Biochimica et Biophysica Acta, 775 (1984) 239–245
Elsevier

BBA 72235

TWO-DIMENSIONAL GEL ELECTROPHORESIS OF MEMBRANE PROTEINS USING ANIONIC AND CATIONIC DETERGENTS

APPLICATION TO THE STUDY OF MITOCHONDRIAL F_0-F_1-ATPase

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(Received January 19th, 1984)
(Revised manuscript received May 28th, 1984)

Key words: Polyacrylamide gel electrophoresis; Detergent; Cationic detergent; Anionic detergent; Membrane protein; F_0-F_1-ATPase; (Pig heart mitochondria)

Polyacrylamide gel electrophoresis in the presence of a cationic detergent, tetradecyltrimethylammonium bromide (TDAB) has been compared to electrophoresis in the presence of an anionic detergent, sodium dodecyl sulfate (SDS). Although, in both systems, the peptides generally migrated as a function of their molecular weight, the TDAB electrophoresis permitted us to obtain a much better resolution of several peptides of the mitochondrial F_0-F_1-ATPase, especially for the α and β subunits and for the oligomycin sensitivity conferring protein (OSCP). The differences between the two electrophoretic profiles have been used to devise a new technique of two-dimensional electrophoresis using successively anionic and cationic detergents. This method could be very useful in the case of membrane proteins, which are generally soluble only in the presence of powerful ionic detergents. It has been particularly successful in resolving the small peptides of the F_0-F_1-ATPase which were difficult to differentiate by other techniques in one- or two-dimensional polyacrylamide gel electrophoresis.

Introduction

The separation of membrane proteins in the presence of anionic detergent by SDS-polyacrylamide gel electrophoresis at neutral or alkaline pH has become a very popular technique [1–3]. More recently, several authors have reported the use of cationic detergents to depolymerize the proteins and perform gel electrophoresis under acidic conditions [4–5]. With this technique, methylation [6,7], esterification or phosphorylation [5] of proteins could be studied without cleavage of the bonds that can be labile in neutral or alkaline conditions.

To resolve complex mixtures of proteins, a two-dimensional system must be used. The general procedure of O'Farrell [8] has often been very efficient. This technique in which a separation by isoelectric focusing precedes the SDS electrophoresis has been applied to membrane proteins [9]. However, Mills and Freedman [10] recently dis-

Abbreviations: SDS, sodium dodecyl sulfate; TDAB, tetradecyltrimethylammonium bromide; Bis, *N*,*N*′-methylene(bis)-acrylamide; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylene diamine; ATPase, adenosine-5′-triphosphatase (EC 3.6.1.3); F_1-ATPase, pig heart mitochondrial ATPase prepared according to the procedure of Penin et al. [17]; OSCP, oligomycin sensitivity conferring protein [27]; IF_1, natural protein inhibitor of mitochondrial F_1-ATPase [25].

cussed the fact that the electrofocusing of membrane proteins raises major difficulties associated with the hydrophobicity of integral membrane proteins. Besides, in isoelectric focusing, a single protein can give several spots that may be scattered over a large area of the gel. This has been observed for example with surface membrane proteins of lymphocytes [11]. The two-dimensional maps obtained are sometimes so complex that computer analysis may become necessary [12]. To overcome this problem, a few authors have used two types of SDS electrophoresis in the presence of different buffers at neutral or alkaline pH values [13,14]. The separation of some proteins was improved in these two-dimensional SDS gel systems but only a few peptides could deviate from the diagonal and the resolution of low molecular weight peptides remained weak. Other two-dimensional gel electrophoresis systems using acidic urea in the first dimension and SDS in the second dimension [15] which have been widely used for ribosomal proteins, have been disappointing for the study of membrane proteins.

We propose here a new two-dimensional polyacrylamide gel electrophoresis technique using SDS, an anionic detergent in the first dimension and TDAB, a cationic detergent in the second dimension. The first dimension is run between neutral and alkaline pH according to Laemmli [1] while the second dimension is made at an acidic pH. These widely different conditions have permitted a very good separation of the peptides of the F_0-F_1-ATPase, especially, in the case of low molecular weight peptides which have always been difficult to resolve.

Materials and Methods

Materials

Acrylamide was purchased from Serva, bisacrylamide from Eastman Kodak, Coomassie blue R and TDAB from Sigma, mixtures of proteins of known molecular weight from Bio-Rad (14000–92500) or from BDH (2512–16949). All other reagents were of the highest purity commercially available. Electrophoreses were run in a Bio-Rad Protean double slab cell equipped with $160 \times 120 \times 1.5$ mm plates.

Methods

Previously described procedures were used to prepare pig heart mitochondria [16], F_1-ATPase [17] and the F_0-F_1-ATPase [18]. The protein contents were estimated by the technique of Lowry et al. [19], modified as in Ref. 20.

SDS-polyacrylamide gel electrophoresis. SDS-PAGE was performed according to Laemmli [1] by using 15% acrylamide in the separating gel and 4% acrylamide in the stacking gel. The samples were prepared by mixing 50 μl of 0.125 M Tris-HCl (pH 6.8) containing 50 to 350 μg of protein with urea (60 mg), β-mercaptoethanol (5 μl), SDS (20 μl of a 10% solution) and pyronine Y (2 μl of a 0.2% solution). The samples were heated for 5 min at 90°C. The electrophoresis was run at 25 mA per slab for 1 h and then 40 mA per slab until the tracking dye reached the bottom of the gel (about 3 h).

TDAB polyacrylamide gel electrophoresis. TDAB-PAGE made in the presence of cationic detergent at acidic pH as described by Amory et al. [5] was slightly modified: the separating gel (12 cm high) was prepared by mixing acrylamide (7.5 g), Bis (190 mg) and ascorbic acid (40 mg) with 25 ml of 0.2 M sodium phosphate buffer (pH 2.0) and 380 μl of freshly prepared 0.03% $FeSO_4$ ($\cdot 7H_2O$) (w/v) in a final volume of 49.2 ml. This solution was filtered through a Whatman GF/C glass fibre paper and mixed under vacuum for 10 min; then 0.5 ml of a 10% TDAB solution (w/v) was added.

Polymerization was initiated with 0.3 ml freshly diluted 0.3% H_2O_2 (v/v). This solution was quickly poured to prepare two gels. Isobutanol saturated with water was layered on the top of each gel in order to obtain a plane upper surface. After polymerisation (about 1 h), isobutanol was removed. The gel surface was carefully dried with a filter paper. The stacking gel solution was made by sequential addition of acrylamide (1 g), bisacrylamide (35 mg), ascorbic acid (20 mg), 15 ml of 0.2 M sodium phosphate buffer (pH 4.0) and 24.5 ml of distilled water. After removing gas under vacuum, the solution was mixed with 0.25 ml of 10% TDAB (w/v) and 0.35 ml of freshly diluted 0.3% H_2O_2 (v/v). The solution was poured onto the separating gel and the combs were settled, leaving 3 cm between the separating gel and the teeth of the comb. The electrode buffer solu-

tion was composed of 85 mM glycine and 0.125% (w/v) TDAB adjusted to pH 3.0 with H_3PO_4. The samples were prepared by mixing 25 μl of 50 mM sodium phosphate buffer (pH 4.0) containing 50 to 350 μg of protein, urea (40 mg) and β-mercaptoethanol (5 μl), with 60 μl of a solution of 10% TDAB (w/v) and Bromophenol blue (2 μl of a 0.1% solution), as a tracking dye. The solution was heated for 5 min at 90°C. This heating step could be replaced by an incubation at room temperature for about 2 h in the case of soluble proteins.

The gel was run at 25 mA for 1 h and at 50 mA until the tracking dye reached 1 cm from the bottom of the gel (about 4 h).

Staining and destaining. Coomassie blue R (250 mg) was dissolved by adding first 10 ml of methanol and then 250 ml of isopropanol. This solution was mixed with 100 ml glacial acetic acid and diluted with distilled water to a final volume of one liter. Both types of gel were stained overnight with this solution and destained with 10% (v/v) acetic acid [3].

Results

Comparison between the separation of proteins by anionic or cationic polyacrylamide gel electrophoresis in one dimension

Fig. 1 compares the results obtained in the separation of soluble or membrane proteins by electrophoresis in the presence of either an anionic detergent, SDS (Fig. 1A) or a cationic detergent, TDAB (Fig. 1B). In the presence of SDS the migration of marker proteins of well established molecular weight (M_r) is correlated with their M_r [2,21]. There is only a small difference between myoglobin fragment I + II and lysozyme which

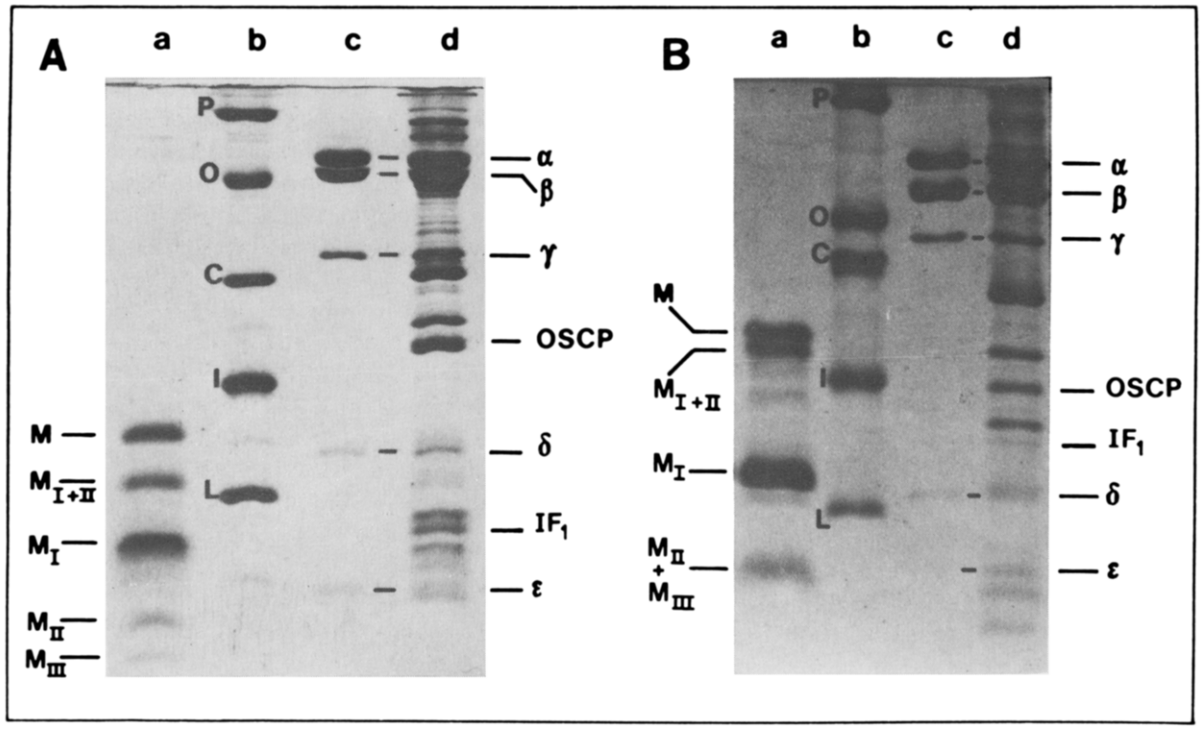

Fig. 1. Polyacrylamide gel electrophoresis in the presence of anionic or cationic deter. (A) SDS; (B) TDAB. Lane a: low M_r marker proteins (15 μg); myoglobin and its cyanogen bromide fragments: M (16949), M_{I+II} (14404), M_I (8159), M_{II} (6214), M_{III} (2512). Lane b: high M_r marker proteins (20 μg); P, phosphorylase *b* (92500); O, ovalbumin (45000); C, carbonic anhydrase (31000); I, soybean trypsin inhibitor (21500); L, lysozyme (14400). Lane c: F_1-ATPase from pig heart mitochondria (12 μg): from top to bottom, subunits α, β, γ, δ and ε. Lane d: F_0-F_1-ATPase (30 μg). IF_1 and OSCP positions were determined by comigration with peptides purified according to Refs. 25 and 30, respectively. Experimental conditions as described in Materials and Methods.

TABLE I

MOLECULAR WEIGHT OF THE SUBUNITS OF F_1-ATPase, OSCP AND IF_1

The apparent M_r of some subunits of the ATPase-ATP synthase complex has been estimated by using the gel presented in Fig. 1. Calibration curves have been constructed by plotting the log of M_r of protein markers as a function of their relative mobility (lanes a and b).

Subunits of F_1-ATPase	Apparent M_r by electrophoresis		Known M_r (Ref.)
	SDS	TDAB	
α	59000	64500	53300 [b] [32]
β	51000	52500	51318 [c] [33]
γ	33000	37500	33160 [b] [32]
δ	16400	15000	16100 [b] [32]
ε	7000	#6000 [a]	5850 [b] [32]
OSCP	24000	21500	20967 [c] [34]
IF_1	10000	18000	9578 [c] [26]

[a] In the case of TDAB the M_r of the ε-subunit has been estimated using the myoglobin fragments as protein markers (lane a) while, for the M_r of the other peptides, the calibration curve used was that of lane b (high M_r protein markers, see the text).

[b] M_r estimated from amino-acid composition of the bovine heart enzyme [32].

[c] M_r determined by amino acid sequence of the bovine heart peptides [33,26].

have a same M_r of 14400 [22,23]. In the presence of TDAB, the migration of proteins of high M_r (lane b, Fig. 1B) is approximately proportional to the M_r while anomalous migrations appeared very clearly in the case of low M_r peptides. For example, myoglobin and myoglobin fragment (I + II) which respectively have a M_r of 16949 and 14400 [22] migrate with a lower relative mobility than the soybean trypsin inhibitor which has a molecular weight of 21500 [24]. In the same way, the myoglobin fragment M_I with a real M_r of 8159 seems to be a larger protein than lysozyme which has a M_r of 14400 [23].

Several differences are also apparent in the separation of the peptides of the F_0-F_1-ATPase in SDS or TDAB electrophoresis. The α and β subunits of the F_1-ATPase have a much better separation with TDAB (lane c, Fig. 1B) than with SDS (lane c, Fig. 1A). The apparent M_r of the subunits of F_1-ATPase have been estimated in the two different gel systems by plotting the log of the M_r of marker proteins as a function of the migration distance [21]. The results are presented in Table I. Although the electrophoretic profiles in SDS or TDAB were not alike, the differences observed in M_r were not very large for most peptides. This shows that the TDAB gel system can be used as the SDS gel system to estimate the apparent M_r of peptides as reported previously [5]. However, some peptides clearly deviate from this rule. For example, the natural ATPase inhibitor, IF_1 [25] migrates at a position which, in TDAB, gives a molecular weight almost twice the value established by amino acid sequence analysis [26].

An advantage of the TDAB system, in the case of the F_0-F_1-ATPase is the very clear separation of the peptide named OSCP [27] from its two neighboring peptides. The identity of OSCP in the TDAB-gel system has been verified by comigration with purified OSCP (not shown).

Another difference between the SDS and the TDAB system can be seen in the area of the low M_r peptides. Below the δ-subunit of F_1-ATPase, the resolution between the various peptides is always difficult. The ε-subunit of F_1-ATPase appears to be one of the smallest peptides in the SDS-gel system (lane c, Fig. 1A) while it is located about half way between the δ-subunit and the fastest moving peptide in the case of the TDAB-gel system (lane c, d, Fig. 1B). These differences have been very useful in improving the separation of the small peptides in two dimensions (see below).

It should be mentioned that a pH of 2.0 in the depolymerisation of F_1-ATPase, as used in the technique of Amory et al. [5] should be avoided because it induced a cleavage of some peptides, in particular the β-subunit of F_1-ATPase (not shown). This acidic cleavage was not observed at the pH of 4.0 used here (Fig. 1B, lane c). In addition the presence of urea was necessary to improve the depolymerisation of mitochondrial membrane proteins.

Two-dimensional gel electrophoresis using anionic and cationic detergents

Fig. 2 shows that the separation of the small peptides was greatly improved when the F_0-F_1-ATPase was resolved in a first dimension by SDS electrophoresis and in a second dimension by

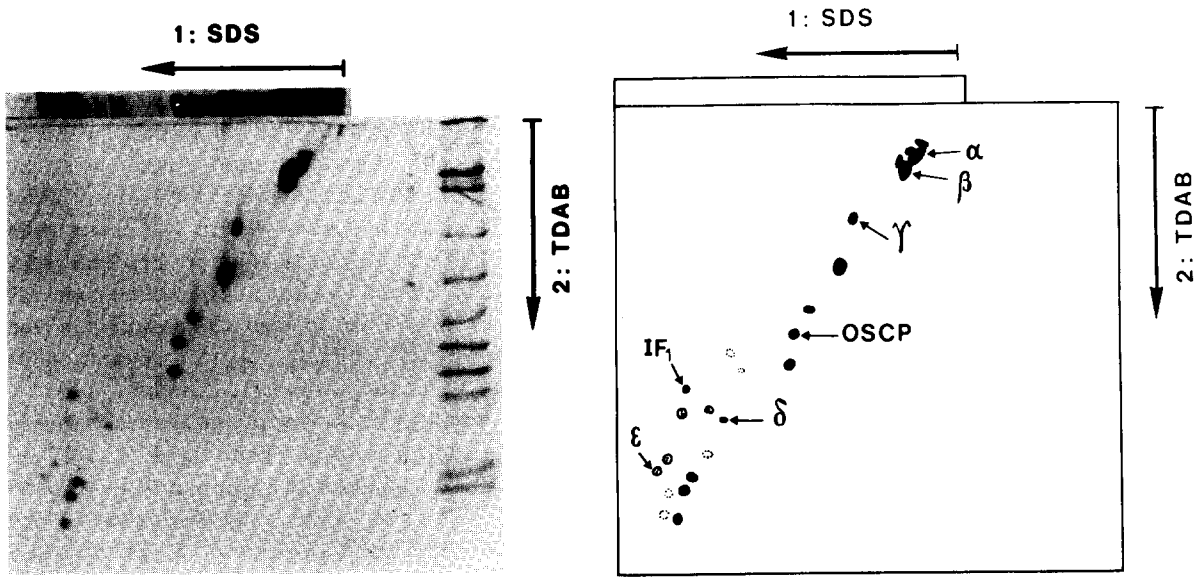

Fig. 2. (A, left) Two-dimensional gel electrophoresis pattern of F_0-F_1-ATPase using SDS electrophoresis in the first dimension and TDAB electrophoresis in the second dimension. The strip of gel run in the first dimension contained about 50 μg of proteins. The lateral well contained 30 μg of F_0-F_1-ATPase depolymerized in the presence of TDAB and urea. The detailed procedures are described in the text. (B, right) Identification of peptides of F_0-F_1-ATPase. F_1 purified as in Ref. 17, IF_1 purified as in Ref. 25, OSCP purified as in Ref. 30 were iodinated as in Ref. 29 according to the procedure of Fracker and Speck [31]. After comigration of the iodinated proteins with the F_0-F_1-ATPase as in (A), the gels were stained and dried. The position of the iodinated protein was determined by autoradoigraphy on Kodak DEF films.

TDAB electrophoresis. The spots of peptides were very clearly separated.

To obtain this clear separation, several precautions have been taken. In the first dimension, a slab gel was prepared in the presence of SDS. A sample of F_0-F_1-ATPase depolymerized as described in Materials and Methods was layered in a 3-cm wide well. After migration, the slab was shaken in 500 ml isopropanol/water (1 : 3, v/v). This solvent was changed 1 h later then 2 h later and finally the slab was left overnight in the bath to remove SDS from the gel. A 5 mm wide strip of gel cut out in the middle of the well was equilibrated in 10 ml of 3% TDAB, 10% glycerol, 2% β-mercaptoethanol and 50 mM sodium phosphate buffer (pH 4.0) for 2 h, with two changes of buffer. The gel should remain clear. If a white precipitate appears, it means that the removal of SDS is not sufficient because SDS precipitates in the presence of TDAB. The overnight shaking described above is normally quite adequate.

The second dimension slab gel (2 mm thick) was prepared as described in Materials and Methods. A 1 cm wide well was formed on one edge of the stacking gel to insert a sample of proteins directly depolymerized in TDAB, as a control. Another 10 cm wide well was formed to insert the strip of gel coming from the first dimension and equilibrated in the TDAB buffer. This gel was maintained in position with 1% agarose melted in the TDAB electrophoretic buffer. The electrophoresis was run for 1 h at 50 mA and 3 h at 80 mA per slab. After each step, a strip of gel prepared in the first dimension was cut out, stained and destained. These controls allowed us to check that the various treatments did not significantly modify the electrophoretic pattern obtained after the first dimension. The intensity of only one band in the low M_r area seemed to decrease after removal of SDS by 25% isopropanol.

If the TDAB electrophoresis was performed in the first dimension instead of SDS electrophoresis, the removal of TDAB by 25% isopropanol induced the solubilizatoin of most of the small peptides. Therefore, it is mandatory to perform the first electrophoresis in the presence of SDS and the

second one in the presence of TDAB. Another advantage of this order is that membrane proteins are easier to completely depolymerize with SDS than with TDAB.

Discussion

In this study, we have first compared the efficiency of anionic and cationic detergent polyacrylamide gel electrophoresis systems for the separation of the peptides of the F_0-F_1-ATPase of mitochondrial membrane. The TDAB electrophoresis as well as the SDS electrophoresis can be used to approximately estimate the molecular weight of the peptides. However, the TDAB electrophoresis must be used with caution since some proteins have an anomalous migration as shown in this report as well as in others [4,5,7]. These anomalous migrations may be related to the acidic or basic nature of the protein which may influence the formation of the detergent-protein complex and/or the migration of this complex [4]. The hydrophobic character of the protein may also play a role in the binding of the detergent to the protein. It is more likely that both charges and hydrophobicity of the proteins are involved in the formation of the protein-detergent micelles. These micelles must have, for some proteins, a larger size than that expected from their molecular weight if the size of the micelle was only correlated to the size of the protein.

The TDAB electrophoresis has been very useful in increasing the separation of some peptides that were difficult to resolve in the SDS electrophoresis. For example, the OSCP could be clearly separated from its two neighboring peptides; this separation was very difficult with SDS (see Ref. 28). In the same way, the α-subunit was more clearly separated from the β-subunit of F_1-ATPase. This has been very useful in identifying the specificity of monoclonal antibodies raised against the F_1-ATPase [29] and in localizing nucleotide binding sites on F_1-ATPase covalently labeled with a radioactive nucleotide analogue (Fellous G., Godinot, C., Baubichon, H., Di Pietro, A. and Gautheron, D.C., Biochemistry, in the press). In the latter case, the TDAB electrophoresis also had the advantage of avoiding the cleavage of the ester bond present in the nucleotide analogue; this cleavage occurs at alkaline pH.

In the second part of this study we have devised a new technique of two dimensional gel electrophoresis well-suited to the separation of membrane peptides. The technique turned out to be especially efficient in the case of the F_0-F_1-ATPase peptides, whose molecular weight was lower than about 20000. In this area, the peptides were very well differentiated while in one-dimension as shown in Fig. 1 or in two dimensions, in the presence of SDS only [13,14], the separation of small peptides was particularly difficult.

The only drawback of applying the technique to membrane proteins other than the F_0-F_1-ATPase could be that it includes a washing of gel with 25% isopropanol which could solubilize some of the peptides. Although Fairbanks et al. [3] reported that peptides of erythrocyte membranes were not solubilized by isopropanol, the lack of solubility in isopropanol must be checked by comparing the possible decrease in the protein bands after isopropanol treatment on the first dimension gels. In addition, it is possible that more powerful cationic detergents should be found to improve the solubilization of some very hydrophobic proteins. For example, the benzyldimethyl-*n*-hexadecylammonium chloride was proved to be superior to dissolve blood platelet proteins and may be used instead of TDAB in the case of other membrane proteins [7]. However, for mitochondrial membranes, the conditions described in this report are convenient.

The two-dimensional polyacrylamide gel electrophoresis in anionic and cationic detergent has several advantages over the isolectric focusing followed by SDS electrophoresis for membrane proteins. The dissociation and denaturation of proteins is easily completed because it is made in the presence of urea and detergents while no single method could be used to solubilize the whole range of microsomal proteins for example, as reported by Mills and Freedman in the absence of powerful ionic detergents [10]. The technique is simple to perform with low cost equipment and reagents as compared to isoelectric focusing where ampholines and sophisticated power supplies are needed. The identification of the peptides are greatly facilitated because the proteins generally migrate as a function of their molecular weight

except for some proteins which exhibit anomalous migrations. Moreover, each peptide gives a unique spot while in isoelectric focusing a single peptide can give a large number of spots that can be scattered on the whole width of gel. In conclusion, the two-dimensional anionic-cationic detergents electrophoresis which has been very efficient to resolve the peptides of the mitochondrial F_0-F_1-ATPase complex could be a general technique applicable to other membrane proteins.

Acknowledgments

The authors wish to thank Dr. A. Di Pietro for stimulating discussions. Thanks are also due to O. Lesbros for his help in preliminary experiments, to C. Van Herrewège for the art work, N. Coudert for the typing and R. Lambrech for improving the english version of the manuscript. This work was supported by the CNRS (LP 5421, ATP No. 8154).

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