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# Positive residues involved in the voltage-gating of the mitochondrial porin-channel are localized in the external moiety of the pore

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The role of positive charges located on the hydrophilic surface of the mitochondrial outer membrane channel was investigated by studying the interaction between LDAO-solubilized porin and a cation-exchanger column. The binding of porin to the column material was inhibited when the elution buffer had a pH of 9 or when 2 mM dextran sulfate was added to the buffer at neutral pH. Interestingly, the addition of a synthetic copolymer of methacrylate, maleate and styrene known as a potent modulator of the voltage-dependence, did not influence the interaction between column material and porin. Incubation of porin with fluorescein isothiocyanate (FITC) resulted in the isolation of a porin fraction in which on average two lysines located on the surface of the pore-forming complex per 35 kDa polypeptide were modified. The voltage-dependence of the fluorescein isothiocyanate modified porin was strongly decreased as compared with the unmodified porin. The experiments presented here give the first biochemical evidence that positively charged lysine residues located on the surface of the channel-forming complex are responsible for the gating of the mitochondrial porin-channel.

Porin-pore; Voltage-dependence; Voltage sensor; Fluorescein isothiocyanate; Lauryl (dimethyl)-amine oxide; Mitochondria

## 1. INTRODUCTION

The outer mitochondrial membrane contains a general diffusion channel which allows the free diffusion of hydrophilic solutes below a well-defined exclusion limit [1-3]. The protein responsible for its permeability properties is the mitochondrial porin, also known as VDAC (voltage-dependent anion channel). The general diffusion channel of the outer mitochondrial membrane is slightly anion selective in the open state. For membrane potential above 20-30 mV, the channel switches into a closed form with completely different permeability properties. The closed state is presumably part of the control of mitochondrial metabolism. Based on experiments at high pH and chemical modifications of mitochondrial porin by succinic anhydride it has been suggested that positively charged amino acids are involved in the phenomenon of voltage-gating [4]. Interestingly, the voltage dependence of the mitochondrial channel can be dramatically increased by polyanions such as dextran sulfate [4] and a synthetic polyanion [5,6], which may interact with one or several positively charged groups within the conductive unit [4].

Two rapid methods are now available for the purification of functional mitochondrial porin to homogeneity [7]. One method uses the detergent Triton X-100 [8] and the other the detergent lauryl (dimethyl)-amine oxide (LDAO) [9]. Triton X-100 has a very long hydrophilic tail which shields the surface-exposed hydrophilic domains of the protein. LDAO, on the other hand, has a very small polar head group which leaves part of the hydrophilic domains, including some positively charged groups of the protein, exposed to the surrounding water [7]. These charged groups are either totally positively charged or predominantly positively charged at neutral pH, since the LDAO-purified protein binds only to cation- and not to anion-exchangers [7,9]. The interaction between the LDAO-porin and the cation exchanger column is thus a suitable system to investigate the positively charged residues located on the external surface of the functionally active pore.

In this paper we describe the influence of chemical modification and different molecules on the interaction between LDAO-porin and a cation-exchange column. Evidence is presented that fluorescein isothiocyanate (FITC), a lysine-specific reagent, blocks positively charged groups exposed to the external surface of the channel and abolishes the voltage-dependence of the porin-pore.

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*Abbreviations:* FITC, fluorescein isothiocyanate, LDAO: lauryl (dimethyl)-amine oxide



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## 2. MATERIALS AND METHODS

### 2.1. *Materials*

Hydroxyapatite (Bio-gel HTP) was obtained from Bio-Rad, Triton X-100 and lauryl (dimethyl)-amine oxide (LDAO) from Serva. Celite 535 was purchased from Roth, FITC (isomer I) from Fluka, and dextran sulfate with an average molecular mass of 5000 Da from Sigma.

### 2.2. *Purification of porin and chromatographies on carboxymethylcellulose*

The purification of bovine heart porin in LDAO was performed as has been described in [9]. The purified protein was ultradialysed against 2% LDAO, 10 mM Tris-HCl (pH 7.0) and 1 mM EDTA (solubilization buffer) and concentrated in an Amicon apparatus (PM 10 membrane). Cation-exchange chromatography was performed across a column filled with carboxymethyl cellulose (CM-52, Whatman). 0.5 ml of porin solution (containing 10–20 μg protein) treated as described below were loaded onto a column with a vol. of 1 ml. The column was pre-equilibrated with the solubilization buffer. The porin retained by the column was eluted with a buffer containing 2% LDAO, 10 mM Tris-HCl, 5 mM $KP_i$ (pH 7.0), 50 mM KCl and 1 mM EDTA.

### 2.3 *Fluorescein isothiocyanate labelling of porin*

The FITC labelling was performed by using a 200 mM stock solution of fluorescein isothiocyanate. Bovine heart mitochondria (10 mg/ml) in a solution containing 250 mM sucrose, 10 mM Tris-HCl (pH 7.2) and 1 mM EDTA were incubated for 1 h at 4°C in the dark with FITC concentrations ranging between 0.05 and 2 mM. Porin (10–20 μg/ml) dissolved in 2% LDAO, 10 mM Tris-HCl, pH 8.5 (or other suitable buffers at other pH values) and 1 mM EDTA was incubated for 1 h at 4°C in the dark at the indicated concentrations of FITC. The reaction was stopped by removing the excess of FITC by filtration on an Sephacryl S-200 (Pharmacia) column, pre-equilibrated with the same buffer at pH 7.4. The amount of FITC bound to porin was estimated by assuming a molar extinction coefficient of 80000 $M^{-1} \cdot cm^{-1}$ at 496 nm [10].

### 2.4. *SDS gel electrophoresis*

Polyacrylamide slab gel electrophoresis of acetone-precipitated samples was performed in the presence of 0.1% SDS. The separation gel contained 14% acrylamide with a ratio acrylamide/bisacrylamide of 30:0.8.

### 2.5. *Lipid bilayer experiments*

The methods used for black lipid bilayer experiments were described previously [11]. The membranes were formed across a circular hole (surface area about 0.1 $mm^2$) in the thin wall separating two aqueous compartments from a 1% (w/v) solution of diphytanoyl phosphatidylcholine (Avanti Biochemicals, Birmingham, AL) in *n*-decane. The current through the membranes was measured with two calomel electrodes switched in series with a voltage source and a current amplifier. The amplified signal was monitored with a storage oscilloscope and recorded on strip chart recorder.

## 3. RESULTS

The channel of the mitochondrial outer membrane forms voltage-gated channels in reconstitution experiments with artificial lipid bilayer membranes. Starting with about 20–30 mV, the channel switches to a low conductance or closed state. The structure that is able to respond to the applied electrical field has been defined as the voltage sensor [4].

We have investigated the interaction of factors and molecules which are known to affect the voltage-dependence of the pore, with the hydrophilic surface of the porin-channel. If the positively charged groups located in the hydrophilic surface of the channel forming unit were neutralized or blocked in part or completely by this treatment, the interaction between the LDAO-porin and the cation-exchanger should be changed. Fig. 1 shows the effect of the pH on this interaction. When porin is applied to the column at pH 7.0, the protein bound to the column and was retained. The same happened with pH 7.5 (Fig. 1A). Porin started to become eluted at pH 8.4 and was completely eluted at pH 9.0 (Fig. 1B–D). This effect caused by low proton concentration (high pH) is most likely due to titration of positively charged groups located on the external hydrophilic domain of the channel. The number of residual positively charged groups was presumably too small to account for a strong protein-cation exchanger interaction. It is interesting to note that the

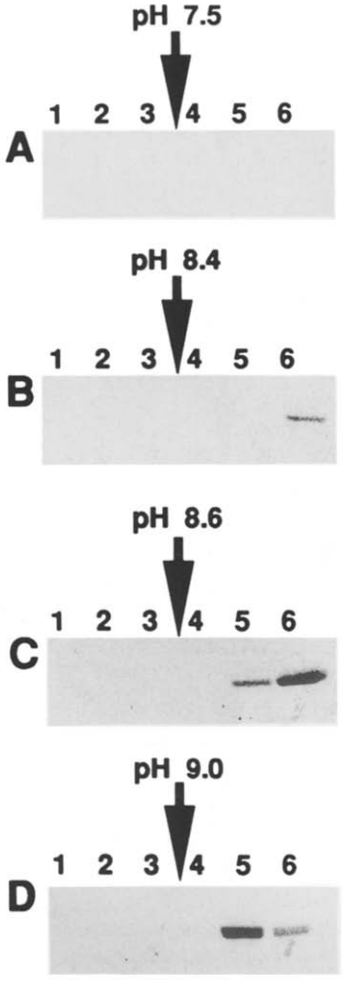


Fig. 1. Elution of porin from a cation-exchanger at different pHs. LDAO-porin was loaded onto a column of carboxymethylcellulose. The column was pre-equilibrated with 50 mM Tris-Cl, pH 7.2. After loading, elution was performed with the same buffer and the first three fractions collected; then the pH of the applied buffer was raised respectively to pH 7.5, 8.4, 8.6 and 9.0 (arrows). Three more fractions were collected and their SDS-PAGE performed.

voltage dependence has been found to decrease at pH 9 but it was not completely eliminated [4]. Fig. 2 shows the influence of dextran sulfate on the elution of porin. The experiments were performed in the following way: the purified LDAO-porin was first incubated with increasing concentrations of dextran sulfate and then loaded onto the cation-exchanger column. In the absence of dextran sulfate, all the protein was retained by the column and only the addition of a salt step allowed its elution. At small concentrations of dextran sulfate, porin was only partially retained by the column. Starting with a concentration of 2 mM, all porin passed through the column. In control experiments we could show that this effect was not caused by a direct interaction of dextran sulfate with the column. In fact, when 2 mM dextran sulfate was loaded onto the CM-52 column prior to the protein, porin was still retained. These experiments indicated that positively charged groups located on the external surface of the conductive unit were responsible for the interaction with the column since they could be shielded by dextran sulfate. It has been reported that dextran sulfate could increase the voltage-dependence of the pore with a maximum effect at 25 mM [4].

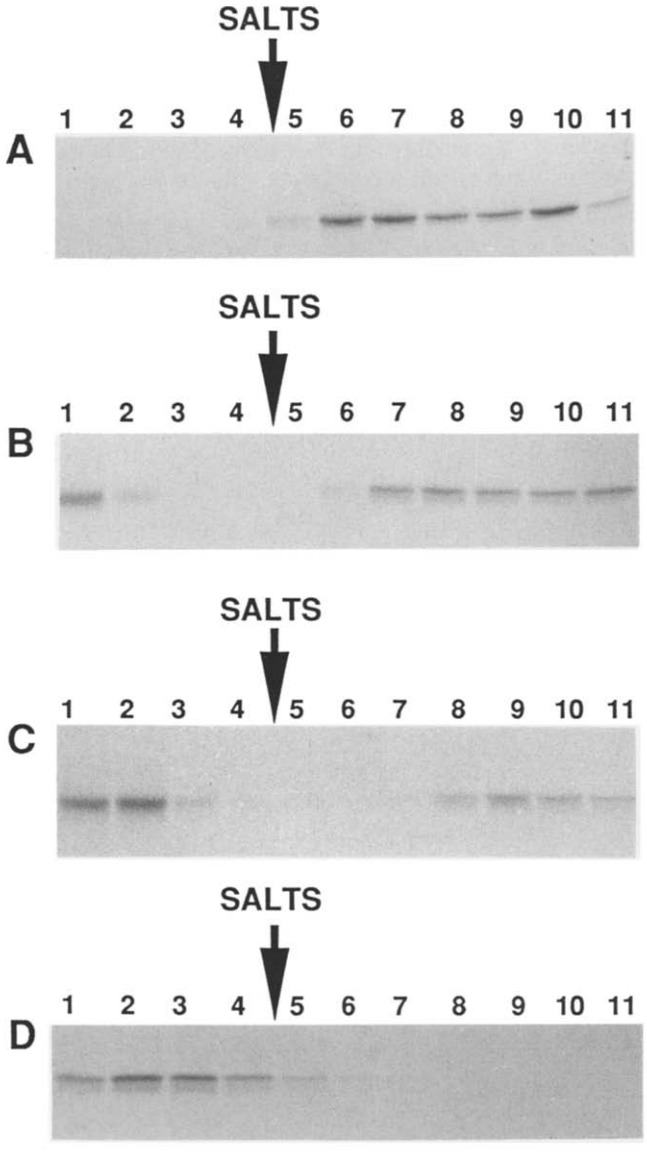


Fig. 2. Elution of porin from a cation-exchanger after preincubation with different concentrations of dextran sulfate. LDAO-porin was incubated with increasing concentrations of dextran sulfate. After 30 min at 4°C the protein was loaded onto a CM-52 column and eluted with the equilibration buffer (first 4 fractions collected) and then, with the equilibration buffer supplemented with 5 mM $KP_i$, 50 mM KCl (see the arrow, 7 more fractions collected). SDS-PAGE of the fractions was performed. (A) LDAO-porin not incubated with dextran sulfate. (B) Incubation with 0.2 mM dextran sulfate. (C) Incubation with 1 mM dextran sulfate. (D) incubation with 2 mM dextran sulfate.

Another polyanion which has been used to modulate the voltage dependence of the porin-channel is a synthetic copolymer of methacrylate, maleate and styrene of molecular mass 10 kDa. We have preincubated LDAO-porin with this polyanion at concentrations as high as 50 μg/ml, much higher than those required to affect the voltage dependence [5,6], but the porin was always retained by the cation-exchanger column (data not shown). This means that different parts of the pore-forming complex were involved in the interaction with dextran sulfate and polyanion, which could also mean that they interact differently with porin in changing the voltage dependence. These data are in agreement with electron microscopic studies of the interaction between porin and the synthetic polyanion. Binding of polyanion should occur primarily outside the channel, in the protein/lipid boundary [12] and not on the mouth of the channel.

A more straightforward approach to the localization of the positively charged groups responsible for the voltage dependence of mitochondrial porin was made by labelling porin with reagents specific for lysines. Among many such compounds, we chose the fluorescein isothiocyanate (FITC) because of its fluorescence and its relative hydrophilicity. Freshly isolated bovine heart mitochondria were incubated with very small concentrations of FITC at pH 7.2. Incubation in 50 μM FITC was already sufficient to obtain a fluorescent band on a SDS gel which corresponded to the electrophoretic mobility of porin (data not shown). Incubation of purified porin for 1 h at 4°C with 1 mM FITC resulted in labelling of the protein (Fig. 3). The results suggested that several lysines, especially those exposed to water, either in the pore or on the surface of the pore-forming unit were modified by FITC. After treatment with FITC, porin was loaded onto the cation-exchange column. Unlike the untreated porin, the FITC-modified porin was only partially retained by the column (Fig. 3A,B). A significant amount of protein was eluted before the addition of salts. This means that the chromatography resulted in the appearance of two 'populations' of FITC-modified porin. In the other

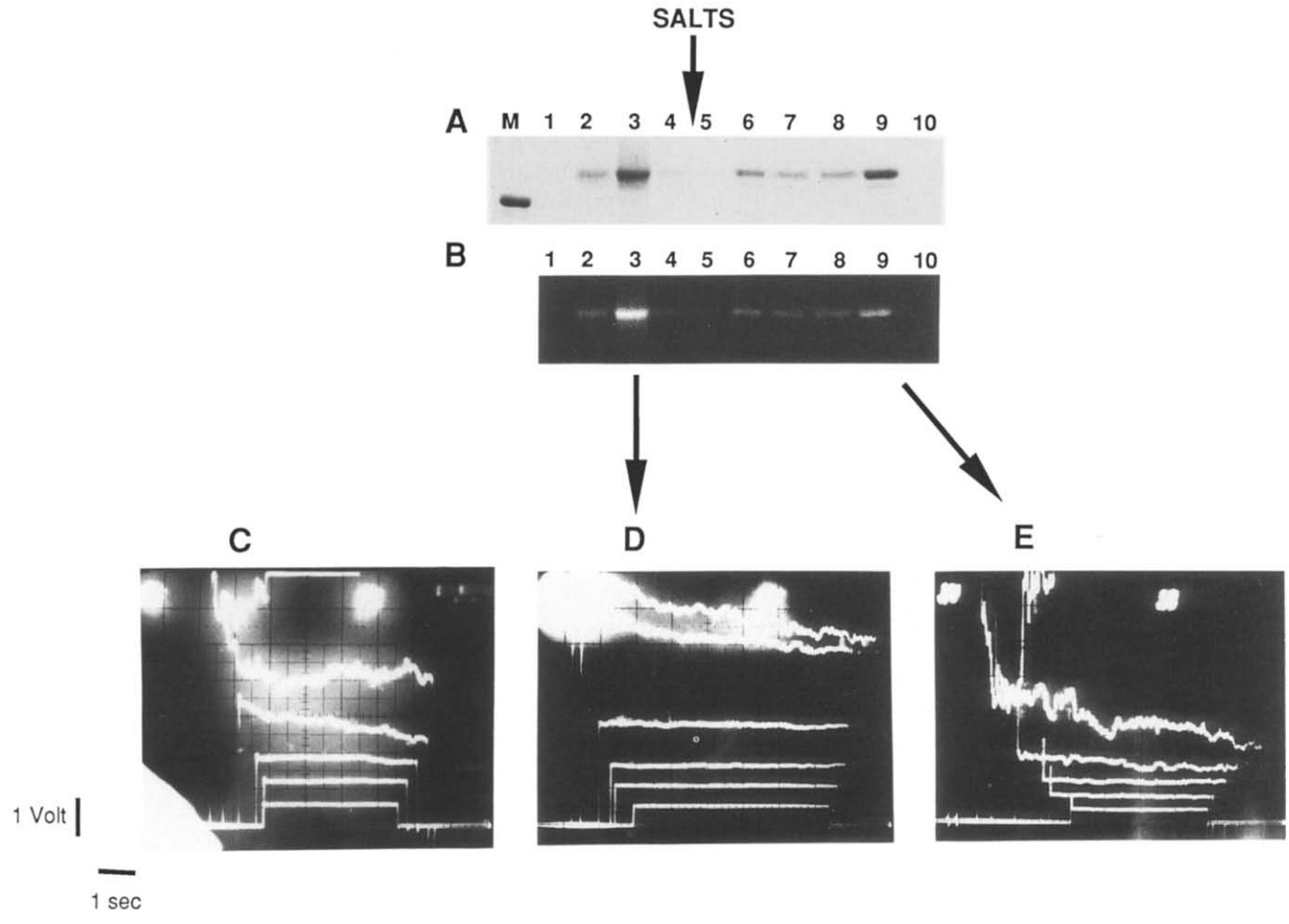


Fig. 3. LDAO-porin was incubated with 1 mM FITC (4°C for 1 h in the dark, pH 8.5). The excess of FITC was eliminated by gel filtration and the pH brought back to 7.4. The labelled porin was loaded onto a CM-52 column and eluted with the equilibration buffer (first 4 fractions collected) and then, with the equilibration buffer supplemented with salts (see the arrow, 6 more fractions collected). Two 'populations' of FITC-labelled porin were separated: porin labelled and unretained by the column (fraction 3) and porin labelled but still retained (fraction 9). The voltage-dependence of these two fractions is shown. (A) Coomassie staining of the gel. M = carbonic anhydrase (630 kDa); 1-10 = fractions collected from the chromatographic column. (B) FITC fluorescence of the gel (C) Current record of a bilayer in which the control preparation of bovine heart porin was inserted. (D) Current record of a bilayer membrane in which modified porin (fraction 3) was inserted. (E) Current record of a bilayer membrane in which modified porin (fraction 9) was inserted. (C,D,E) The transmembrane potentials applied were: +10, +20, +30, +50, +100, +120 mV.

part which was retained by the column, the lysines were probably predominantly modified in the interior of the pore.

We examined the functional properties of the two 'populations' of FITC-modified porin in reconstitution experiments with lipid bilayer membranes. Both FITC-modified porin preparations showed pore-forming activity; the single channel conductance in 1 M KCl was $4.0 \pm 0.5$ nS in both cases. However, their voltage dependences were found to be very different. With respect to the untreated protein (Fig. 3C), porin whose external lysines were modified by FITC exhibited no or only very little voltage dependence; in fact, a membrane potential of 100 mV caused only a small decrease of the membrane conductance (Fig. 3D) while the fraction retained on the column (and still labelled by FITC) showed the same voltage dependence as the control (Fig. 3E).

The amount of FITC bound to porin which is not retained by the column is shown in Fig. 4 as a function of the pH of incubation. From this titration it was derived that approximately 2 lysines per 35 kDa polypeptide are modified by FITC at the pH values (8.2-9.2) used in the reconstitution experiments to study the voltage dependence.

## 4. DISCUSSION

The voltage dependence of the mitochondrial outer membrane channel has been explained by the movement of 1-4 positively charged groups through the entire membrane as a consequence of the applied electrical field [3,4]. However, amidation of negatively charged

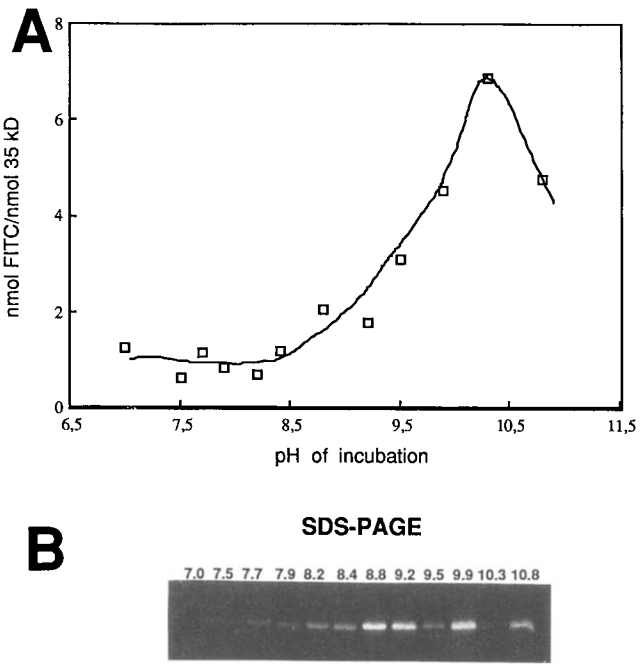


Fig. 4. Titration of amino groups accessible to FITC in porin. Purified LDAO-porin was incubated (30 min – at 4°C in the dark) at the indicated pHs with 1 mM FITC. The unreacted FITC was separated from the labelled protein by gel filtration. In the meantime the pH of the protein solutions was brought back to 7.4. The porin fractions coming from Sephacryl were loaded onto cation-exchange columns and only the protein unretained was collected. The FITC and the protein content were estimated spectrophotometrically, at 496 nm for FITC and at 280 and 260 nm for protein. (A) Plot of the molar ratio FITC/35 kDa polypeptide against the pH of incubation of the protein. (B) FITC fluorescence of the labelled protein. The low intensity of the band at pH 10.3 corresponds to a low protein content of the fraction.

groups [13] has presented evidence that as many as 22 positively charged groups could be involved in channel gating. This would mean that almost all basic amino acids present in the primary structure are involved in some 'delocalized' gating and that no cluster of gating charges exist within the channel-forming unit. In the light of our experimental data we consider such a possibility as rather unlikely.

In this paper, in fact, we have shown that several factors and conditions interacted with positively charged groups, either lysines or arginines, located on the hydrophilic surface of the pore-forming unit. Specific FITC-labelling of porin suggested that these positively charged groups were lysines. Interestingly, the FITC-induced modification of two lysines per porin monomer was already sufficient to induce a drastic change of the voltage-dependence of the channel and to reduce the interaction between column material and protein. The modification of lysines located inside of the channel is, on the other hand, not sufficient to affect the voltage dependence. The FITC effect on one of the populations of the modified porin is presumably caused by the modification of the voltage-sensor as demonstrated by the reconstitution experiments. The experimental data presented here gave the first evidence for the localization of positively charged residues, responsible for voltage-gating, on the hydrophilic surface of the conductive unit. Further work is in progress at present to localize the FITC-modified lysines within the primary structure of the mitochondrial porin.

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