Biochimica et Biophysica Acta 813 (1985) 230–242
Elsevier

BBA 72480

The 35 kDa DCCD-binding protein from pig heart mitochondria is the mitochondrial porin

Vito De Pinto [a], Massimo Tommasino [a], Roland Benz [b] and Ferdinando Palmieri [a,*]

[a] *Department of Pharmaco-Biology, Laboratory of Biochemistry, University of Bari and CNR Unit for the Study of Mitochondria and Bioenergetics, Via Amendola 165/A, 70126 Bari (Italy) and* [b] *Fakultat für Biologie, Universität Konstanz, D-7750 Konstanz (F.R.G.)*

(Received October 26th, 1984)

Key words: Porin; DCCD-binding protein; Mitochondrial membrane; Membrane reconstitution; (Pig heart)

The protein which can be labelled by low concentrations of dicyclohexylcarbodiimide in the M_r region of 30000–35000 has been purified from pig heart mitochondria with a high yield and as a single band of apparent M_r 35000 in dodecyl sulphate-containing gels. The protein is not identical with the phosphate carrier as suggested before, since the two proteins behave differently during isolation. Incorporation of the isolated 35 kDa dicyclohexylcarbodiimide-binding protein into lipid bilayer membranes causes an increase of the membrane conductance in definite steps, due to the formation of pores. The specific pore-forming activity increases during the purification procedure. The single pore conductance is about 4.0 nS, suggesting a diameter of 1.7 nm of the open pore. The pore conductance is dependent on the voltage across the membrane. Anion permeability of the pore is higher than cation permeability. These properties are similar to those described for isolated mitochondrial and bacterial porins. It is concluded that the 35 kDa dicyclohexylcarbodiimide-binding protein from pig heart mitochondria is identical with porin from outer mitochondrial membrane.

Introduction

Low concentrations of dicyclohexylcarbodiimide (DCCD) inhibit oxidative phosphorylation by reacting covalently with a hydrophobic polypeptide of the H^+-ATPase complex, the proteolipid or subunit c, first isolated as [^{14}C]DCCD-labelled polypeptide [1–5]. In several laboratories, it was found that when mitochondria are incubated with low concentrations of [^{14}C]DCCD and subsequently subjected to SDS gel electrophoresis, three protein bands appear to be labelled with radioactivity, i.e., the DCCD-binding component of the F_0 part of the H^+-ATPase, its dimer, and a protein of M_r about 33000 [3,6–8]. Initially, the last protein was considered to be an aggregate of the DCCD-binding proteolipid [6,7]. Subsequently, however, it was shown not to belong to the ATPase, because it is not extracted by chloroform/methanol and it is not present in the immunoprecipitate H^+-ATPase complex [8]. More recently, it was suggested that the DCCD-binding protein of about 33 kDa is identical with the phosphate carrier of the inner mitochondrial membrane, on the basis of a similar electrophoretic mobility and behaviour in the isolation [9]. Furthermore, by applying a higher resolution SDS gel

* To whom correspondence should be addressed.

Abbreviations: DCCD, dicyclohexylcarbodiimide; EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; SDS, sodium dodecyl sulphate.

electrophoretic system, the apparent molecular weight of the protein, which can be labelled by low concentrations of DCCD and does not belong to the ATPase, was more accurately determined to be 35 kDa in pig heart mitochondria [10]. A prerequisite in the elucidation of the function of the 35 kDa DCCD-binding protein is its isolation.

In this paper, we have purified the 35 kDa DCCD-binding protein from pig heart mitochondria and have functionally identified it with porin. Evidence is given that the 35 kDa DCCD-binding protein of mitochondria is not identical with the phosphate carrier. On the contrary, the purified protein, reconstituted in artificial lipid bilayer membranes, forms pores having common features with those produced by the mitochondrial porin from rat liver [11] and *Neurospora crassa* [12].

The outer mitochondrial membrane is permeable to solutes of small molecular weight but not of large molecular weight [13]. Responsible for these permeability properties is the presence of a polypeptide of M_r around 30000 [11,14]. This protein was named mitochondrial porin in analogy to the bacterial porins and in agreement with the endosymbiotic theory [14,15]. The outer mitochondrial membrane contains only one type of porin [11]. Therefore, ADP-ATP exchange with the cytosol across the outer membrane is only possible through the pore formed by the mitochondrial porin. Furthermore, it has been shown that the hexokinase-binding protein present in the outer mitochondrial membrane is identical with the mitochondrial porin [16,17]. This finding indicates that the mitochondrial porin could play an important role in the metabolism of the cell and the mitochondria.

Materials and Methods

Materials. Affi-gel 501 (an organomercurial agarose gel), Dowex AG 1-X8 (100–200 mesh) and hydroxyapatite (Bio-Gel HTP) were obtained from Bio-Rad; [^{14}C]DCCD from CEA, France; [^{32}P]phosphoric acid from Amersham International (Amersham, U.K.); ENHANCE from New England Nuclear; acrylamide, *N*,*N*′-methylenebisacrylamide, SDS, Triton X-100 and *N*-ethylmaleimide from Serva; cardiolipin from Serdary; egg yolk phospholipids (L-α-phosphatidylcholine, type X-E), asolectin (L-α-phosphatidyl type II-S from soybean) and Triton X-114 from Sigma; diphytanoylphosphatidylcholine from Avanti Biochemicals Birmingham, AL; *n*-decane from Fluka (Buchs, Switzerland, purum). Oxidized cholesterol was prepared as described earlier [18] from a 4% (w/v) solution of cholesterol (Eastman-Kodak) in *n*-octane.

Incubation with radioactive DCCD. Pig heart mitochondria prepared according to Ref. 19 were incubated (5 mg protein/ml) with [^{14}C]DCCD (2 nmol/mg protein) for 16–20 h at 0°C in a buffer comprising of 0.25 M sucrose/1 mM EDTA/10 mM Tris-HCl (pH 7.4). [^{14}C]DCCD-labelled mitochondria were washed twice and suspended in the same medium.

Purification of the 35 kDa DCCD-binding protein. Pig heart mitochondria (110–130 mg protein) were solubilized with 3% Triton X-100/20 mM KH_2PO_4 (pH 6.5)/20 mM KCl/1 mM EDTA at a protein concentration of 10 mg/ml. Where indicated, the solubilization buffer contained Triton X-114 instead of Triton X-100 and/or cardiolipin (2 mg/ml). After 20 min incubation at 0°C, the unsolubilized material was spun down at 147000 $\times g$ for 30 min. 600 μl of supernatant (Triton extract) were applied on hydroxyapatite columns (Pasteur pipettes filled with 600 mg of dry material) and the elution was performed with the solubilization buffer. The first 500 μl eluted from several columns were pooled and applied (1.5–2.5 mg protein) on an Affi-Gel 501 column (0.7 × 8 cm) equilibrated with the solubilization buffer. The unretarded proteins from the Affi-Gel column were pooled, applied (600 μl) on hydroxyapatite/celite (1 : 1, w/v) columns (Pasteur pipettes filled with 600 mg of dry material) and eluted with the solubilization buffer. The first 500 μl were collected.

SDS gel electrophoresis. Polyacrylamide slab gel electrophoresis of acetone-precipitated samples was performed in the presence of 0.1% SDS according to Laemmli [20] with the following modifications: the separation gel contained a linear gradient of acrylamide from 14 to 20% and 15% sucrose (acrylamide/*N*,*N*′-methylenebisacrylamide, 49 : 1). Alternatively, the separation gel contained 17.5% acrylamide and an acrylamide/bisacrylamide ratio of 150. Both systems give a high resolution of polypeptides of M_r close to 30000 [21]. Where

indicated, the electrophoretic systems generally applied for the resolution of the cytochrome b-c_1 complex [22] and of the cytochrome c oxidase complex [23] were also used. Staining was performed with the silver nitrate method [24]. The molecular weights were determined with the help of Pharmacia low-molecular-weight markers. Fluorographies were performed making use of ENHANCE and following the producer's instructions; screens from Du Pont and Kodak X-OMAT S films were used. Gel slices were digested and counted according to Ref. 25.

Assay of reconstituted phosphate transport. It was performed essentially as described previously [21,26]. Liposomes were prepared by sonicating 80% egg yolk phospholipids and 20% mitochondrial phospholipids in 50 mM KCl/10 mM KH_2PO_4/20 mM Hepes/1 mM EDTA (pH 6.5). After incorporation of the protein samples (50 μl) into liposomes by the freeze-thaw-sonication procedure, the assay was started by the addition of 10 μl 10 mM [^{32}P]phosphate (100 000–160 000 counts/min) and terminated with 10 μl N-ethylmaleimide (final concentration 2 mM). The external phosphate was removed by applying the samples to Dowex AG 1-X8 columns (0.5×4 cm, chloride-form, equilibrated with 170 mM sucrose) and the radioactivity of the eluted proteoliposomes was counted.

Incorporation of the 35 kDa DCCD-binding protein into planar bilayers and conductance measurements. Artificial lipid bilayer membranes were obtained as described previously [18] from a 1–2% (w/v) solution of the indicated lipids in n-decane. The cell used for bilayer formation was made of Teflon. The circular hole in the wall separating the two aqueous compartments had an area of either 2 mm^2 (for the macroscopic conductance measurements) or 0.1 mm^2 (for the single-channel experiments). The aqueous solutions were used unbuffered with a pH of 6. The protein was added from stock solutions to the aqueous salt solutions either immediately before membrane formation or after the membrane had completely turned black in order to prevent protein inactivation. Calomel electrodes with salt bridges were inserted into the aqueous compartments on both sides of the membrane. The macroscopic conductance measurements were performed with a Keithley 610 C electrometer. A Keithley 427 current amplifier was used for the single-channel experiments. The amplified signal was monitored with a Tektronix 5115 storage oscilloscope and recorded with a strip-chart recorder. Zero-current membrane potentials were measured with a Keithley 610 C electrometer 5–10 min after the application of a salt gradient across the membranes, as described earlier [27].

Amino acid analysis. The protein was precipitated by 90% cold acetone. The precipitate was washed twice with aqueous acetone and lyophilized. Samples were hydrolyzed for 24 h in 6 M HCl at 110°C and analyzed on the BIOTRONIK Amino Acid analyzer LC 5000. Cysteine was determined as cysteic acid according to Ref. 28.

Other methods. Protein was determined by the Lowry method modified for the presence of Triton [29]. When 2-mercaptoethanol was present, the method was adapted by using iodoacetic acid [30]. To measure [^{14}C]DCCD binding, samples were precipitated with cold acetone (9 : 1). The pellets were suspended in 0.5 ml 5% trichloroacetic acid, centrifuged again and solubilized in SDS. The radioactivity was counted by liquid scintillation.

Results

Identification of the 35 kDa DCCD-binding protein in the hydroxyapatite pass-through

High-resolution gradient SDS gel electrophoresis has revealed that the pass-through of hydroxyapatite obtained from Triton X-114 solubilized mitochondria contains five protein bands in the M_r region of 30 000–36 000 [21]. It has also been shown that, among these proteins, band 5 corresponds to the ADP/ATP carrier and band 3 is (or comprises) the phosphate carrier [21].

In the experiment of Fig. 1, [^{14}C]DCCD-labelled pig heart mitochondria solubilized with Triton X-100 were applied to hydroxyapatite and the pass-through was analyzed by SDS gel electrophoresis. Fig. 1 shows that the five protein bands present in the hydroxyapatite pass-through are differently distributed in the various fractions. The first fraction contains bands 2–5, whereas band 1 appears only in later fractions. The comparison between silver staining (Fig. 1a) and fluorography (Fig. 1b) of the various protein bands present in the hy-

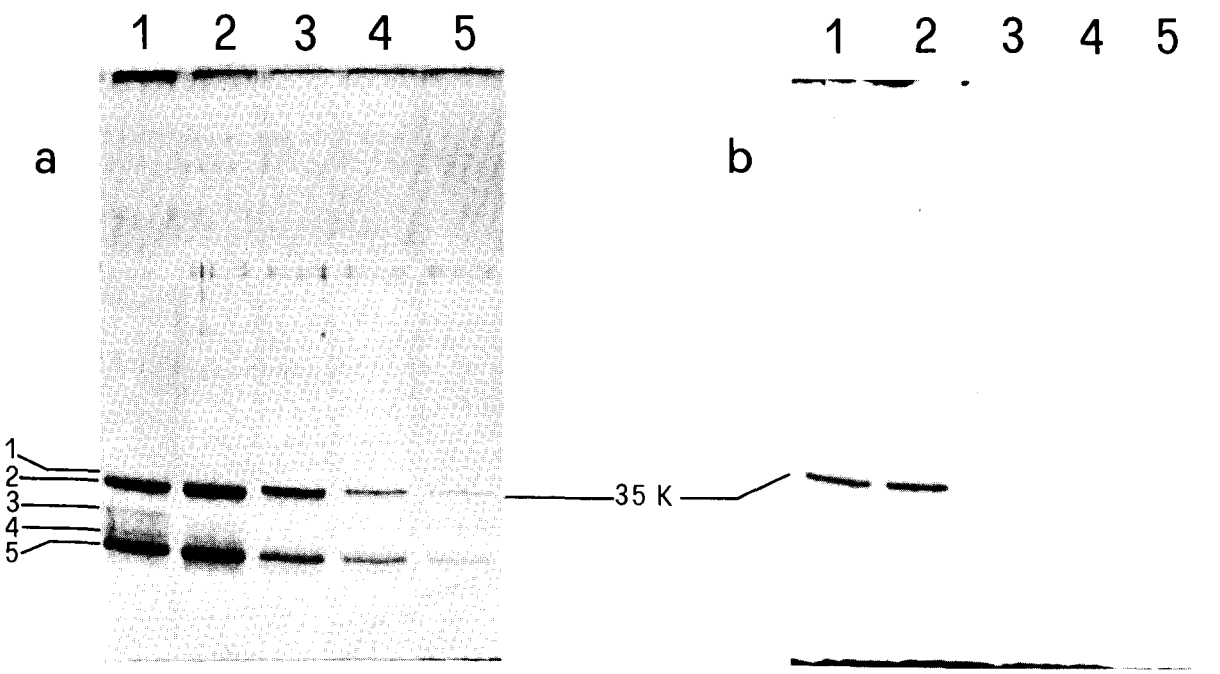


Fig. 1. Identification of the 35 kDa DCCD-binding protein in the hydroxyapatite pass-through of [^{14}C]DCCD labelled and Triton X-100 solubilized pig heart mitochondria. (a) Staining and (b) fluorography of the SDS gel electrophoresis of fractions 1–5 of the hydroxyapatite pass-through.

droxyapatite pass-through demonstrates that the DCCD labelling is only associated to band 2 of M_r 35 000. Band 1 is definitely not labelled by [^{14}C]DCCD since the radioactivity is decreased in the third fraction, which contains more band 1 than band 2, and is absent in the fourth and fifth fractions of the pass-through which contain band 1 but not band 2. Furthermore, bands 3–5 are clearly not labelled.

In further experiments using [^{14}C]DCCD-labelled mitochondria (not shown), we found that with both Triton X-100 and Triton X-114, band 2 (i.e., the 35 kDa DCCD-binding protein) largely exceeds the amount of band 3 in the first fractions (0.5 ml) of the hydroxyapatite pass-throughs without added cardiolipin (cf. lane 1 of Fig. 1). When, on the other hand, cardiolipin was added to the solubilization buffer, the hydroxyapatite pass-throughs with both Triton X-100 and X-114 show an inverse situation: band 3 (i.e., the phosphate carrier) is increased and band 2 is drastically diminished. The amount of labelling, which is only associated with band 2 as demonstrated by fluorography, is much more pronounced in the hydroxyapatite pass-throughs obtained in the absence than in the presence of cardiolipin. These results indicate that the 35 kDa DCCD-binding protein (i.e., band 2) and the phosphate carrier have different properties, since cardiolipin causes elution of band 3 (see Ref. 21), whereas it prevents that of band 2. The striking difference between the 35 kDa DCCD-binding protein and the phosphate carrier as regards elution from hydroxyapatite is directly demonstrated by measurements of the reconstitutable phosphate transport activity and the DCCD radioactivity in the hydroxyapatite eluates. As reported in Table I, the reconstituted phosphate transport activity of the hydroxyapatite pass-throughs is approx. 4-times higher with added cardiolipin. In contrast to this, the [^{14}C]DCCD radioactivity associated with band 2 is higher without added cardiolipin.

Purification of the 35 kDa DCCD-binding protein

The 35 kDa DCCD-binding protein, present in the first fraction of the hydroxyapatite pass-

TABLE I

DIFFERENT ELUTION OF THE 35 kDa DCCD-BINDING PROTEIN AND THE PHOSPHATE CARRIER FROM HYDROXYAPATITE

Triton X-114 or Triton X-100 solubilized mitochondria, in the presence or absence of 2 mg/ml DPG, were applied on HTP columns. The first 500 μl of the pass-throughs were collected and analyzed. Other conditions as described in Materials and Methods. HTP, hydroxyapatite; DPG, cardiolipin.

	Phosphate exchange (nmol·4 min^{-1}·mg^{-1})	^{14}C radioactivity (cpm in band 2)	Eluted protein (μg)
HTP pass-through (Triton X-114 + DPG)	36300	220	97.5
HTP pass-through (Triton X-114 − DPG)	8150	850	72.5
HTP pass-through (Triton X-100 + DPG)	23400	185	165
HTP pass-through (Triton X-100 − DPG)	7575	655	132.5

through, was separated from the phosphate carrier by means of an organomercurial agarose gel (Affi-Gel 501) column with a high capacity for SH-containing proteins. As shown in Fig. 2, on applying the hydroxyapatite pass-through to this column, about half of the proteins passes through unretarded and the others can be eluted by a 2-mercaptoethanol gradient. Only the unretarded proteins are labelled by [^{14}C]DCCD, whereas only the first part of the peak eluted with 2-mercaptoethanol possesses reconstitutable phosphate transport activity. The analysis of the pass-through of the Affi-Gel column by SDS gel electrophoresis (Fig. 3, lane 3) reveals that the Affi-Gel chromatography eliminates mainly one band (band 3), previously identified with the phosphate carrier.

In order to fractionate further the unretarded proteins obtained from the organomercurial

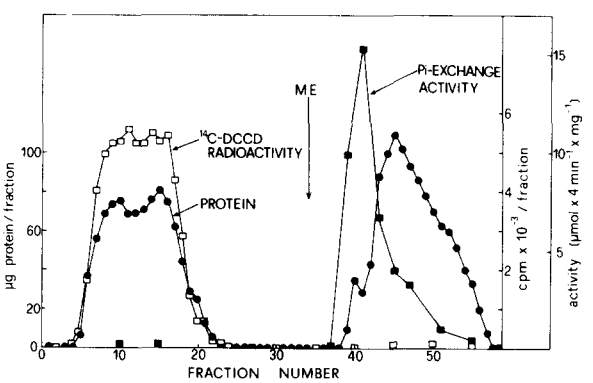


Fig. 2. Separation of the 35 kDa DCCD-binding protein from the phosphate carrier by Affi-Gel 501 chromatography of the hydroxyapatite pass-through obtained from Triton X-100 solubilized mitochondria. The hydroxyapatite pass-through (6.0 ml) was applied to the Affi-Gel column which was washed with the solubilization buffer. 1 mg/ml cardiolipin was present in the preequilibrated column and in the washing. The arrow shows the point where a 0–15 mM mercaptoethanol (ME) gradient was added to the elution buffer. Aliquots of 50 μl were used for protein determination (●———●), for measurements of reconstituted phosphate transport activity (■———■) and for measurements of [^{14}C]DCCD radioactivity (□———□).

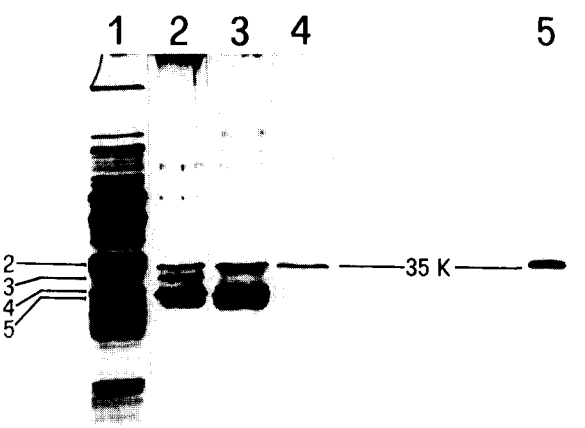


Fig. 3. SDS gel electrophoresis of fractions obtained during the purification of the 35 kDa DCCD-binding protein. 1, Triton X-100 extract; 2, hydroxyapatite pass-through; 3, Affi-Gel 501 first peak from 2; and 4, hydroxyapatite/celite pass-through from 3; 5, fluorography of the 35 kDa protein purified from [^{14}C]DCCD-labelled mitochondria.

agarose column, various adsorbents and ion exchangers were tested. With celite alone, band 5 is completely retained, but band 4 is still present in the eluate together with band 2. With the combined use of celite and hydroxyapatite in the ratio of 1 : 1 (Fig. 3, lane 4), the 35 kDa DCCD-binding protein is eluted alone and thus completely separated from the other proteins present in the pass-through of the Affi-Gel column. The isolated 35 kDa DCCD-binding protein is electrophoretically pure not only in our gel system, but also in two other systems generally applied to the resolution of the cytochrome b-c_1 complex (complex III) and the cytochrome c oxidase (complex IV), respectively (Fig. 4). Fig. 4 also shows that the 35 kDa DCCD-binding protein migrates differently from any components of complex III and IV. Furthermore, it does not aggregate in any of the electrophoretic systems used.

The purification steps of the 35 kDa DCCD-binding protein from pig heart mitochondria are presented in Table II. The amount of bound DCCD radioactivity is used to measure the degree of purification. Starting from 111 mg of mitochondrial protein, 0.265 mg of pure 35 kDa protein is obtained with a recovery of 0.2% and a purification factor of 233. Direct application of the Triton X-100 extract or the hydroxyapatite eluate to the hydroxyapatite/celite column results in a purification factor of about half that reported in Table II. Thus, in this case, i.e., without the Affi-Gel step, the phosphate-carrier protein is also

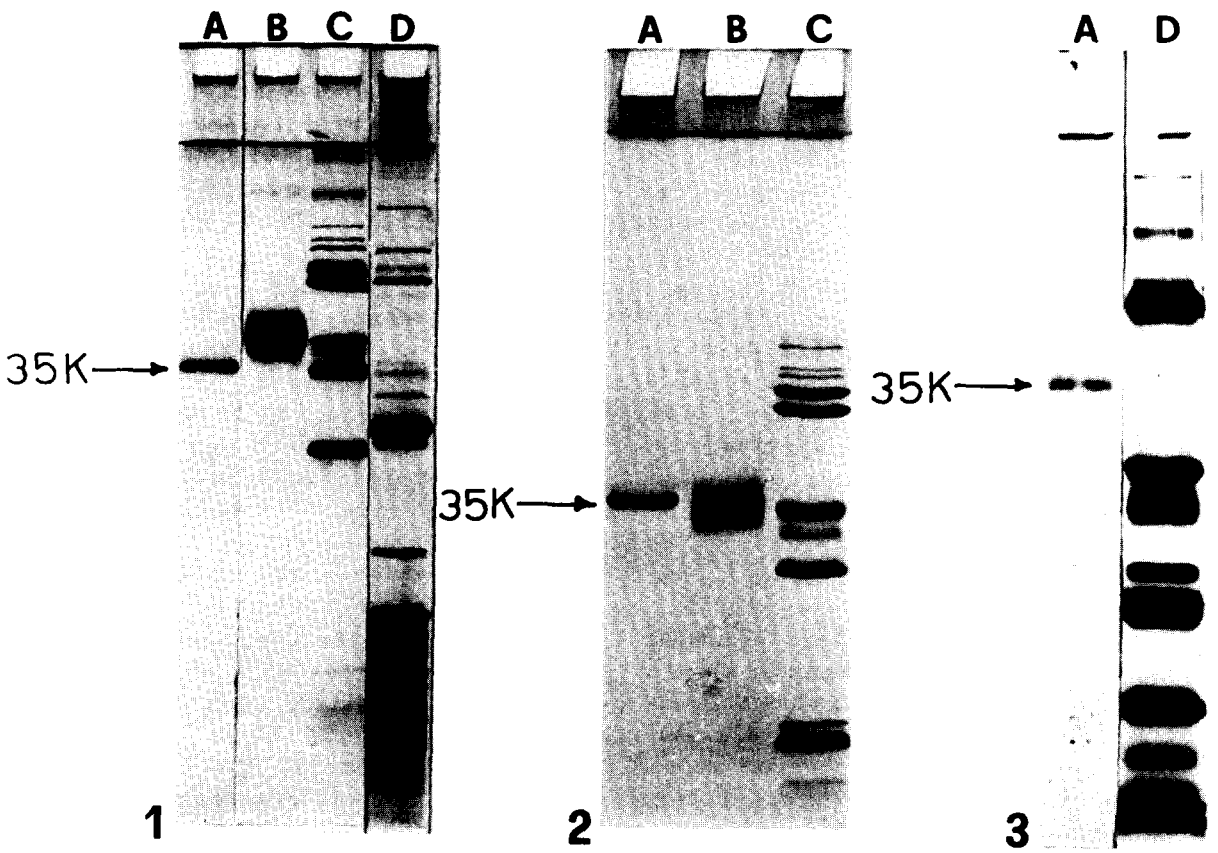


Fig. 4. Electrophoretic analysis of 35 kDa DCCD-binding protein, isolated cytochrome b, cytochrome b-c_1 complex and cytochrome c oxidase by three different systems. 1, Our electrophoretic system (17.5% acrylamide) as described in Materials and Methods; 2, electrophoretic system used for cytochrome b-c_1 complex (10–15% linear gradient of acrylamide) [22]; 3, electrophoretic system used for cytochrome c oxidase (18.75% acrylamide in the presence of 6 M urea) [23]. Samples applied to the gel: isolated 35 kDa DCCD-binding protein (A), isolated cytochrome b (B), cytochrome b-c_1 complex (C), and cytochrome c oxidase (D).

TABLE II

PURIFICATION, PROTEIN YIELD AND SPECIFIC [^{14}C]DCCD-BINDING OF THE 35 kDa DCCD-BINDING PROTEIN

Conditions as in Materials and Methods. HTP, hydroxyapatite. Samples of each purification step were subjected to SDS gel electrophoresis and fluorography. Densitometric traces of the radioactivity of the gels revealed by fluorography were obtained by means of a LKB 2202 Ultroscan Laser Densitometer coupled to a Shimadzu C-R 1 B. The specific [^{14}C]DCCD binding to the 35 kDa protein is given as the ratio between the integrated area of radioactivity associated to the 35 kDa protein and the total protein applied to the gel.

	Protein (mg)	Protein recovery (%)	Specific [^{14}C]DCCD binding ($mV \cdot s \cdot mg^{-1}$)	Purification (fold)
Mitochondria	111	100	5226	1
Triton X-100 extract	35.6	32.1	20138	3.8
HTP	2.33	2.1	366084	70.0
Affi-Gel (1st peak)	1.04	0.9	533433	102.0
HTP/celite	0.27	0.2	1220283	233.5

present in the hydroxyapatite/celite eluate in agreement with previous reports [31,32].

Amino acid composition

The amino acid composition of the 35 kDa DCCD-binding protein from pig heart mitochondria is presented in Table III. The protein does not have a particular excess of hydrophobic amino acids, and the polarity index calculated according to Capaldi and Vanderkooi [33] is 49.7%, a value very similar to that calculated for mitochondrial and bacterial porins [12,34,35]. The amino acid composition of the 35 kDa DCCD-binding protein is also similar to that of mitochondrial and bacterial porins although the 35 kDa protein contains somewhat more serine and less aromatic amino acids.

Incorporation of the 35 kDa DCCD-binding protein into lipid bilayer membranes

The conductance of lipid bilayer membranes increased by several orders of magnitude when the purified 35 kDa DCCD-binding protein was added in a small concentration to the aqueous phase bathing a membrane. The conductance increase was a function of time similar to that observed before for bacterial [36] and mitochondrial [11,12] porins. After addition of the protein to a black membrane or after the formation of a membrane in protein-containing aqueous phase, the membrane conductance increased rapidly within 20–40 min. A much slower increase of the membrane conductance was observed after that time. The addition of the detergent Triton X-100 alone in a similar concentration to that used in the presence of the protein had only little effect if any on the membrane conductance. The addition of the purified 35 kDa DCCD-binding protein to lipid bilayer membranes from diphytanoylphosphatidylcholine/*n*-decane showed a specific membrane conductance of $3.1 \cdot 10^{-5}$ $\lambda/S \cdot cm^{-2}$. In these measurements, the aqueous phase contained 0.1 M KCl (pH 6), 40 ng/ml protein and less than 100 ng/ml Triton X-100; the conductance values were taken 30 min after blackening of the membranes. The specific membrane conductance of the purified protein was 43.7-times higher than that obtained by the addition of the Triton X-100 extract of whole mitochondria. It should be noted that this figure of 43.7 is 1.4-times smaller than the value obtained when using the specific [^{14}C]DCCD binding as a means of following the purification of the 35 kDa DCCD-binding protein (see Table II). This result indicates that a partial loss of the pore-forming activity occurs during the purification of the 35 kDa DCCD-binding activity. Elsewhere, a similar but more pronounced decrease in the specific activity has been observed in the course of the purification of porins from rat liver and mung bean mitochondria [11,14].

Single-channel experiments

Experiments with small amounts of the 35 kDa DCCD-binding protein using membranes of small

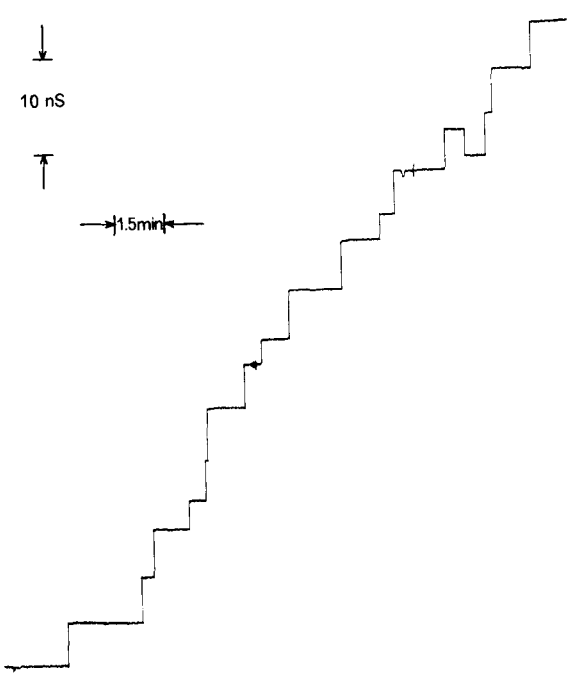
TABLE III

AMINO ACID COMPOSITION OF THE 35 kDa DCCD-BINDING PROTEIN COMPARED TO THAT OF VARIOUS PORINS

The composition presented is the mean of four analyses. n.d., not determined.

	35 kDa DCCD-binding protein from pig heart (mol%)	Porins		
		Rat liver [a] (mol%)	*Neurospora crassa* [b] (mol%)	*OmpC* from *Escherichia coli* [c] (mol%)
Asx	10.37	11.61	11.17	18.21
Thr	6.06	8.96	9.89	6.94
Ser	10.69	5.83	7.77	4.62
Glx	10.73	8.29	2.94	9.25
Pro	2.96	2.51	2.64	0.87
Gly	14.77	11.65	9.47	13.58
Ala	7.08	7.38	13.42	6.94
Cys	2.08	n.d.	n.d.	0
Val	4.70	5.93	7.63	6.07
Met	2.03 [d]	0.59	2.05	0.87
Ile	3.45	3.65	3.89	2.89
Leu	7.82	9.97	5.98	6.36
Tyr	1.87	4.41	3.35	8.38
Phe	3.61	6.16	6.7	5.49
Trp	n.d.	n.d.	n.d.	1.16
Lys	5.59	9.44	8.62	4.34
His	1.81	1.35	2.92	0.29
Arg	4.57	2.27	2.39	3.76
Polarity:	49.6%	47.8%	45.7%	47.4%

[a] From Ref. 34.
[b] From Ref. 12.
[c] From Ref. 35.
[d] Mean of two analyses.



surface area and a high current resolution showed that the conductance increase is caused by the formation of pores in the membrane. Fig. 5 shows an experiment of this type. The 35 kDa DCCD-binding protein was added to a black lipid bilayer membrane from a 1:1 mixture of asolectin/cholesterol dissolved in *n*-decane in a final concentration of 10 ng/ml. The current at 10 mV transmembrane potential increases in a stepwise fashion which is typical of the previously isolated bacterial and mitochondrial porins. Most current fluctuations are directed upwards at this transmembrane potential and terminating events are only rarely observed (Fig. 5). A histogram of the

Fig. 5. Stepwise increase of the membrane current after the addition of the 35 kDa DCCD-binding protein to the aqueous phase bathing a black membrane from asolectin/cholesterol (molar ratio 1:1) dissolved in *n*-decane. The aqueous phase contained 1 M KCl, 10 ng/ml protein and 10 ng/ml Triton X-100 (pH 6); $T = 25°C$. The applied voltage was 10 mV.

conductance of all the events at 10 mV is given in Fig. 6a. Fig. 6b shows for comparison a histogram obtained for the mitochondrial porin from rat liver under identical conditions [11]. Both histograms show a similar distribution for the channel sizes. The most frequent value for the single-channel conductance is about 4.0–4.5 nS. A second peak in the distribution of single-channel conductances is observed in the range between 1.5 and 2.5 nS which contains about 20% of the total number of single events. It has been suggested earlier that the smaller single-channel conductance corresponds to a substate of the pore which eventually may revert to the open state [11]. It has to be noted, however, that in a preparation of mitochondrial porin from *Paramecium tetraaurea*, only the smaller channel size of 2.0–3.0 nS has been observed (Ludwig, O., Benz, R. and Schultz, J.E., unpublished results). From this we may suggest that the mitochondrial pore exists in two stable conformations with different single-channel conductances and different permeability properties for hydrophilic solutes.

At 10 mV transmembrane potential, the terminating events represent a minor fraction of the current fluctuations. At larger voltages, the terminating events become more and more frequent. The terminating events are always smaller than the initial on-steps. This finding indicates that the pore is not completely closed at high voltages but is susceptible to many different conductance states depending on the magnitude of the transmembrane potential. The voltage dependence of the 35 kDa DCCD-binding protein is shown in Fig. 7. The voltage across a membrane containing two channels is switched to +50 mV and then to −50 mV. The two channels switch to substates in a somewhat asymmetric fashion as has also been observed for mitochondrial porin from rat liver [11] and *Neurospora crassa* [12].

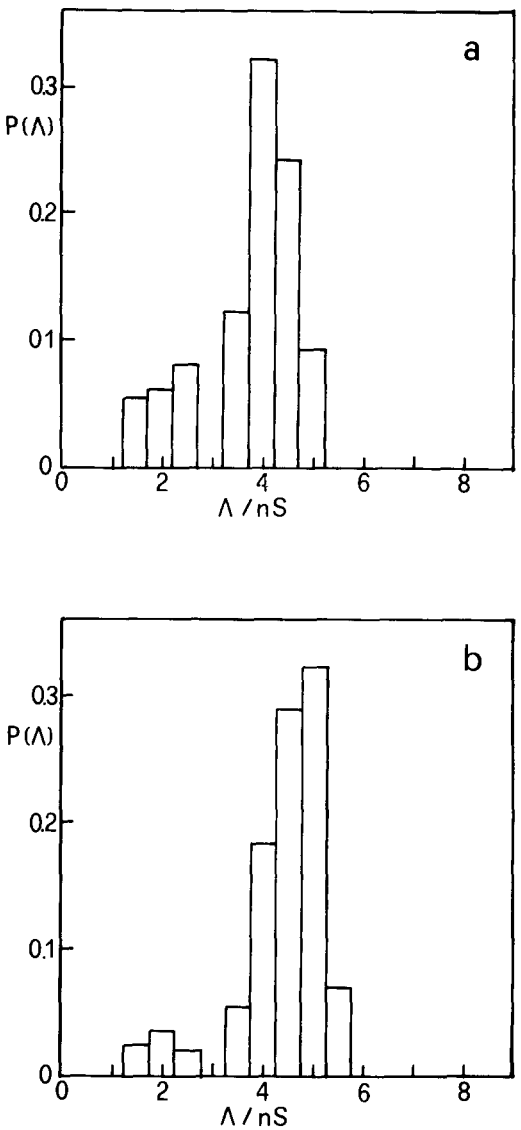


Fig. 6. Histogram of conductance fluctuations observed with membranes from asolectin/cholesterol (molar ratio 1:1) dissolved in *n*-decane in the presence of (a) 35 kDa DCCD-binding protein ($\bar{\Lambda}$ = 4.0 nS, 236 conductance fluctuations); (b) rat liver mitochondrial porin (n = 189, $\bar{\Lambda}$ = 4.3 nS [11]). The aqueous phase contained 1 M KCl (pH 6); T = 25°C. The applied voltage was 10 mV.

Zero-current membrane potentials

Further information about the structure of the pore formed by the 35 kDa DCCD-binding protein was obtained by zero-current membrane potential measurements. The protein was added in a final concentration of 50 ng/ml to a $1 \cdot 10^{-2}$ M KCl solution bathing a black membrane from asolectin/*n*-decane. After the increase of the conductance over a period of 20–30 min, the instrumentation was adjusted to the measurements of zero-current potential and the salt concentration was raised 10-fold on one side of the membrane by adding small amounts of concentrated KCl solution. The dilute side became negative with a voltage of about −10 mV, indicating preferen-

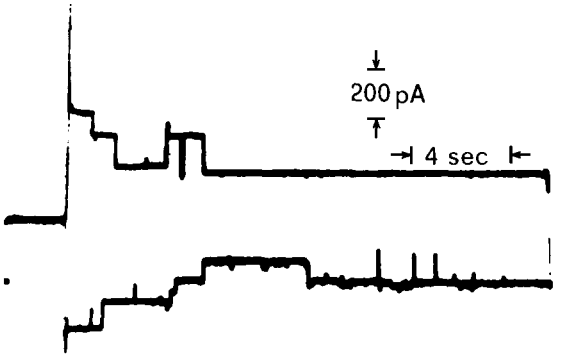


Fig. 7. Decay of the membrane current in response to transmembrane potential of +50 mV and −50 mV applied to a membrane from asolectin/cholesterol (molar ratio 1:1) dissolved in *n*-decane. The membrane contained two channels induced by the 35 kDa DCCD-binding protein of pig heart mitochondria. The aqueous phase contained 1 M KCl (pH 6); $T = 25°C$.

tial movement of anions through the pore. Table IV shows a comparison of the zero-current membrane potential obtained in this study with that published earlier for mitochondrial porin from rat liver [11] and for hexokinase-binding protein [16] which has been shown to be identical with the mitochondrial porin [16,17]. The ratio of the anion permeability P_a divided by the cation permeability P_c was calculated by using the Goldman-Hodgkin-Katz equation as described earlier [27]. The results accord well with those reported from another laboratory for rat liver porin [37].

TABLE IV

ZERO-CURRENT MEMBRANE POTENTIAL (V_m) OBSERVED IN THE PRESENCE OF THE 35 kDa DCCD-BINDING PROTEIN OF PIG HEART MITOCHONDRIA

The data were obtained in the presence of a 10-fold KCl gradient across membranes from asolectin/cholesterol (molar ratio 1:1) dissolved in *n*-decane; $T = 25°C$. V_m is the potential on the dilute side minus the potential on the concentrated side. The ratio of the permeability P_a/P_c was calculated according to the Goldman-Hodgkin-Katz equation. The results for mitochondrial porin and hexokinase binding protein from rat liver as derived earlier [16] are given for comparison.

Protein	V_m/mV	P_a/P_c
35 kDa DCCD-binding protein	-10 ± 3	1.6 ± 0.2
Rat liver mitochondrial porin	-2 ± 2	1.1 ± 0.12
Rat liver hexokinase binding protein	-3 ± 2	1.2 ± 0.12

Discussion

We have previously shown the presence of the 35 kDa DCCD-binding protein in the hydroxyapatite pass-through obtained from Triton X-100 solubilized bovine or pig heart mitochondria [9,10] and have suggested that it is identical with the phosphate carrier [9]. We have demonstrated that, upon application of high-resolution gradient SDS gel electrophoresis, the hydroxyapatite pass-through contains five protein bands and the phosphate carrier is identical with band 3 [21]. The functions of the other bands, however, remained to be established; furthermore, it remained to be checked whether the phosphate-carrier protein is indeed labelled by DCCD. The data reported in this paper show that only one protein band of the hydroxyapatite pass-through is labelled by DCCD and that this is not the phosphate carrier (i.e., band 3) but band 2 of M_r 35000 (Fig. 1). This result not only identifies the 35 kDa DCCD-binding protein in the hydroxyapatite pass-through, but also rules out the possibility of the identity of the 35 kDa DCCD-binding protein with the phosphate carrier. This non-identity is also supported by other experimental data: (a) cardiolipin prevents the elution of both [^{14}C]DCCD radioactivity and band 2 from hydroxyapatite, whereas it causes elution of the phosphate carrier (Table I, see also Ref. 21), and (b) the phosphate carrier can be completely separated from the [^{14}C]DCCD radioactivity and band 2 by chromatography on Affi-Gel 501 (Fig. 2). Our data also rule out the possibility that the 35 kDa DCCD-binding protein is a component of the cytochrome b-c_1 complex or of the cytochrome c oxidase (Fig. 4), which are both known to be labelled by DCCD [38–40], although at higher concentrations than those used in the present paper.

The 35 kDa DCCD-binding protein, functionally identified with the mitochondrial porin (see below), has been purified here for the first time from heart mitochondria. The purification, involving solubilization of mitochondria with the non-ionic detergent Triton X-100 and three chromatographic steps (hydroxyapatite, Affi-Gel 501 and celite) results in an extremely pure preparation as tested by three different electrophoretic systems (Fig. 4). The same purification procedure has been

applied to different tissues of mammalia with comparable results (not shown). The yield of the purified protein is about 0.2% of the total mitochondrial protein (cf. Table II), i.e., much higher than that obtained from isolated outer mitochondrial membranes [11,12,34] and in the same order as that obtained from whole mitochondria of *N. crassa* [41]. The method is more simple and rapid than those used to purify the mitochondrial porin from isolated outer mitochondrial membranes of rat liver [11,34] and *N. crassa* [12]. However, when using mammalian tissues in this study, in contrast with the method applied to purify porin from whole mitochondria of *N. crassa* [41], chromatography on Affi-Gel 501 was found essential to remove completely the phosphate carrier (Figs. 2 and 3). This agrees with previous observations that celite adsorbs completely the ADP/ATP carrier and other proteins present in the hydroxyapatite pass-through from mammalian mitochondria but not the phosphate carrier [31,32].

The purified 35 kDa DCCD-binding protein from heart mitochondria shares many properties with previously isolated mitochondrial as well as bacterial porins [11,12, 34–35]. They all have an apparent molecular weight ranging between 30 000 and 40 000, a similar chemical composition (Table III), a polarity of 45–50% and are relatively rich in threonine. The 35 kDa protein purified from heart mitochondria, like all porins, has a large number of charged amino acids in its amino acid composition. Most of these charges are presumably used to stabilize the pore-forming complex via internal ion bridges. Only a few charges may be exposed on the surface of the protein and/or inside the pore. An excess of positively charged groups is responsible for the anion selectivity of the mitochondrial porin and its gating process [42].

In reconstitution experiments with artificial lipid bilayers, the purified 35 kDa DCCD-binding protein functions as a pore with properties strikingly analogous to those of mitochondrial porins isolated from rat liver [11] and *N. crassa* [12]. Furthermore, the specific pore-forming activity increases during the purification procedure. Thus, we feel confident in concluding that the 35 kDa DCCD-binding protein is identical with the mitochondrial porin in pig heart mitochondria. This indicates that the mitochondrial porin has a negatively charged group in a hydrophobic environment. Similar groups with a high affinity for DCCD have been identified in the F_0 part of the H^+-ATPase located in the inner mitochondrial membrane [5] and in the proton pump of *N. crassa* cytoplasmic membrane [43].

Insertion of the 35 kDa DCCD-binding protein into lipid bilayer membranes at very low concentrations results in an increase of membrane conductance due to the formation of pores. The single-channel conductance is about 4.0 nS in 1 M KCl, and with a variety of salts was found to be closely related to the specific conductance of the salts in the aqueous phase (Benz, R., De Pinto, V. and Palmieri, F., unpublished results). This result indicates that the mitochondrial porin (or the 35 kDa DCCD-binding protein) from pig heart forms large water-filled channels in lipid bilayer membranes. Assuming that a channel is a hollow cylinder with a circular cross-section and that the cylinder is filled with an aqueous salt solution of the specific conductance Λ, the pore conductance σ is given by the following formula: $\Lambda = \sigma\pi r^2/l$, where r is the radius and l is the length of the pore. Assuming the length of the pore to be 6 nm (corresponding to the thickness of the outer mitochondrial membrane) and assuming that the pore interior has the same specific conductance as the aqueous phase ($\sigma = 110$ mS/cm), the average pore diameter may be calculated to be around 1.7 nm. Such a diameter of the pore in the outer mitochondrial membrane is consistent with the results of research into the permeability of isolated mitochondria [13] and with those of research into the electron microscopy of outer mitochondrial membranes [44,45]. X-ray diffraction patterns of plant outer mitochondrial membranes have suggested that a protein of about 30 kDa forms a prominent subunit structure with an inner low-electron-density core of approx. 2 nm [46,47]. On the other hand, experiments, in which the swelling of liposomes inserted with a channel from *N. crassa* mitochondria was measured, indicated that the diameter of this channel could be as large as 4 nm [48]. However, this result probably represents an overestimation of the size of the pore, because conductance data always gave diameter values very similar to those obtained by other methods [49].

The mitochondrial porin from pig heart forms voltage-dependent pores in lipid bilayer membranes. This result is in agreement with what was observed earlier for mitochondrial porins from rat liver [11] and *N. crassa* [12]. The pore is reduced to a smaller diameter at higher voltages. This would result in the restriction of the permeability of larger solutes. So far, it is not clear how such a transmembrane potential could be created across the outer membrane, because an ionic gradient across this membrane cannot generate a potential difference for a longer time than a few milliseconds. The generation of an intrinsic potential in the outer membrane would probably be too slow to account for the control of the mitochondrial metabolism. In fact, no evidence for such a mechanism has been detected in in vitro experiments with mitochondria. On the other hand, it has to be borne in mind that in situ mitochondria have a different structure from that of in vitro mitochondria. In situ mitochondria show a close contact between inner and outer membrane and no intracristae space [50]. From this it can be suggested that the permeability of the outer membrane may be controlled by electric coupling between inner and outer membrane.

Acknowledgements

The authors wish to thank Mr. Luigi Anzivino and Miss Angela Schmid for excellent technical assistance, Dr. Pasquale Petrilli for helping in the determination of the amino acid composition and Dr. Dieter Brdiczka and Mr. Jürgen Krause for many helpful discussions. This work has been supported by the Consiglio Nazionale delle Ricerche (CT. 83.446) and by the Deutsche Forschungsgemeinschaft (Be 865/1-2 and Be 865/3-1).

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