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Isolation and Purification of Bacterial Membrane Proteins by the Use of Organic Solvents: The Lactose Permease and the Carbodiimide-Reactive Protein of the Adenosinetriphosphatase Complex of Escherichia Coli

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Techniques for the solubilization and fractionation of integral membrane proteins have been developed in recent years. A small portion of membrane protein (about 2%, proteolipid fraction) will partition into chloroform or 1-butanol, and, in several cases, these proteins retain functional activity. A virtually complete solubilization can be achieved at neutral pH by use of aprotic solvents, like hexamethylphosphoric triamide or N-methylpyrrolidone.

At relatively low concentrations (< 3 M) aprotic solvents inhibited β-D-galactoside transport by whole cells and the derivative membrane vesicles of Escherichia coli, but this inhibition could be largely reversed by a simple washing procedure. At higher concentrations of aprotic solvent (5–6 M), 50–80% of the total protein of lactose transport-positive membrane vesicles was solubilized. When these extracts were added to intact lactose transport-negative membrane vesicles, lactose transport was reconstituted, the required energy being provided by either respiration (e.g., addition of D-lactate) or by a K^+ diffusion potential established with the aid of valinomycin.

The dicyclohexylcarbodiimide (DCCD)-reactive subunit of the E. coli ATPase complex was found to partition into chloroform, and to be amenable to further purification in organic solvent. Ether precipitation and chromatography on DEAE-cellulose and hydroxypropyl-Sephadex G-50 yielded an homogeneous polypeptide of an apparent molecular weight of 9,000.

The purified and unlabeled DCCD-reactive protein was incorporated into K^+-loaded liposomes, and a membrane potential was generated by the addition of valinomycin. There are indications that the DCCD-reactive protein alone made the membrane specifically permeable for protons.

Key words: Escherichia coli, lactose permease, carbodiimide-reactive protein, Ca^{2+}, Mg^{2+}-ATPase, aprotic solvents, organic solvents, integral membrane proteins, bioenergetics

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INTRODUCTION

Many peripheral membrane proteins (1) of bacterial membranes, like the periplasmic binding proteins of various transport systems (2) and the BF_1 portion of the ATPase complex (3), have been purified to homogeneity. However, great difficulties still remain in the solubilization and purification of integral membrane proteins (1) in their active forms. The solubilization of these proteins by detergents has, in a number of cases (4, 5), been an important step in the purification procedure. However, in other cases detergents are highly inhibitory. For example, binding of a high-affinity β-D-galactoside to the E. coli lactose permease is completely abolished in the presence of 0.1% Trition X-100 (6). This protein has therefore not yet been solubilized in a functional form.

Two alternative methods for the solubilization of integral membrane proteins also have been used. Chaotropic agents effectively solubilize E. coli membrane proteins (7), and the D-lactate dehydrogenase has been solubilized in active form by 0.75 M guanidine hydrochloride (8). The present paper will describe, however, a further method for the solubilization of internal membrane proteins using organic solvents.

RESULTS AND DISCUSSION

Solubilization by Organic Solvents

Isolation of proteolipids. The solubilization of certain membrane proteins like the enzyme II of the phosphotransferase transport system (9) and phospholipase A_1 (10) of E. coli, requires the addition of 5 and 15% (vol/vol) 1-butanol, respectively. When more than about 20% (vol/vol) 1-butanol is added to aqueous buffer, a 2-phase system results. Proteolipids can therefore be defined as polypeptides which, in a water/organic solvent 2-phase system, will partition into the organic phase. This definition leaves open the question of whether partitioning is due to the hydrophobicity of the polypeptide proper, or to complex formation with lipids.

Protein fractions soluble in organic solvent (chloroform) were initially discovered in a number of tissues (11), and the myelin proteolipid has been particularly well studied (12). Recent reviews are available (13, 14).

The proportion of the protein of various biological membranes which, at pH values greater than 4, will partition into a chloroform or 1-butanol phase is typically around 2%. Functional activity may be retained under such conditions. Solubilization with 1-butanol, and a number of newly-developed methods applicable to organic solvents, were used for the purification to homogeneity of the extremely hydrophobic C_{55}-isoprenoid-alcohol kinase of Staphylococcus aureus (15, 16). Similar techniques were also successfully applied to the following catalytic membrane proteins: the glycosyl-transferases (17) and C_{55}-isoprenoid-alcohol kinase (18) of Klebsiella aerogenes, the proline transport system of E. coli (19) and the dicyclohexylcarbodiimide (DCCD)-reactive subunit of the E. coli ATPase complex (see below). In addition, the diglyceride kinase activity of E. coli membranes (20) has recently been found to partition in high yield into the 1-butanol phase when the standard procedure (16) for 1-butanol extraction (at pH 4.2) was used (E. Bohnenberger and H. Sandermann Jr., unpublished results). Proteolipids important in cellular energetics may also be extracted using organic solvents. For example, extremely hydrophobic polypeptides which partition from water into chloroform or 1-butanol phases have been isolated from the mitochondrial ATPase (21) and sarcoplasmic Ca^{2+}-ATPase (22), and the latter proteolipid appears to act as an ionophore (23).

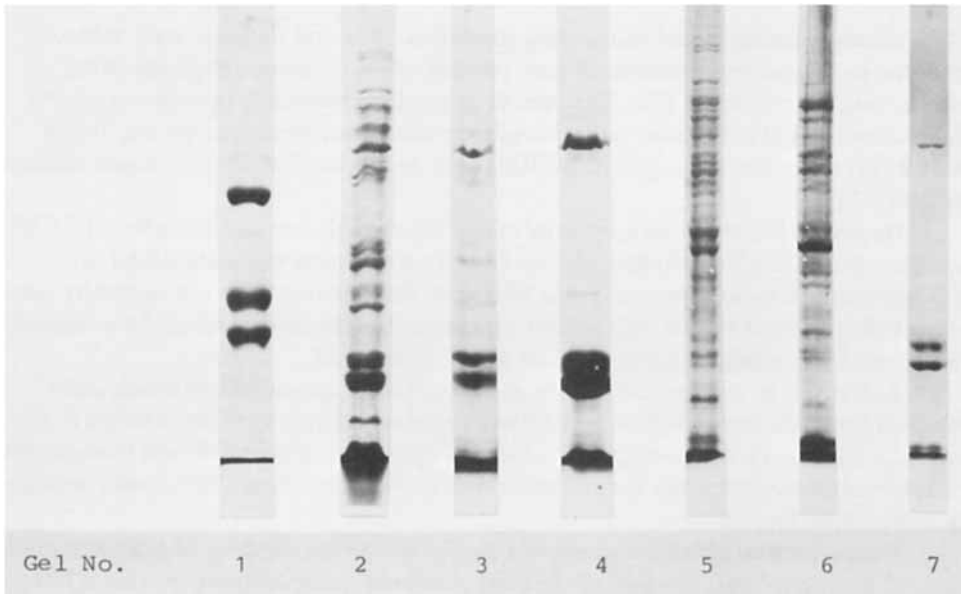

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of polypeptides extracted with aprotic solvents from E. coli membrane preparations. Polyacrylamide gels were run according to Laemmli (54). Staining was by Coomassie Blue. Gel No. 1: Standard proteins (from top: bovine serum albumin, mol wt 67,000; ovalbumin, mol wt 43,000; aldolase, mol wt 40,000; chymotrypsinogen, mol wt 25,000). Gel No. 2: Total membrane preparation of E. coli prepared according to Osborn and Mason (53). This material was used for preparing the extracts of gels no. 3 and 4. Gel No. 3: Extract from total membrane (53) prepared using 90% (vol/vol) HMPT at pH 7. The extracts were in all cases the supernatants after ultracentrifugation (100,000 × g, 120 min). Gel No. 4: Extract of total membrane (53) prepared using 90% (vol/vol) MP at pH 7. Gel No. 5: Membrane vesicle protein prepared according to Kaback (35). Gel No. 6: Extract of membrane vesicles prepared using 90% (vol/vol) HMPT at pH 7. Gel No. 7: Extract of membrane vesicles prepared using 90% (vol/vol) MP at pH 7.

A virtually complete solubilization of membrane proteins in solvents like chloroform or 1-butanol can be achieved at strongly acidic pH values (pH < 2), but under such conditions functional activities are lost, and membrane reconstitution appears to lead to artifacts (24).

Aprotic solvents. Aprotic solvents, in particular hexamethylphosphoric triamide (HMPT), N-methylpyrrolidone (MP), and dimethylsulfoxide, bring about extensive solubilization (up to 80%) of E. coli membrane proteins under mild conditions (25, 26). These solvents are all freely miscible with water, and solubilized a number of polypeptides (Fig. 1). Depending on the pH and solvent concentration, some selectivity of solubilized polypeptides can be achieved. For example, the prominent outer membrane polypeptides of molecular weight (mol wt) 30,000–40,000 (27) appear to be enriched under certain conditions (gel no. 4, and perhaps no. 7, of Fig. 1).

The retention of certain enzyme activities in aprotic solvent has been known for some time (28), and a recent application of aprotic solvents is in the field of cryoenzymology (29, 30). Aprotic solvents are also known to induce the differentiation of certain mammalian cells, and to produce phase separation phenomena with artificial phospholipid bilayers (31).

The Lactose Permease System of E. coli

Characterization under inactivating conditions. Fox and Kennedy were able to label the lactose permease protein (y-gene product of the lac-operon) by an indirect affinity labeling procedure (32). The protein, labeled at an essential cysteine-residue by radioactive N-ethyl-maleimide, was subsequently solubilized by the detergents, Triton X-100 (32) or sodium dodecyl sulfate (SDS) (33), and its mol wt was determined as about 30,000 (33).

Reversible inhibition by aprotic solvents. At relatively low concentrations (< 3 M), aprotic solvents like N-methylpyrrolidone (Fig. 2) or dimethyl sulfoxide inhibited β-D-galactoside transport by whole cells of E. coli. The inhibition was not caused by membrane leakiness since even at high solvent concentrations the control values for nonspecific hydrolysis by intracellular β-galactosidase were not increased.

In contrast to detergents like SDS or Triton X-100, aprotic solvents were easily removed by washing with buffer, and cellular transport activity was fully restored in this way (25, 34; Fig. 2). Lactose uptake by isolated membrane vesicles (35) was more sensitive to aprotic solvents, but again the inhibition could be largely reversed by a simple washing procedure (34; Fig. 3).

Reconstitution of lactose-transport negative membrane vesicles. At high concentrations of aprotic solvent, 50–80% of the total protein of transport-positive vesicles from E. coli ML 308–225 were solubilized. For solubilization of membrane proteins the vesicles were washed once with 100 mM potassium phosphate buffer (pH 8.0), collected by centrifugation, and solubilized at 25°C for 5 min by incubation in 90% hexamethylphosphoric triamide containing 100 mM LiCl, 50 mM Tris-SO_4 (pH 7.5), and 0.3 mM dithiothreitol.

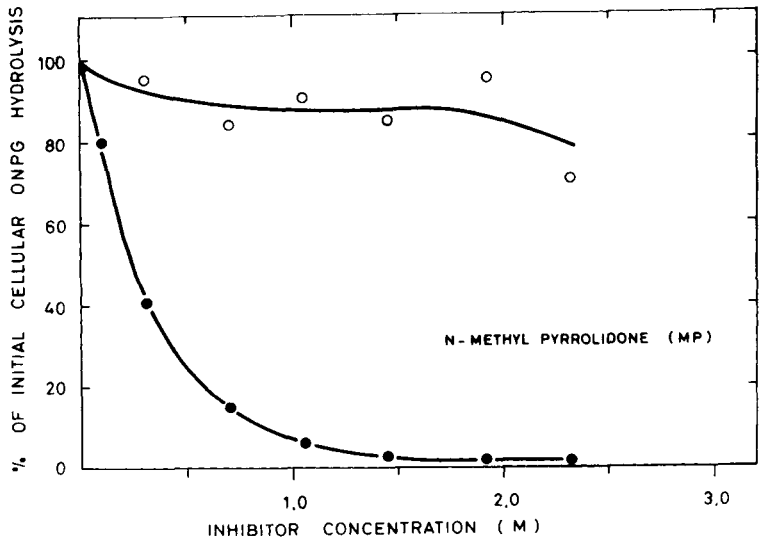

Fig. 2. Reversible inhibition of β-D-galactoside transport by aprotic solvents. Inhibition of the cellular hydrolysis of o-nitrophenyl-β-D-galactopyranoside in the presence of N-methylpyrrolidon (•——•), and after removal of the aprotic solvent by pelleting the cells and resuspending in buffer (reactivation, o ——o). Experimental details are given in Ref. 34.

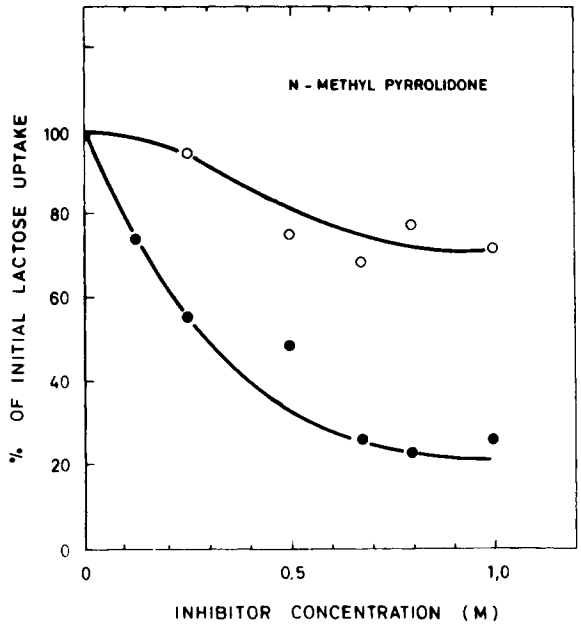

Fig. 3. Inhibition of [^{14}C] lactose uptake of membrane vesicles (35) of E. coli ML 308-225 by N-methylpyrrolidone (●——●), and after removal of the aprotic solvent by pelleting the vesicles and resuspending in buffer (reactivation, ○ ——○). Experimental details are given in Ref. 34.

After ultracentrifugation (140,000 × g, 2 hr) this extract (R-extract) was used in the reconstitution experiments. For that purpose membrane vesicles of the transport-negative strain E. coli ML 35 were suspended in 50 mM Tris-SO_4 (pH 7.5), 2 mM $MgSO_4$, and 0.3 mM dithiothreitol (0.7 mg of protein in 0.5 ml). The mixture was sonicated (Branson sonifier, model S 125, microtip attachment) in an ice-bath for 10–15 sec, during which time 50 μl R-extract was added (0.46 M final solvent concentration). The vesicles were washed once with the above buffer and assayed for [^{14}C] lactose uptake (42). Lactose accumulation was observed in the presence of D-lactate, whereas no uptake could be detected in the presence of uncoupler (1 μM CCCP) or of high-affinity inhibitor (5 mM thio-βD-digalactoside) (Fig. 4). It should be emphasized that the steady-state uptake of lactose (12 nmol/mg vesicle protein) by the reconstituted vesicles was significantly above the 1:1 binding stoichiometry expected if the transport protein added had been completely reconstituted (2 nmol/mg protein, e.g., Ref. 36). Lactose uptake could also be reconstituted by using a potassium diffusion potential, which was established with the aid of valinomycin (34, 42; Fig. 5). This technique of energization is, in principle, also applicable to reconstitution in artificial liposomes which are free of the respiratory enzymes of E. coli ML 35 membrane vesicles. Such simplified systems may yield answers to as yet open questions, such as the energy requirement for binding rather than transport of D-galactosides (36, 37), or the mechanism of symport of protons and β-D-galactosides (38).

It has been possible to fractionate the lipid and polypeptide components of the aprotic solvent extracts employed for reconstitution on columns of Sephadex LH-20, Sephacryl S-200, or Controlled Pore Glass CPG-10. Phospholipids appeared to occur in monomeric form in these extracts and were clearly separated from the eluted polypeptides.

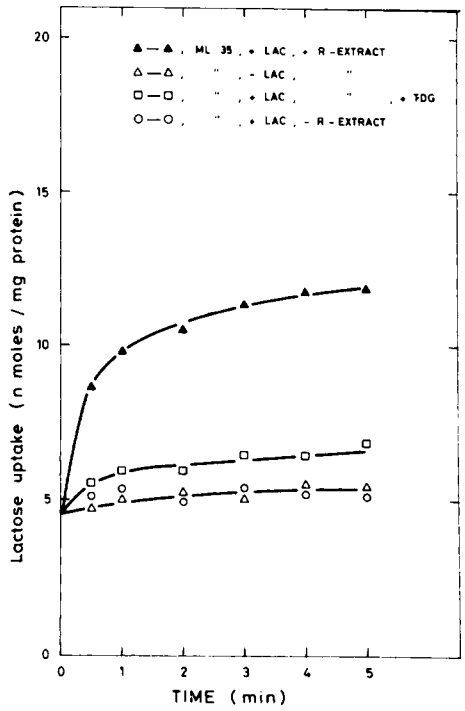

Fig. 4. Reconstitution of [^{14}C] lactose transport in membrane vesicles (35) prepared from cells of the lactose-transport negative strain, E. coli ML 35. The following method was applied for the preparation of the extract used for the reconstitution. Membrane vesicles prepared from E. coli ML 308-225 (transport positive, 12 mg protein) were solubilized in 1 ml of 90% (vol/vol) hexamethyl phosphoric triamide, containing 100 mM LiCl, 50 mM Tris-SO_4 (pH 7.5), 0.3 mM dithiothreitol. For the reconstitution, the above extract (50 µl, after ultracentrifugation, 140,000 × g, 2 hr) was added with constant sonication (10–15 sec, Branson Sonifier, model S 125; temperature ~ 2°C) to a suspension (0.5 ml) of membrane vesicles (0.7 mg protein) of E. coli ML 35 in 50 mM Tris-SO_4 (pH 7.5), 2 mM $MgSO_4$, 0.3 mM dithiothreitol. The membrane vesicles were reisolated by centrifugation, and assayed for [^{14}C] lactose uptake in the presence (▲——▲) or absence (△——△) of D-lactate, or in the presence of D-lactate with the addition of 5 mM thio-β-digalactoside (□——□). No uptake was observed when the addition of R-extract was not included in the above procedure (○——○). Further experimental details are given in Ref. 34.

Hopefully, these studies will finally allow the definition and characterization of the minimum number of components required for lactose permease activity.

The Carbodiimide-Reactive Protein of E. coli

The membrane-bound Ca^{2+}, Mg^{2+} stimulated adenosine triphosphatase complex from bacteria plays a crucial role in energy-conserving reactions (39). It has been demonstrated that this complex is composed of 2 structurally distinct entities. One component is the ATPase (BF_1) which actually catalyzes hydrolysis of ATP and which belongs to the category of peripheral proteins. The other component (BF_0) is buried within the cytoplasmic membrane and thus belongs to the category of integral proteins. The hydrolysis of ATP by the bacterial ATPase complex is coupled to the translocation of protons (39–41). Therefore, it was reasonable to assign the BF_0 component a role in the translo-

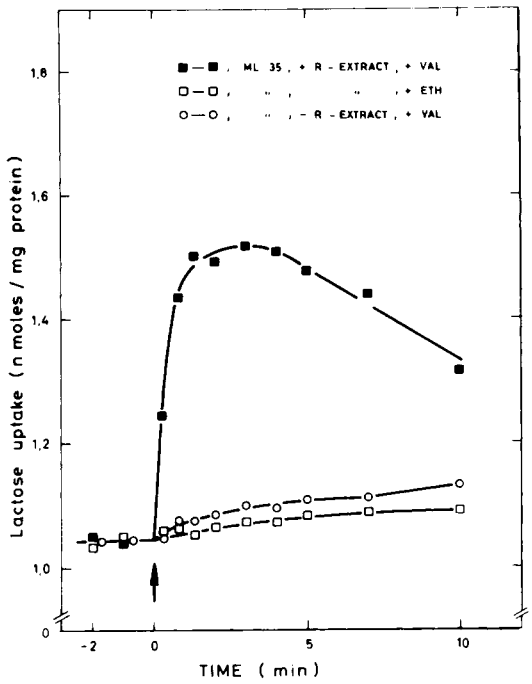

Fig. 5. Lactose transport in reconstituted ML-35 membrane vesicles in response to an artificial membrane potential. Membrane vesicles (35), prepared from cells of E. coli ML 35 were loaded with K^+ as previously described (42), and, in a control experiment, assayed for [^{14}C] lactose uptake after addition of valinomycin (○——○). The transport assay was also performed after prior addition of aprotic solvent extract (■——■), and a control, in this experiment, was obtained by omission of valinomycin (□——□). Experimental details are given in Ref. 34.

cation of protons. This view was supported recently by several lines of experimental evidence (42–46).

Energy-transducing reactions carried out by the ATPase complex from bacteria are inhibited by DCCD, as is also the case in mitochondria and chloroplasts. It is now well established that the inhibitor exerts its irreversible effect on the BF_0 part.

Since the DCCD-reactive protein is the only component so far which has been implicated in the proton translocation process directly, it was conceivable that this protein alone might be required for the translocation of protons. Therefore, the characterization and purification of the DCCD-reactive protein seemed warranted.

Characterization and purification of the DCCD-reactive protein. For the identification of the DCCD-reactive protein it was necessary to demonstrate that the reaction of DCCD with that protein was related to inhibition of the ATPase activity. This was accomplished by using mutants where the ATPase activity was no longer inhibited by DCCD (47, 48). After treatment of E. coli membranes derived from wild type and mutant with [^{14}C] DCCD, the proteolipids were extracted with chloroform/methanol (2:1). After precipitation of the proteolipids with diethyl ether from the washed chloroform/methanol extract, it could be demonstrated by SDS-gel electrophoresis, that one protein was labeled by [^{14}C] DCCD in membranes of the wild type, but not in the mutants (Fig. 6; for further details see Refs. 47 and 48). A direct correlation between the reaction of [^{14}C] DCCD

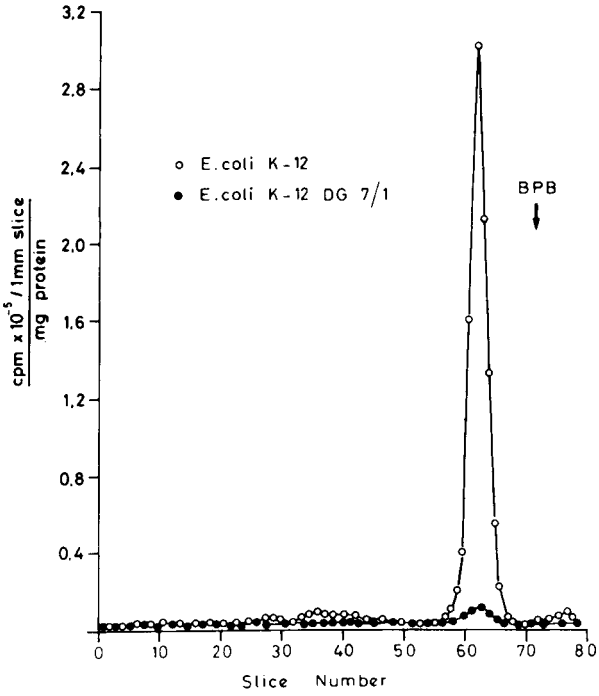

Fig. 6. Sodium dodecyl sulfate-acrylamide gel electrophoresis of [^{14}C]-labeled proteolipid preparations of E. coli K 12 (wild type) and E. coli K 12 DG 7/1 (mutant strain). The preparation of membranes from both wild type and mutant strain, the labeling of the membranes with [^{14}C] DCCD (14.5 mCi/mmol), and the extraction of the membranes with chloroform/methanol (2:1) were performed as already described (51). The proteolipids were precipitated from the chloroform/methanol extract with diethyl ether and subsequently taken up in chloroform/methanol (2:1). Samples (~ 5 μg of protein) from the wild type and the mutant strain were electrophoresed on gels (13% acrylamide, 0.65% N,N'-diallyltartardiamide, for further details see Ref. 51) containing 0.2% sodium dodecyl sulfate, and 1 mm slices were dissolved in 2% periodic acid and counted. The position of the tracking dye, bromophenol blue (BPB) is marked by an arrow.

with that protein and the inhibition of the ATPase activity was thus established. Less than 10% of the membrane protein was soluble in chloroform/methanol, achieving in this first step a greater than 10-fold purification of the DCCD-reactive protein. It should be emphasized that this extraction procedure was performed without the addition of acid.

Despite the fact that about 80–85% of radioactivity from [^{14}C] DCCD incorporated into membranes was due to unspecific labeling, further purification of the DCCD-reactive protein could be followed by monitoring radioactivity, since this protein was the only radioactively labeled component extracted into chloroform/methanol and subsequently precipitated with diethyl ether (47, 48).

The crude proteolipid fraction still contained several protein components and a considerable amount of phospholipids. Since DEAE-cellulose column chromatography has proven to be a powerful separating system for proteins (16) and lipids (49) in the presence of organic solvents, the crude proteolipid fraction was applied to such a column (50, 51). Sodium dodecyl sulfate-gel electrophoresis and determination of the phosphorus content revealed that DEAE-cellulose chromatography separated the DCCD-reactive protein from

other contaminating proteins as well as from phospholipids. The final purification of this protein was achieved by chromatography on hydroxypropyl-Sephadex G-50 in the presence of chloroform/methanol containing ammonium acetate (50, 51).

Reconstitution of the unlabeled DCCD-reactive protein. For the reconstitution experiments, it was necessary to apply the DCCD-reactive protein in an unlabeled form. The unlabeled protein was purified in the same way as the labeled one (50, 51). To demonstrate that the purified, unlabeled DCCD-reactive protein had retained at least some of its biological activity, the following method was used: The protein, together with a phospholipid mixture in chloroform from a thermophilic bacterium (52), were evaporated to dryness by rotary evaporation. The residue was dispersed in buffer and sonicated. The liposomes were loaded with potassium by heat treatment in the presence of high concentrations of potassium phosphate (53). These K^+-loaded liposomes containing the DCCD-reactive protein were washed with sucrose to remove the potassium outside. Addition of valinomycin resulted in an efflux of K^+. Since K^+ ions carry a positive charge, an electrical potential difference was thus generated across the membrane of the liposomes. Following the pH changes in the medium there are indications that the DCCD-reactive protein makes the membrane of the liposomes specifically leaky for protons and treatment with DCCD reduces the high permeability. This indicates that the DCCD-reactive protein purified by this method retains at least part of its biological activity.

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