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Purification of VDAC (Voltage-Dependent Anion-Selective Channel) from Rat Liver Mitochondria

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Summary. The outer membrane of rat liver mitochondria contains a channel-forming protein known as VDAC (voltage-dependent anion-selective channel). This protein has been functionally purified by a combination of ion exchange chromatography, gel filtration and affinity chromatography on a Concanavalin A-containing column. An estimated 300-fold purification was achieved over the specific activity in mitochondrial membranes. When the purified protein is run on an SDS polyacrylamide gel, essentially only one band is present at a position consistent with a molecular weight of 32,000. The resulting protein is functional and behaves normally based on channel size, selectivity and voltage dependence.

Key Words channel · voltage-dependence outer membrane · mitochondrion · VDAC

Introduction

It is generally agreed that voltage-dependent channels are the basic structures responsible for membrane excitability, including nerve conduction and muscle excitation. In order to understand the molecular basis for the voltage-dependence of certain channel-forming proteins, knowledge of their structures is very important. This paper describes the purification of a voltage-dependent channel-forming protein called VDAC isolated from mitochondria (voltage-dependent anion-selective channel).

VDAC's properties were first described by Schein, Colombini and Finkelstein (1976) when the channel-forming protein was inserted into a planar phospholipid bilayer. It has characteristic properties (Colombini, 1979, 1980*b*, *c*) which distinguish VDAC from other transport proteins and channels. The major ones are single-channel conductance, ion selectivity and the voltage needed to close the channels. By these functional criteria VDAC was separated from other proteins and purified in detergent solution. This purification method was presented in part at a mini-symposium and has appeared in print as an abstract (Colombini, 1980*a*).

Materials and Methods

Isolation of Mitochondrial Membranes

Mitochondria were isolated from the livers of rats (Sprague Dawley and other albino lines) essentially according to the procedure of Parsons, Williams and Chance (1966). Either sex was used. The rats were given only water the night before they were to be sacrificed. Four rats were usually used, yielding on the order of 50 g wet weight of liver. During all subsequent steps the preparation was kept between 0 and 5 °C. The liver was minced, homogenized and the mitochondria were purified by differential centrifugation as described previously (Parsons et al., 1966). Outer membranes were not isolated. Instead, the mitochondria were suspended in a hypotonic medium (1 mM KCl, 1 mM TrisHCl, pH 7.5) and centrifuged at 27×10^3 *g* for 10 min. The supernatant was discarded, the pellet resuspended in the same solution and the centrifugation repeated. This process was performed once more, and finally the pellet was suspended in approximately an equal volume of the same hypotonic medium. The protein content of the suspension was determined by the method of Lowry et al. (1951). The mitochondrial membrane suspension was diluted to a final protein concentration of about 15 mg/ml.

Solubilization and Elution Through an Ion Exchange Column

Sufficient detergent was added to make the mitochondrial membrane suspension 2% (vol/vol) in Triton X-100. The solution was allowed to sit on ice for 30 min prior to centrifugation in a Beckman Ti 50 rotor at 40,000 rpm. The golden liquor was removed and applied to the ion exchange column which had been prepared in a 4 °C cold room.

The ion exchange column was prepared by using a combination of DEAE Sephadex and CM Sephadex. Seven grams dry weight of each of these were allowed to swell in 20 mM KCl, 0.5% Triton X-100 in separate containers. After the fines were removed, the CM Sephadex was packed into a 25-mm diameter column. The DEAE Sephadex was then packed on top of the CM column.

The eluate from the ion exchange column was collected in two batches. The first was the void volume of the column and the second was a volume equal to the volume of sample applied to the column. VDAC was not bound to the column and was located in the second batch. The time at which the second batch should be collected could be determined both from the yellow color reaching the base of the column and

from the schlieren produced when the solution of different composition issues from the column and mixes with the previously eluted solution. The second batch thus collected was applied to the Sepharose CL6B column.

Elution Through a Sepharose CL6B Column

About 1.8 liters of Sepharose CL6B packed in a 90-cm column was used for this step. The column was maintained at room temperature and equilibrated with 0.5 M KCl, 1 mM HEPES (Na^+ salt), 0.5% Triton X-100 and 0.05% Na azide, pH 7.0. After the sample was applied, the column was eluted at about 1 ml/min. (The column was run overnight.) Fifteen to 20 ml fractions were collected. The refractive index of the eluate was monitored with a Refracto Monitor Model 1107 (Laboratory Data Control, Riviera Beach, Fla.). The fractions were assayed for VDAC activity as described below. Normally, only fractions at and around the expected location for VDAC elution were actually assayed for VDAC activity. This location coincided with the start of the elution of the colored material. The fractions containing the highest activity were pooled, supplemented to contain 20% (vol/vol) dimethylsulfoxide and stored frozen $\leqq -20$ °C. This material is designated as fraction 1.

Final Purification with Con-A Affinity Column

Concanavalin A attached to beads was purchased from Sigma Chemical Co. (St. Louis, Mo.). The Con-A attached to Agarose or to Sepharose 4B worked equally well. The Con-A beads were stored in 1 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.02% merthiolate (at ~4 °C). The beads were warmed to room temperature and all subsequent steps were done at room temperature. The beads were washed with 100 mM KCl, 5 mM NaHEPES, pH 7.0, 0.1% Triton X-100 prior to use. (The fines were also removed at this point.) Fifteen to 20 ml of packed gel were used to form a column. Fraction 1 was diluted approximately 10-fold with water to bring the Triton concentration to 0.1%. This can be determined spectrophotometrically by monitoring the absorbance at 280 nm. The diluted solution was applied to the column and eluted at 1 to 1.5 ml/min. The solution which eluted from the column was labeled the effluent. The column was then washed with 20 ml of 100 mM KCl, 5 mM NaHEPES, pH 7.0 and 0.1% Triton X-100. The solution that eluted was labeled wash. Finally, the column was eluted with 0.3 M α-methylmannoside made up with the same ingredients as in the wash medium. This elution was allowed to flow rather slowly (~0.5 ml/min). The eluate was collected in approximately 10-ml fractions and assayed for VDAC activity[1] (*see below*). Fractions with high activity were pooled. This material is designated as fraction 2.

Generation of Membranes and Assay of VDAC

Planar phospholipid bilayer membranes were generated as described previously (Schein et al., 1976) following the method of Montal and Mueller (1972). The aqueous phase of the cis side (i.e. the side to which the sample was to be added) was 1.0 M KCl + 5 mM $CaCl_2$ while that on the trans side was 0.1 M KCl + 5 mM $CaCl_2$. The voltage across the membrane was controlled by means of a voltage clamp similar to that described previously (Schein et al., 1976). The voltage on the cis side was controlled with respect to the trans side (the trans side was virtual ground). The diameter of the membrane was approximately 0.15 mm.

An aliquot of the fraction to be tested for VDAC activity was added to the cis side under constant stirring. Ten microliters of a solution containing 1% Triton were added, while 50 µl were used if the solution contained 0.1% Triton. The volume of the aqueous phase on the cis side was approximately 4 ml. Channel insertion was usually monitored in the absence of an external electric field. The number of channels inserting per unit time was measured by actually counting channels (for samples with low activity) or by dividing the observed current by the single-channel current for high activity samples.

Other Methods and Materials

SDS polyacrylamide gel electrophoresis was performed essentially according to the method of Laemmli (1970). Protein was assayed according to Lowry et al. (1951), and by the fluorescamine method of Udenfriend et al. (1972). Sepharose CL6B was purchased from Pharmacia Fine Chemicals, Piscataway, N.J. Ammonyx LO was a generous gift from the Onyx Chemical Co., Jersey City, N.J. Asolectin, a crude mixture of soybean phospholipids, was purchased from Sigma Chemical Co., St. Louis Mo. and treated as described by Kagawa and Racker (1971).

Results and Discussion

Measurement of Channel-Forming Activity

Activity was measured by determining the rate of incorporation of channels into a phospholipid membrane. As was shown previously (Colombini, 1979), VDAC inserts into phospholipid membranes from the aqueous phase in the presence of very small amounts of the detergent, Triton X-100. A zwitterionic detergent, Ammonyx LO[2], also works well.

The permeability of the planar phospholipid membrane was very low ($<10^{-10}$ S) prior to addition of the sample to the aqueous phase. After sample addition the permeability increased if ion-conducting pathways entered the membrane. More activity was obtained if the sample was allowed to equilibrate to room temperature prior to its addition to the membrane's bathing solution. VDAC pathways were distinguished from other pathways by two criteria[3]: 1) ion selectivity, 2) voltage dependence. Under the conditions described in Materials and Methods (1 M KCl + 5 mM $CaCl_2$ *vs.* 0.1 M KCl + 5 mM $CaCl_2$) the selectivity of a membrane containing essentially only VDAC permeability

[1] At this stage one sometimes encounters difficulty in getting VDAC to insert into the bilayer. This may be overcome by supplementing the sample (or an aliquot thereof) with 0.1% asolectin sonicated in water and 0.5% ammonyx LO.

[2] Ammonyx LO may be a better choice than Triton because, unlike Triton, it can be easily removed by dialysis and absorbs very poorly in the UV region.

[3] Although the single channel size (i.e. permeability due to single channels) is a good criterion, it is not useful when large permeabilities have been induced in the membrane.

was such that a reversal potential of ~11 mV positive on the high salt side was observed[4]. Lower voltages or voltages of the opposite sign indicated the presence of other pathways in that membrane. In practice, the presence of other pathways was rarely seen. Their occurrence was usually attributable to membrane instability[5]. The voltage dependence was considered normal if, when the voltage in the cis side was changed from zero to −40 mV, the current decreased to one-half the instantaneous value in about 30 sec.

The rate of channel insertion into the membrane rather than the number of channels inserted was chosen as a measure of the VDAC concentration of a sample. The rate of channel insertion should be proportional to the VDAC concentration in the aqueous phase just as the total number of channels inserted after some long period of time. However, the latter is more prone to error due to scavenging of channels by the chamber walls and the monolayer. Also, if the membrane broke before insertion into the membrane had ceased, the rate of insertion was still obtainable while the total number of channels inserted prior to breakage would underestimate the sample activity.

Figure 1 shows a typical experiment. There is a lag time, due perhaps in part to the unstirred layer and in part to some cooperativity between channels[6]. Cooperativity is also indicated by the S-shape of the initial insertion. The rate of insertion rapidly reaches a fairly constant value which is maintained for several minutes. This quasi-constant rate was used as a measure of medium VDAC content and hence VDAC content of the sample.

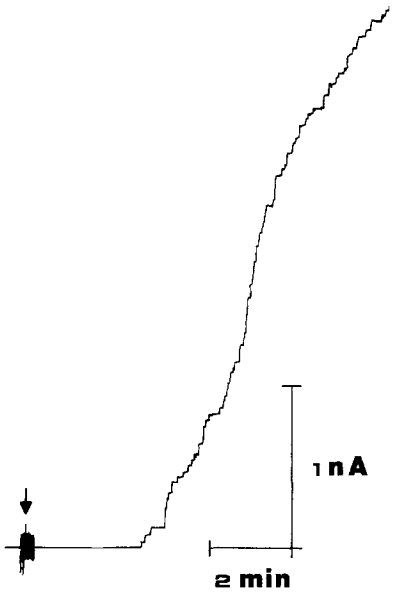

Fig. 1. Assay of VDAC activity in a sample. The Figure shows a time course of channel insertion into a planar phospholipid membrane after the addition of 10 μl of sample at the point indicated by the arrow. The parameter on the vertical axis is current in nanoamperes. The membrane was generated as indicated in Materials and Methods. No field was applied, as the salt gradient provided the driving force for ion flux. The sample was added to the high salt side

The assay is more qualitative than quantitative since the result obtained varies considerably from assay to assay. The presence of other constituents in the sample may also influence the results. For example, the Triton concentration has very strong influence. Triton was required for insertion, and a strong correlation seemed to exist between the amount of Triton added along with the sample and the amount of VDAC insertion[7] (*data not shown*). For example, the addition of one 10-μl aliquot of a sample to the aqueous phase bathing the membrane induces many more channels to insert than the sequential addition of two 5-μl aliquots. In view of these results, other material in the sample may influence VDAC insertion, so that the specific activity used in Table 1 may be a poor indication of true VDAC content.

Purification of VDAC

Generation of Partially Purified Fraction 1. Mitochondrial membranes obtained as described in Ma-

[4] The value of 11 mV was determined for the reversal potential of a single channel. Although this value appears to differ markedly from previously reported values (Schein et al., 1976), in those experiments the reversal potentials were corrected for all nonvoltage-dependent membrane permeability including the closed state conductance of the channels. If this is not done, very similar results are obtained (note where the curve intersects the voltage axis in Fig. 5*b* of Schein et al., 1976).

In doing the measurement of the reversal potential it is important to pulse from zero voltage and measure the initial current value. This is repeated until a voltage is found which generates an initial current equal to zero. With time after the voltage has been applied, some channels close. Since the closed state has a rather different selectivity, the overall reversal potential drops to lower values.

[5] When old samples (or samples which had been stored in an inappropriate way) were tested, one could observe current changes (in the absence of applied electrical field and the presence of a salt gradient) in the direction opposite to that produced by the insertion of "normal" VDAC channels. This indicates the insertion of channels with cation selectivity. These events were interpreted as due to the insertion of partially denatured VDAC channels.

[6] One has the impression that it is harder to get the first channel to insert, and once the first has inserted others insert more easily.

[7] In our hands Triton X-100 by itself does not introduce VDAC-like permeabilities in membranes. It does at times increase membrane permeability but this is usually followed by membrane breakage.

Table 1. Fractionation by Con-A affinity column

Fraction	VDAC activity (Channels min^{-1} μg^{-1})	Volume (ml)	Protein content[e] (μg/ml)
input[a]	57	920	21
effluent[b]	4	915	30
wash[c]	12	20	26
tube 1[d]	N.D.	9.8	30
tube 2	7	9.9	35
tube 3	11	9.8	39
tube 4	72	12.1	30
tube 5	3	9.8	15
tube 6	0	9.9	11
tube 7	6	10.2	7

[a] Tenfold dilution of fraction 1 prior to addition to column.
[b] Solution which flowed out of the column as the "input" was applied.
[c] Solution which flowed out of the column as the buffer described in the methods was applied.
[d] Solution which flowed out of the column when buffer supplemented with α-methylmannoside was applied. The tubes were collected in sequence from 1 to 7.
[e] Protein was estimated by the fluorescamine method (*see* section 6 of Materials and Methods).

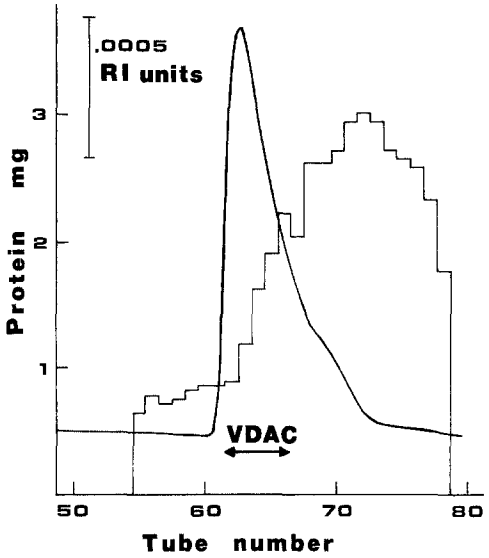

Fig. 2. Fractions eluted from the Sepharose CL6B column. Each tube contained 18 ml of eluate. The smooth line represents the output of a refractive index monitor and the scale in refractive index units is shown. The histogram represents the results of protein determinations on the tubes (following the method of Lowry et al., 1951). Triton precipitates during the assay but the precipitate can be removed by centrifugation without significant effects on the color of the supernatant. Some of the color is due to material leaching off the column and not protein. This correction was not made in the Figure. The tubes containing a considerable amount of VDAC activity are indicated by the arrow with points on both ends (i.e. tubes 62 to 66)

terials and Methods were extracted in 2% Triton X-100. The Triton/protein ratio was kept above 1:1. After removal of the Triton-insoluble material by centrifugation, the gold-colored liquid was applied to the combined ion exchange column.

The size and shape of this column is crucial. A tall narrow column will rapidly clog while a very short wide column with a fast flow rate will remove only a portion of the removable proteins. A 25-mm-diameter column worked well. If the solution exiting the column was cloudy or became cloudy, the sample was eluted too rapidly and had to be centrifuged prior to the following step.

The eluate from the ion exchange column was applied to the Sepharose CL6B column. This column was run at room temperature since comparable activity was obtained when the column was run at room temperature or in a 4 °C cold room. The yellow color elutes just after VDAC (there is some overlap) and with the majority of the protein. Figure 2 shows the protein profile and the output of the R.I. monitor in relation to the VDAC activity. Tubes containing most of the VDAC activity were pooled, adjusted to contain 20% (vol/vol) DMSO and stored frozen either at −20 or −70 °C. At −20 °C the preparation had activity for many months. This is a useful partially purified fraction designated as fraction 1.

The vessel used to store fraction 1 should be made of plastic. Teflon or polypropylene work well. If glass is used, although VDAC activity seems normal, VDAC loses its ability to bind to the Con-A affinity column used for the final purification step. This puzzling observation, which cost a great deal of time and labor, has not been explained.

Final Purification Step. The Con-A Sepharose column was run at room temperature and pretreated as described in Materials and Methods. Fraction 1 was diluted 10-fold with water and applied to the column. A slow flow rate ensured good binding but required overnight to complete. VDAC's binding to Con-A appears to be weak, because some VDAC does not bind and some is eluted with buffer alone. However, much of the VDAC elutes when α-methylmannoside is added to the buffer. In view of the above-mentioned effect of storage in glass, perhaps a conformational change in VDAC makes the molecule unable to bind to Con-A, and this may explain what appears to be weak binding. SDS-solubilized VDAC does not bind Con-A (Gordon Shore, *personal communication*).

Table 1 shows a typical experiment in which the various fractions collected from the Con-A af-

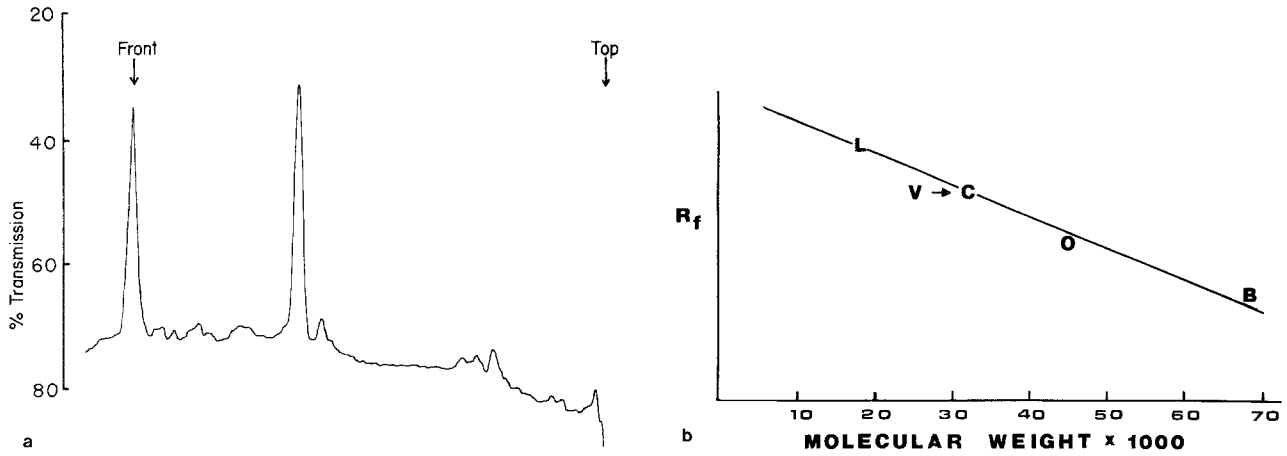

Fig. 3. SDS polyacrylamide gel electrophoresis of fraction 2. Fig. 3*a* is a tracing of the percent transmission of light at 570 nm as a function of distance along the gel. The gel was 10% acrylamide stained with Coomassie blue. The 3% stacking gel was not scanned and contained no visible bands. The 3 to 10% interface is indicated by "Top" in the Figure. The point to which low molecular weight materials migrated is indicated by "Front" in the Figure. On the same slab gel but in a different well, protein standards were run for calibration. Figure 3*b* shows how the major band in fraction 2 migrated as compared to the standards. The symbols signify: *B*, bovine serum albumin; *O*, ovalbumin; *C*, carbonic anhydrase; *L*, lactoglobulin, and *V* indicates the location of the fraction 2 band (the putative VDAC) on the line

finity column were analyzed for protein content[8] and VDAC activity. The highest specific activity of VDAC was found in tube 4, while most of the protein does not stick to the column and ends up in the effluent.

In this highly purified state channel-forming activity decays in a few days. Addition of phospholipid helps to slow down the decay.

Proof of Purity

Although absolute proof of purity is very difficult, the following experiments indicate a high degree of purity. When fraction 2 was treated with SDS under reducing conditions and subjected to polyacrylamide gel electrophoresis, only one major band is seen (Fig. 3*a*). When the gel is calibrated with molecular weight standards (Fig. 3*b*) the band's location indicates a molecular weight of 32 kD. There is Coomassie blue staining material at the leading edge. The nature of this low molecular weight material has not been determined.

The observation of one major band on an SDS polyacrylamide gel does not prove that the protein represented by that band was responsible for the activity. The purified protein was subjected to electrophoresis under nondenaturing conditions, solubilized in Triton X-100. Although VDAC activity was detected which co-migrated with the protein stain, the activity recovered from the gel was very low. Therefore the experiment was not conclusive.

[8] Since the fluorescamine assay was used to estimate protein content, the values obtained may be high. Fluorescamine reacts with all primary amino groups.

Table 2. Properties of VDAC at different stages of purification

	Whole mitochondria[a]	Fraction 1	Fraction 2
Single channel size (nS) (in 1 M KCl, 5 mM $CaCl_2$)	4.3±0.2	4.1±0.1	4.2±0.1
Steepness of voltage dependence (n)[a]	4.4±0.8	4.7±0.4	4.5±0.4
Voltage at half-maximal closure[a] V_o (mV)	37 ±2	33 ±2	31 ±1
Reversal potential[b] (mV) (1 M KCl, 5 mM $CaCl_2$) *vs.* (0.1 M KCl, 5 mM $CaCl_2$)	11 ±4	11 ±9	11 ±2

[a] Prepared and measured as described previously (Schein et al., 1976).

[b] The potential necessary to reduce the current across the membrane to zero (*see also* footnote 4). The sign refers to the high salt side.

The degree of purification achieved is also hard to estimate because of the variability of the assay method. The specific activities achieved with the final material, fraction 2, are around 100 channels min^{-1} μg^{-1} (the range is from 50 to 250). This compares with 0.3 channels min^{-1} μg^{-1} for rat liver mitochondrial membranes. Hence, perhaps a 300-fold purification was achieved.

That VDAC was not significantly altered by the purification process is shown in Table 2. VDAC's fundamental properties are virtually the same before and after purification.

Comparison with Other Channel-Formers Isolated from Mitochondrial Outer Membrane

Zalman, Nikaido and Kagawa (1980) have reported the purification of a 30,000 dalton protein from mung bean mitochondria, which when inserted into liposomes induces permeability to saccharides up to 2000 to 8000 daltons. Although we have not looked for VDAC activity in mung bean mitochondria, Zalman et al. (1980) probably purified VDAC for the following reasons: a) VDAC was found in mitochondria from very diverse species (Colombini, 1979); b) the properties described in their work are consistent with VDAC's known properties.

Roos, Benz and Brdiczka (1982) have recently published a partial purification of a pore-forming protein from rat liver mitochondria. Its properties are very similar but not identical to those of VDAC. The differences are probably not due to the isolation of a different protein but rather to different methods of analysis. Roos et al. (1982) report a marked asymmetry in the voltage-dependent behavior of the channels which we do not see even when we observe the properties of single channels. This difference may have arisen as a result of the different method of isolation or perhaps as a result of using solvent containing planar bilayers. We find no pronounced asymmetry either in channels transferred from mitochondria and placed into planar bilayer membranes without detergent treatment at anytime (following the method previously described, Schein et al., 1976) or with purified VDAC isolated with detergent. Hence the basically symmetrical behavior which we observe is probably not an artefact of purification.

The activity reported by Roos et al. (1982) is about three orders of magnitude less than that reported here (correcting for membrane area and concentration of protein in the aqueous phase). This is most likely a result of the use of solvent containing membranes by the above investigators and not a good measure of the relative degree of purity. A better estimate of relative purity can be made by examining the degree of purification which Roos et al. (1982) obtained above the level observed in whole outer membranes. Correcting for 32 kD peptide content they report a threefold loss in activity. However, since they obtained a 14-fold enhancement of the 32 kD peptide in their preparation, I calculate a 4.8-fold increase in specific activity of channel-inserting ability. By contrast we reported (Colombini, 1979) the activity of VDAC insertion using whole outer membranes to be 1.2 channels min^{-1} μg^{-1}. This is roughly 1% of the activity observed with pure VDAC. Hence the procedure reported here seems to purify VDAC 20-fold more than that reported by Roos et al. (1982).

Although the paper of Roos et al. (1982) shows only a few minor bands contaminating the main 32 kD band, it is important to realize that the 32 kD band could be heterogeneous. We did not try to purify the 32 kD peptide but instead followed the VDAC activity.

Freitag, Neupert and Benz (1982) have purified a pore-forming protein from *Neurospora crassa* outer mitochondrial membranes. These membranes are known to contain VDAC (Colombini, 1980*c*) and this may be by far the predominant protein in these membranes (Mannella, 1982; Mannella, Cognon & Colombini, 1982). Hence most likely this pore-forming protein is VDAC. The small differences in observed properties may be explained as above for the protein isolated from rat liver.

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