ultraviolet absorption and by the method of Lowry et al (3). Determination of organic phosphorus was used as a measure of phospholipids. Phosphate was measured in hydrolyzed samples by the method of Fiske and Subbarow (8). Radioactivity was measured with a Nuclear-Chicago counter.
RESULTS AND DISCUSSION

Figure 1 shows the elution pattern of the membrane proteins extracted with 5 mM BRIJ-36 T, 1 mM EDTA, in 20 mM Tris-hydrochloride buffer, pH 7.5, and eluted with the same buffer. Both enzymes appear as discrete peaks separated from each other. The use of other nonionic, cationic, and anionic detergents brought about the inactivation of the formate dehydrogenase. When the insoluble material was not eliminated by centrifugation, a significant portion of the membrane components absorbing at 280 nm were eluted in the excluded volume. The activities of the formate dehydrogenase and nitrate reductase eluted in this position were almost negligible (see Fig. 2 and 3).

Even though the nitrate reductase appeared nearly as one peak, it was possible that it might be heterogeneous and contain several forms of the enzyme (Fig. 2). During the gel filtration procedure, the majority of the membrane lipids appeared to be separated from the membrane proteins (Fig. 2) and to elute as a distinct major peak and one minor one. No phospholipid could be detected in the position where the nitrate reductase was eluted. The concentration of cytochromes in the eluates from the 4% agarose column were too low to determine their position and achieve their identification. To localize the general region where they might elute, an attempt was made to label them by the use of *Fe²⁺.

Figure 3 shows that the pattern of the radioactive entities was rather sharp, a small fraction appearing in the excluded volume, one in the position where the nitrate reductase appears, and a sharp peak centered in tube 155 in the vicinity of the internal volume. No conclusion can be reached about the nature of these components; however, the highest peak appearing at or beyond the VI could be a basic element since these are retained by the gel matrix. After solubilization of the membranes with 5 mM BRIJ-36 T, cytochrome b, was no longer reduced by formate nor oxidized by nitrate, and its alpha band changed from 558 to 562 nm. This change suggests that a physical separation of cytochrome b, from other membrane components has taken place.

The majority of the phospholipids elute as low-molecular-weight components. It is probable that the detergent substitutes for the lipid in maintaining the proteins active as discrete entities in a "solubilized" form (C. Gitler,
Fig. 2. Elution pattern of nitrate reductase and phospholipids at room temperature. Solubilization of membranes was carried out as described for Fig. 1, but insoluble material was not eliminated. Elution from a Sepharose 4B column (2.5 by 80 cm) was carried out at room temperature, and 1.5-ml samples were recovered. Symbols: ○, absorbance at 280 nm; O, nitrate reductase expressed as nanomoles of nitrite formed per minute; x, phosphate, as absorbancy at 660 nm.

Fig. 3. Elution pattern of 59Fe-containing proteins from solubilized membrane of E. coli. Bacteria were grown at 37 °C for 6 h in a medium containing 0.6 nmol of 59FeCl₃ per liter (1.6 μCi/μmol). Cells were centrifuged and washed. Membranes were prepared as usual and subjected to chromatography as described for Fig. 2. Symbols: ○, absorbance at 280 nm; x, nitrate reductase as nanomoles of nitrite formed per minute; O, radioactivity (counts per minute x 10³).
Annu. Rev. Biophys. Bioeng., in press). In view of the high molecular weight of the formate dehydrogenase and nitrate reductase entities obtained, and from the finding that a $^{55}$Fe-containing component is eluted in the region of the nitrate reductase, it is conceivable that the entities isolated represent perhaps those aggregates present in the intact membrane. If random reaggregation occurred on adding the detergent, the enzymatic activity would be expected to be dispersed throughout the elution profile. However, the fact that electron transport is lost implies either that the actual topological relation of the aggregate elements is lost or, alternatively, that some low-molecular-weight components are removed by the detergent. Experiments are in progress on the recombination of different fractions to test this latter possibility.

Our results open the possibility of purifying the individual components of the nitrate reductase complex from the membrane of E. coli and of studying their aggregation under controlled conditions. They also offer new methodology to study the biochemical alterations of pleiotropic mutants such as those described by Ruiz-Herrera and De Moss (5).

ACKNOWLEDGMENTS

This work was supported by a grant to one of us (J.R.H.) from the Research Corporation, Brown Hazen Fund, and by the C.O.F.A.A. of the Instituto Politécnico Nacional.

LITERATURE CITED