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ISOLATION AND PURIFICATION OF DICYCLOHEXYLCARBODIIMIDE-REACTIVE PROTEOLIPID FROM *BACILLUS SUBTILIS* MEMBRANE

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The membrane-bound ATPase activity of *Bacillus subtilis* was inhibited by dicyclohexylcarbodiimide (DCCD). The DCCD-reactive proteolipid of *B. subtilis* was extracted, from labelled or untreated membranes contaning F_1 or depleted of F_1, with neutral or acidic chloroform/methanol. Purification of the [^{14}C]DCCD-binding proteolipid was attempted by column chromatography on methylated Sephadex G-50 and on DEAE-cellulose. The maximal amount of DCCD which could be bound to the purified proteolipid was found to exceed the amount bound by the purified proteolipid extracted from membranes labelled with the lowest [^{14}C]DCCD concentration required for maximal inhibition of the membrane-bound ATPase activity. The radioactive protein peaks eluted by gel filtration and ion-exchange chromatography were analysed by urea-SDS polyacrylamide slab gel electrophoresis and autoradiography. Radioactivity was incorporated into two components of M_r 18 000 and 6000 when proteolipid was purified by methylated Sephadex. The 6000 polypeptide was always present, whatever the extraction and purification procedures. However, the 18 000 polypeptide was present in largest quantity only when proteolipid was extracted from membranes containing F_1 and purified by methylated Sephadex. When proteolipid was purified on DEAE-cellulose this [^{14}C]DCCD binding component of M_r 18 000 was absent.

Introduction

It has been proposed in two reviews [1,2] that the ATPase complex of mitochondria, chloroplasts and bacterial membranes possesses a structure composed of two parts: a hydrophylic component (F_1) and a hydrophobic component (F_0). The membrane-bound or the solubilized form of the F_1 component is able to hydrolyse ATP. The polypeptides of F_0 could be envisaged as forming a pore through the membrane, so as to allow the passage of H^+ and H_2O to and from F_1. The action of DCCD on ATPase activity provides evidence for a relationship between these two parts. Thus, DCCD inhibits membrane-bound ATPase activity, but does not inhibit soluble ATPase activity.

Catell and co-workers [3] have shown that a specific mitochondrial proteolipid bound the DCCD covalently. In the case of *Escherichia coli*, Patel and Kaback [4] have shown that the inhibition of proton movement by DCCD is directly related to its binding to the DCCD-reactive proteolipid, although it would be possible that other subunits of F_0 mediate the proton translaction [5].

Proteolipids were characterized by Folch and Lee [6] as a class of hydrophobic membrane proteins. Their solubility in organic solvents permitted the isolation of the apoprotein of the proteolipids and then their characterization. The DCCD-binding proteolipid has been isolated from membrane preparations of various mitochondria [3,7,8] and chloroplasts [9,10]. In bacteria, the DCCD-reactive proteolipid only of *E. coli* [11,12] and of a thermophilic bacterium [13] has been purified and some molecular

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; F_1, hydrophylic and catalytic part of the ATPase complex; F_0, hydrophobic part of the ATPase complex; SDS, sodium dodecyl sulfate.

properties have been determined [14].

In a previous study [15], we detected with DCCD an 80% inhibition of membrane-bound ATPase activity of the strict aerobe, *B. subtilis* but DCCD did not inhibit the soluble ATPase activity. This work reports: (1) the isoltation and purification of DCCD-binding proteolipid of *B. subtilis* membranes, under different extraction conditions; (2) the optimal conditions for the inhibition and labelling of ATPase activity and proteolipid with DCCD and [^{14}C]DCCD, respectively; (3) the best method for the extraction of this bacterial proteolipid which was determined by an extensive analysis of the results in urea-SDS polyacrylamide slab gel electrophoresis and autoradiography.

Materials and Methods

Chemicals

Unlabelled DCCD and [^{14}C]DCCD were obtained respectively from Pierce Chemical Company and Commissariat à l'Energie Atomique. All other compounds were obtained from Sigma, Serva and Merck.

Microorganism and preparation of cytoplasmic membrane

B. subtilis (leu$^-$met$^-$thr$^-$) was grown in an enriched medium described previously [15]. The cells were harvested at the end of the log phase.

For the study of the *B. subtilis* DCCD-binding proteolipid, two different cytoplasmic membrane materials were used.

(a) Membranes containing F_1 were prepared as previously reported [15]. The membranes were washed three times in a 50 mM Tris-HCl (pH 7.5) buffer containing 0.1 mM phenylmethanesulfonyl fluoride.

(b) Membranes depleted from F_1. The hydrophylic segment (F_1) was removed by washing the above membranes with 5 mM Tris-HCl (pH 7.5) buffer containing 0.1 mM phenylmethanesulfonyl fluoride as described previously [15]. The membranes were stored at −20°C, suspended in a 50 mM Tris-HCl (pH 7.5) buffer containing 0.1 mM phenylmethanesulfonyl fluoride and 50% glycerol.

Assay of the inhibition of the membrane-bound ATPase

Inhibition by unlabelled DDCD of membrane-bound ATPase activity was studied as a function of time, DCCD concentration and membrane protein concentration. ATPase activity was measured according to the procedure of Baginski et al. as previously reported [15]. The initial reaction rate at each different DCCD and membrane protein concentration was measured by the P_i released at three times (1, 2.30 and 4 min).

Labelling of membranes

Membranes at a protein concentration of 1.5–12 mg/ml were suspended in 50 mM Tris-HCl (pH 7.5) containing 1 mM $CaCl_2$, 0.1 mM phenylmethanesulfonyl fluoride and 20% glycerol. [^{14}C]DCCD was added as an ethanolic solution to a final concentration of 100 μM. The suspension was stirred for 16 h at 4°C and centrifuged at 100000 × g for 30 min. The membrane pellet was washed twice with the above buffer.

Isolation of crude proteolipid

A combination of the methods of Catell et al. [3] and Folch et al. [16] as described by Fillingame [11] was used and for comparative studies the extraction was made under neutral and acidic conditions with chloroform/methanol (2 : 1) and chloroform/methanol/12 M HCl (2 : 1 : 0.01), respectively.

Proteolipid purification

Two different procedures for purification of the crude proteolipid were attempted:

(1) Chromatography on methylated Sephadex G-50. Methylated Sephadex G-50 (medium) was prepared in our laboratory according to the procedure of Nystrom and Sjövall [17]. The methylation controlled by analysis of OCH_3 group was found to be 28–29%. Methylated Sephadex was equilibrated overnight with the solvent used (chloroform/methanol/0.1 M HCl) (1 : 1 : 0.1) and poured onto a 100 × 3 cm column. The crude proteolipid sample was applied in a small volume of 90% formic acid and the column eluted with 1 l of the above solvent. Bound [^{14}C]-DCCD served as a marker during the purification. Column fractions were used for determination of radioactivity and proteins. The radioactive protein fractions were concentrated by precipitation with 4 vol. diethylether. After 24 h at −20°C the precipi-

tate was removed by centrifugation at 2000 × *g* for 2 h at −20°C.

(2) Chromatography on DEAE-cellulose. DEAE-cellulose (Cellex D, Bio-Rad) was prepared and packed as described by Rouser et al. [18]. The column was then equilibrated and the proteolipid was layered and eluted as reported by Fillingame [11]. Radioactivity and proteins were estimated on column fractions. Protein fractions were concentrated as indicated by Fillingame [11].

Analytical procedures

(1) Protein concentration. Protein was determined by the method of Lowry et al. [19] with sodium dodecyl sulfate in the assay as described by Fillingame [20].

(2) Radioactivity. The radioactivity was measured in a liquid scintillation counter. Samples of 100–200 μl were dissolved in 10 ml Atomlight (New England Nuclear).

(3) Polyacrylamide slab gel electrophoresis. The urea-SDS system of Swank and Munkres [21] for low molecular weight polypeptides was used. The general procedure was slightly modified for polyacrylamide slab gels of 1.5 mm thickness. The concentration of acrylamide was 3% for the upper gel and 13% for the lower gel. Gels were stained in a solution of 0.25% Coomassie brillant blue R. Gels were calibrated for apparent molecular weight determination using cytochrome *c* (12400) and a calibration kit containing six proteins: α-lactalbumin (14400), soybean trypsin inhibitor (20100), carbonic anhydrase (30000), ovalbumin (43000), bovine serum albumin (67000) and phosphorylase *b* (94000). The gel after staining and destaining was dried under vacuum and was autoradiographed by contact with Kodak X-OMAT XR 1 film which was processed according to standard procedures. The film was scanned with a Vernon densitometer possessing an integrator.

Results

Inhibition of ATPase activity by DCCD

As a preliminary, it was necessary to determine exactly the minimal concentration of DCCD giving maximal inhibition. Assays for the inhibition of membrane-bound ATPase activity showed than an 84% maximal inhibition was obtained in about 30 min by DCCD at a concentration greater than 10 μM with a membrane suspension at a protein concentration of 1.5 mg/ml. The protein concentration of membrane suspension is a very important factor for determining maximum inhibition. For membrane protein concentrations of 10.2, 6 and 2.2 mg/ml high concentrations of DCCD (0.1–0.5 mM) inhibited the membrane-bound ATPase activity by 50, 60 and 80%, respectively (Fig. 1). An increase of the inhibition as a function of time was noted: at higher membrane protein concentrations (12 mg/ml), the inhibition was increase by 10% in about 16 h.

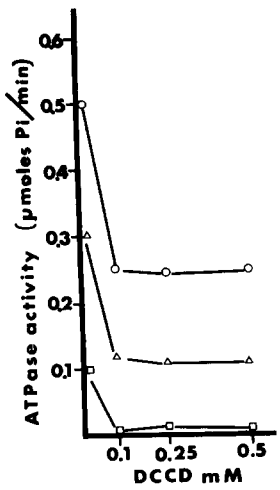

Fig. 1. Study of ATPase activity as a function of unlabelled DCCD and membrane protein concentrations. Membrane protein concentrations (mg/ml): o———o, 10.2; Δ———Δ, 6; □———□, 2.2.

Purification and labelling of the [^{14}C]DCCD-proteolipid

Crude proteolipid was obtained either from membranes with bound F_1, with a specific ATPase activity of 0.055 ± 15% unit/mg protein, or from soluble ATPase-depleted membranes with a residual specific activity of 0.017 ± 15% unit/mg protein. These values indicated a decrease of specific membrane ATPase activity near to 70% after F_1 solubilization. Crude proteolipid was extracted either with acidic or neutral chloroform/methanol. Each crude proteolipid sample obtained in these different conditions was purified by chromatography on methylated Sephadex G-50 as described in Material and Methods and the results were compared. In all cases the radioactivity and the

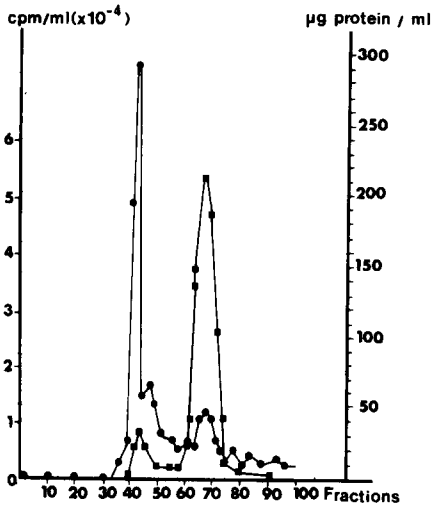

Fig. 2. Methylated Sephadex G-50 chromatography of a crude proteolipid obtained from 16 g (wet wt.) membrane pellet depleted from F_1. These membranes were suspended at a protein concentration of 2 mg/ml and labelled with 0.1 mM [^{14}C]DCCD (2.5 mCi/mmol). Fractions of 3.3. ml were collected. Protein (•———•) and radioactivity (▪———▪) were determined.

protein profiles were identical. 85% of the total radioactivity was eluted in fractions which correspond to the small protein peak (Fig. 2). In this figure fractions 60 to 70 have constant and optimal specific activity. When the labelling of the proteolipid was done with a membrane protein concentration of 12 mg/ml and 0.1 mM [^{14}C]DCCD for 16 h, a spec. act. of 40 nmol [^{14}C]DCCD/mg proteolipid was found. On the other hand, if the membrane protein concentration were about 2 mg/ml, the labelling ratio of proteolipid was 130–140 nmol [^{14}C]DCCD/mg proteolipid (Fig. 2). As a function of membrane protein concentration there was a correspondence between the amount of [^{14}C]DCCD bound and the ATPase inhibition. However, if the labelling of proteolipid was made with a membrane protein concentration of 1.5 mg/ml and 10 µM [^{14}C]DCCD (lowest inhibitor concentration necessary to attain maximum inhibition) only 30 nmol [^{14}C]DCCD/mg proteolipid were bound. Then, in this case no correspondence existed between ATPase inhibition and [^{14}C]DCCD bound and a 10-times higher concentration of [^{14}C]DCCD was necessary to obtain maximum [^{14}C]DCCD binding.

When crude proteolipid was purified by DEAE-cellulose, the ammonium acetate gradient eluted a radioactive protein peak between 0–10 mM and an unlabelled protein peak between 30–50 mM (data not shown) and the yield was reduced by this purification method. For the same crude proteolipid sample, only 10% of the amount of [^{14}C]DCCD-binding proteolipid eluted by methylated Sephadex G-50 was eluted in the ammonium acetate gradient.

Electrophoresis analysis

The radioactive peaks eluted from the methylated Sephadex G-50 column and the DEAE-cellulose col-

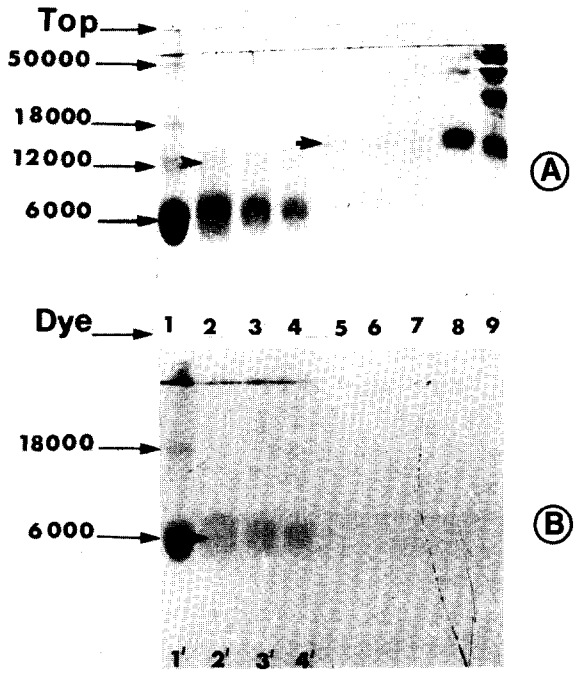

Fig. 3. Urea-SDS polyacrylamide slab gel electrophoresis and autoradiography. *B. subtilis* membranes at a protein concentration of 12 mg/ml containing F_1 were labelled with [^{14}C]-DCCD (4.5 mCi/mmol). Crude proteolipid extract was layered onto a methylated Sephadex column and onto a DEAE-cellulose column. (A) Stained gel. 1: 150 µg of the concentrated radioactive protein peak eluted from methylated Sephadex G-50 column. 2–7: polypeptides eluted from DEAE-cellulose by the ammonium acetate gradient. 2, 3, 4: 50, 40 and 30 µg, respectively, of the concentrated radioactive protein peak eluted between 0 and 10 mM. 5, 6, 7: 20, 15 and 10 µg, respectively, of the unlabelled protein peak eluted between 30–50 mM. 8: 30 µg cytochrome *c*; and 9: 2.5 mg of proteins of the calibration kit. The arrowheads indicate faintly staining bands. (B) Autoradiography of gel A exposed for 7 days. The arrowheads indicate faintly radioactive bands.

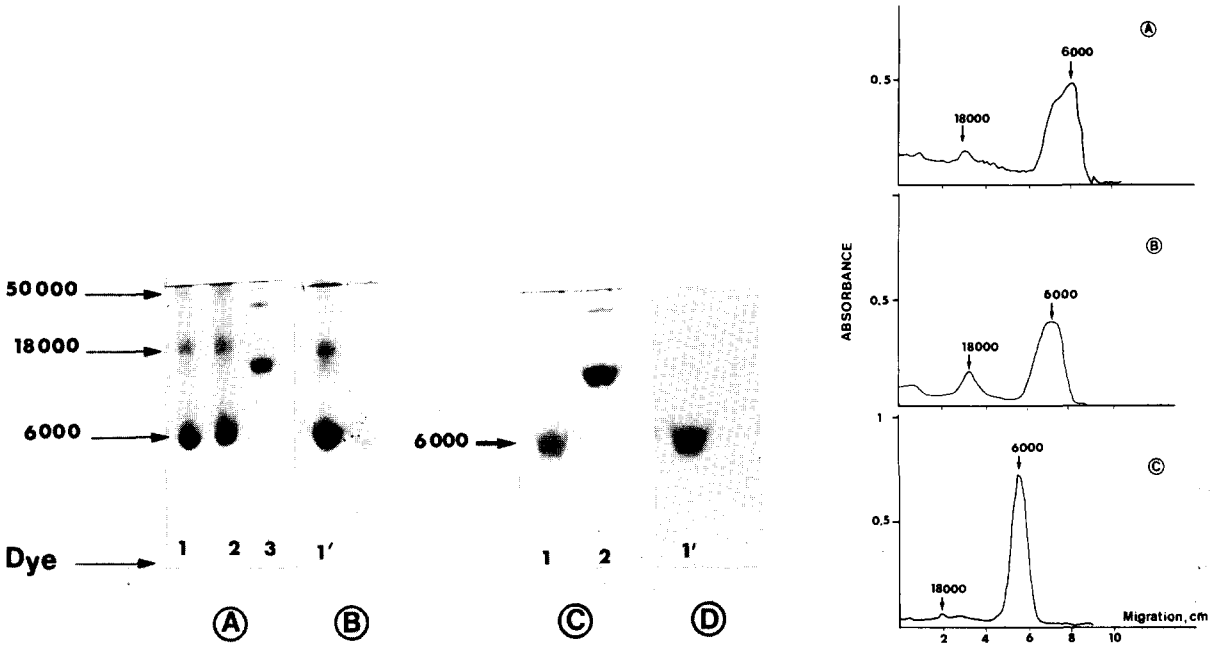

Fig. 4. Urea-SDS polyacrylamide slab gels electrophoresis and autoradiography of the fractions of the top of the radioactive protein peak eluted from methylated Sephadex G-50 column. (A) Stained gel. 1 : 50 μg proteolipid originating from membranes containing F_1 which at a protein concentration of 12 mg/ml were labelled with [^{14}C]DCCD (5 mCi/mmol); neutral extraction. 2: 50 μg proteolipid originating from unlabelled membranes containing F_1; acidic extraction. 3: 30 μg cytochrome *c*. (B) Autoradiography of gel A exposed for a month. (C) Stained gel 1: 50 μg proteolipid originating from F_1-depleted membranes which at a protein concentration of 4 mg/ml were labelled with [^{14}C]DCCD (2.5 mCi/mmol); acidic extraction. 2: cytochrome *c*. (D) autoradiography of gel C exposed for 7 days.

Fig. 5. Densitometric trace of the [^{14}C]DCCD-purified proteolipid run on a urea-SDS polyacrylamide slab gel. (A) 100 μg [^{14}C]DCCD (10 mCi/mM) proteolipid extracted from membranes containing F_1; acidic extraction. (B) 50 μg [^{14}C]DCCD (5 mCi/mmol) proteolipid extracted from membranes containing F_1, neutral extraction. (C) 50 μg [^{14}C]DCCD (2.5. mCi/mmol) proteolipid extracted from membranes depleted of F_1; acidic extraction.

umn were analyzed by urea-SDS polyacrylamide slab gel electrophoresis as described in Material and Methods.

When crude proteolipid was obtained from membranes with bound F_1, and purified by methylated Sephadex G-50 column three or four bands were observed (Fig. 3A1 and Fig. 4A1,2) of M_r 50000, 18000, 12000 and 6000. This last band was the major one. Autoradiography of the gels showed that the 6000 and 18000 polypeptides were labelled by the [^{14}C]DCCD (Fig. 3A1, B1′ and Fig. 4A1, B1′). About 60% of the total radioactivity was recovered in the 6000 band and between 15 and 25% in the 18000 band (Fig. 5A and B). The neutral or acidic conditions of the extraction caused no modification of the ratio and the number of these bands (Fig. 4A1 and 2). [^{14}C]DCCD binding proteolipid and untreated proteolipid have the same electrophoretic components (Fig. 4A1 and 2).

When crude proteolipid was obtained from membranes with bound F_1 and purified by DEAE-cellulose column chromatography, the radioactive protein peak showed only one radioactive band (with the same relative mobility than the 6000 polypeptide eluted by the methylated Sephadex G-50 column) and one faintly unlabelled band of M_r 12000 (Fig. 3A 2–4 and B 2′–4′). The unlabelled protein peak consisted of a polypeptide of M_r 14000 (Fig. 3A 5–7).

When crude proteolipid was obtained from F_1-depleted membranes and purified by methylated Sephadex G-50 chromatography we observed an

increase in the homogeneity and purity of the lowest molecular weight proteolipid fraction (Fig. 4C, D and Fig. 5, C) 80% of the total radioactivity was recovered in this fraction, whereas the M_r 18 000 fraction contained only 5%.

Discussion

The results concerning inhibition of membrane-bound ATPase activity and labelling DCCD-reactive proteolipid showed that, at the lowest inhibitor concentration (10 μM) necessary to reach maximum inhibition, only 0.18 mol [^{14}C]DCCD was bound by 1 mol *B. subtilis* DCCD-binding proteolipid (considering a minimal M_r of 6000, see below). But, with an excess of 100 μM DCCD, 0.84 mol [^{14}C]DCCD was bound by 1 mol of this proteolipid. Similar results were obtained by Sebald et al. [8] with *Neurospora crassa* DCCD-reactive proteolipid. Fillingame [11] reported that one-third of the total *E. coli* DCCD-reactive proteolipid was labelled by [^{14}C]DCCD under conditions which maximally inhibited ATPase activity. On the other hand, Sone et al. [13] reported that only 0.27 mol DCCD was sufficient to block H^+ conduction of 1 mol thermophylic bacterium DCCD-reactive proteolipid. These results could be explained by the following hypothesis which suggested that the DCCD-reactive proteolipid was organized in vivo as an oligomer (trimer or hexamer) and the modification of one of the three or six subunits of the oligomer was sufficient to block the enzymatic activity of the complex. Using higher DCCD concentrations, the whole DCCD-reactive proteolipid could be saturated in the case of *Neurospora crassa* mitochondria [8] and almost completely in the case of *B. subtilis*. We think, as already pointed out Sebald et al. [8], that when DCCD proteolipid binding sites are determined only under conditions which maximally inhibit ATPase activity, misleading values may be obtained.

By autoradiography, we have visualized two labelled polypeptides with apparent molecular weights of 18 000 and 6000, respectively. This result is surprising, because in *E. coli* [11,12] and in the thermophylic bacterium [13] only a single radioactive component, of apparent molecular weight of about 8000 and 6000, respectively, was extracted into chloroform/methanol and subsequently precipitated with diethylether. We believe, for many reasons, that the smaller labelled polypeptide of *B. subtilis* (M_r 6000) corresponds to the DCCD-binding proteolipid subunit of *E. coli* [11,12] and that of the thermophylic bacterium [13]. It has a molecular weight close to that of the DCCD-binding proteolipids of these bacteria. It was the major labelled polypeptide of the radioactive peak eluted by chromatography on methylated Sephadex G-50 column, when crude proteolipid was extracted from membranes containing F_1. If this crude proteolipid was purified on a DEAE-cellulose column as in the case of *E. coli* [11,12] only one labelled polypeptide, of M_r 6000, was eluted. However, it was the unique labelled polypeptide of the radioactive peak eluted by methylated Sephadex G-50 chromatography, when the crude proteolipid was extracted from F_1-depleted membranes.

Glaser et al. [22,23] obtained very similar results concerning the two labelled polypeptides. Thus, these authors detected, by autoradiography of the gels of F_0 from beef-heart mitochondrial DCCD-sensitive ATPase, two labelled components poorly stained with Coomassie blue, Their molecular weights have been estimated to be in the range of 4500–5000 and 16 000–18 000, respectively. These two proteins can easily be extracted from mitochondria with chloroform/methanol. Glaser et al. [22,23] pointed out that the two polypeptides probably represent monomeric and oligomeric forms of the [^{14}C]DCCD-binding protein. But if the 18 000 labelled component is an oligomeric form of the M_r 6000 band, it should be interesting to know why it was principally present when chloroform/methanol extraction was performed on *B. subtilis* membranes containing F_1.

This work has shown that different proteolipid subunits may be obtained, depending on the cytoplasmic membrane material and the purification procedure used, and that some parts of *B. subtilis* membrane proteolipid are more hydrophobic than others. These results will be taken into consideration during further studies on proton transport across artificial membranes and the role of each component remains to be determined for reconstitution of proton translocation activity.

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