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Characterization and partial purification of the VDAC-channel-modulating protein from calf liver mitochondria

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Abstract

The mitochondrial channel, VDAC, mediates metabolic flux across the mitochondrial outer membrane. When reconstituted into planar phospholipid membranes, VDAC is voltage-dependent, existing in multiple conformational states with different selectivities and permeabilities. At low membrane potentials, these channels are in the open state and are anion-selective. VDAC channels switch to lower-conductive closed states at high membrane potentials. The VDAC modulator, a soluble mitochondrial protein, has been demonstrated to dramatically increase the voltage dependence of VDAC channels and induce the channels to enter closed states even at low membrane potentials. We have isolated and partially purified this modulating protein and the activity is associated with a 54 kDa protein on SDS-PAGE. Under native reduced conditions the activity eluted around 100 kDa from a gel filtration column. As little as 200 ng/ml of the partially purified protein was sufficient to modulate reconstituted VDAC channels. This protein had a p*I* of 5.1. A second activity with a p*I* of 4.8 was far more potent, making VDAC-channel-containing membranes virtually non-conductive in some experiments. The effects of both modulator activities could be completely reversed by the addition of pronase. Simple perfusion of the chamber did not reverse the effect of the modulator on VDAC. By controlling the gating of VDAC channels, the VDAC modulator could play an important role in regulating cellular metabolism.

Key words: VDAC; Mitochondrion; VDAC modulator; Membrane channel; Protein purification; Intermembrane space; (Calf); (*N. crassa*)

1. Introduction

Mitochondria not only act as the major site of cellular energy transduction, but also participate in the synthesis of heme, pyrimidines, amino acids, and many other key metabolites. Even in an anaerobic environment, mitochondria are essential to the growth and survival of cells. Thus far, there is no evidence that eukaryotic cells can survive without this organelle (or endosymbiotic bacteria) [1,2]. The mitochondrial outer membrane channel, VDAC, exists in all eukaryotic species examined, from fungi to mammals [3–5]. In addition to the known location in the mitochondrial outer membrane, there is evidence that this channel may exist in plasma membranes as well [6,7]. When reconstituted into planar phospholipid membranes, VDAC channels are voltage-dependent [3,4,8] and can exist in multiple conformational states with different selectivity and permeability. At low transmembrane potentials, these channels are in the highly-conductive, open state and are anion-selective, providing the permeability pathway for the flux of negatively-charged metabolites across the mitochondrial outer membrane. At higher voltages (such as 40 mV in soybean phospholipid membranes), VDAC channels switch to low-conducting, closed states. The closed-state channel not only has a smaller pore size and pore volume [9–11], but also reverses its selectivity (from anion-selective to cation-selective) [12–14]. Therefore, the closed states are much less permeable to small ions and probably impermeable to multiple-negatively-charged metabolites, such as ATP and ADP [13,15]. The electro-

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physiological properties of VDAC channels are very conserved in all VDACs studied so far [3,16], which may suggest an important regulatory role of VDAC channels in cellular functions.

There is considerable evidence that VDAC channels are also the binding sites of both hexokinase and glycerol kinase to mitochondria [17–21]. The free and bound states of these kinases may reflect metabolic regulation in response to changes in cellular energy transduction [22–24]. Changes in the conformational state of VDAC could affect the permeability of the outer membrane [13,15] and may alter the binding of the kinases to mitochondria. These effects should have far-reaching consequences on metabolism.

The conformational change (gating) of VDAC channels is greatly influenced by a variety of substances, including various polyanions [11,14], trivalent metals [25–27], and a modulating protein, called the VDAC modulator [28,29]. The VDAC modulator was first found to increase the voltage sensitivity of VDAC channels in *Neurospora crassa* [28]. Modulator proteins with similar activity were found in a variety of species, from plants to mammals [29]. In the reconstituted system, the VDAC modulator increases the voltage dependence of VDAC channels, induces VDAC to assume a closed state of even lower conductance, and keeps some channels in closed states even at very low potentials. A 3-fold increase in the steepness of voltage dependence (n) of VDAC was observed at low concentrations of the modulating protein [30]. With intact mitochondria, a crude modulator preparation has been demonstrated to partially inhibit mitochondrial functions, including the ADP-stimulated respiration (state 3 respiration) and adenylate kinase activity [15]. This inhibition was not observed if the outer membrane had been damaged, indicating a specific action of VDAC modulator on outer membrane permeability (presumably the closure of VDAC channels).

In this paper we report the characterization and partial purification of this VDAC modulator and also describe a novel and more potent VDAC modulating activity. This may represent an activated form of the VDAC modulator or perhaps the existence of a second modulating protein.

2. Materials and methods

Materials and reagents. A wall-less mutant of *N. crassa* (ATCC 32360) was used for the preparation of VDAC channels. Fresh calf liver from a local slaughterhouse was used as the tissue source for purification of the VDAC modulator.

Isolation of mitochondria and mitochondrial subfractions. Mitochondria were isolated from fresh calf liver using a modification of the method of Rickwood et al. [31]. Due to the large amount of tissue to be processed (about 2.5 kg), the liver was first minced in a 4°C cold-room by using a meat-grinder. The material was then homogenized using a Waring blender (1 gallon capacity) twice for 2 s at low speed, diluted with cold buffer to approximately 0.5 g/ml, and then filtered through a double layer of cheesecloth to remove tissue debris. The mitochondria were isolated from other cell organelles by differential centrifugation. The supernatant after $2500 \times g$ for 10 min was pelleted down by centrifuging at $4000 \times g$ for 8 min and then $10000 \times g$ for 2 min. The pellet was collected as crude mitochondria. This process was repeated three to four times to minimize the contamination of other organelles. The shorter than usual time for the high-speed centrifugation helped reduce microsomal contamination and broken mitochondria. The mitochondria were suspended in a hypotonic buffer solution (15 mM MOPS (pH 7.2), 0.25 mM EDTA) to break the mitochondrial outer membrane selectively, thereby releasing the intermembrane space proteins. The optimum lysing conditions were monitored using marker enzymes: succinate : cytochrome c oxidoreductase and malate dehydrogenase [32] as well as total protein released. The following proteinase inhibitors were added to the hypotonic solution: 2 μg/ml aprotinin, 10 μg/ml chymostatin (dissolved in DMSO), 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 20 μM phenylmethylsulfonyl fluoride (PMSF). This mixture halted all proteinase activity in the comparable fraction from *N. crassa* (M. Holden, unpublished observation). After the mitochondria had been suspended in the hypotonic buffer (on ice) for 30 min, the suspension was centrifuged at $17500 \times g$ for 30 min to remove remaining mitochondria and other particulates.

Ammonium sulfate fractionation. The preparation enriched in intermembrane space proteins was fractionated by ammonium sulfate precipitation on the same day as the mitochondrial isolation. Proteins that precipitated when the ammonium sulfate (HPLC grade) concentration was raised from 40 to 60% contained VDAC modulator activity. The pellet following the centrifugation of this solution (at $12000 \times g$ for 20 min) was resuspended in 25 ml of 10 mM MOPS, 1 mM DTT, 0.25 mM EDTA (pH 7.2), plus the above proteinase inhibitors. This material was frozen in 4-ml aliquots at −85°C. This was used as the starting material for two further purification processes. Loss of total activity during each separation procedure usually limited the numbers of separation steps after the ammonium sulfate precipitation to two.

Preparative isoelectric focusing. For preparative isoelectric focusing, the modulator preparation (one of the frozen 4-ml aliquots, see above) was dialyzed overnight against 0.1% glycine, 1 mM DTT, and centrifuged at $12000 \times g$ for 30 min to remove the precipi-

tated proteins. 5 ml of the supernatant (total protein conc. was about 50 mg and came from about 500 mg of total mitochondrial protein) was used in BioRad's Rotofor IEF system. Bio-Lyte ampholyte (1.5% final conc.) (pH range 4–6), was used to set up the pH gradient. To reduce protein precipitation during focusing, 10% glycerol and 1% glycine was used. After focusing, ampholyte was removed from proteins by bringing the samples' salt concentration to 1 M KCl, 50 mM phosphate buffer (final conc.) (pH 7.0), followed by gel filtration.

Size exclusion chromatography, HPLC, and protein purification. The active fractions collected from preparative IEF were pooled together, concentrated by Amicon centriprep-10, and subjected to gel filtration on a Sephacryl S-200 HR column. Alternatively, modulator-containing samples from ammonium sulfate precipitation were directly applied to a Sephacryl S-300 HR column (1.5 × 100 cm). The running buffer was 50 mM phosphate buffer (pH 7.0), supplemented with 1 mM DTT. For HPLC chromatography, Rainin's Hydropore strong anion exchange column (10 cm × 4.7 mm) was used. The low ionic strength buffer (solution A) was 20 mM phosphate buffer (pH 7.0), 1 mM DTT, and the high ionic strength solution (solution B) contained 1 M KCl, 0.1 mM EDTA in addition to the above ingredients. The flow rate was 1 ml/min. A linear gradient from 0% solution B to 60% solution B in 30 min was used to elute the modulator-containing sample. Eluted proteins were monitored by using a UV detector at 280 nm. The fractions corresponding to individual peaks were collected, concentrated, and dialyzed for further analysis.

Gel electrophoresis. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and nondenaturing gel electrophoresis were performed as described previously [33,34]. The non-denaturing gel was run in a neutral pH system at 4°C. The final concentrations of the buffer solutions were: stacking gel buffer, 100 mM Tris-phosphate (pH 5.5); resolving gel buffer, 70 mM Tris-HCl (pH 7.5); running buffer, 30 mM Tris-diethylbarbiturate (pH 7.0). Either 7.5% or 10% acrylamide concentrations were used depending on the proteins to be resolved. Following gel electrophoresis, the proteins were visualized by staining either with Coomassie blue or silver (BioRad silver staining kit).

Protein and marker enzyme assay. Protein concentrations were assayed using BCA kit (Pierce) or absorbance at 280 nm. Bovine serum albumin (BSA) fraction V was used as a standard in protein assay. Mitochondrial marker enzymes, succinate : cytochrome *c* oxidoreductase and malate dehydrogenase, were measured according to the methods used by Mannella [32].

Isolation and reconstitution of VDAC channels into planar phospholipid membranes. Mitochondrial membranes isolated from *Neurospora crassa* were the source of VDAC channels. The membrane pellet was suspended in 10 mM KCl, 10 mM MOPS, 1 mM EDTA, 15% dimethylsulfoxide (DMSO) (pH 7.2) and stored frozen at −20°C. Prior to the experiments, the channels were solubilized by using 1% Triton X-100 (final conc.). Planar phospholipid membranes were made across a 0.1 mm hole in a Saran partition by the monolayer method [45] as previously described [46]. The phospholipid membrane was generated using 1% diphytanoyl phosphatidylcholine (DPyPC; Avanti Biochemicals, Birmingham AL). VDAC channels were inserted into the membrane by adding a small aliquot (3–8 μl) of the Triton-solubilized channels to the *cis* compartment. The gating properties of VDAC channels were examined under voltage-clamp conditions [8,46]. All voltages refer to the *cis* compartment with *trans* being held at virtual ground.

3. Results

Measurement of the modulator activity on VDAC channels

The modulator activity was measured on reconstituted VDAC channels. The responses of VDAC channels to different voltages were tested before the addition of the modulator-containing fraction. Following modulator addition, the changes in (1) the rate of channel closure and (2) the decrease in total current, were used as two parameters to assess the activity of the VDAC modulator. Fig. 1 shows a typical experiment in which the modulator greatly increased the

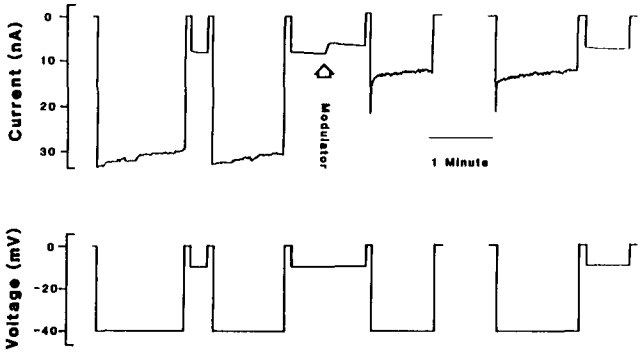

Fig. 1. The VDAC modulator from calf liver mitochondria greatly increased the rate of channel closure and the voltage dependence of VDAC channels. VDAC channels were isolated from mitochondrial membranes of *N. crassa* and the modulator was a fraction obtained from preparative isoelectric focusing. Top record: membrane current through VDAC channels before (first two pulses) and after the addition of the modulator-containing fraction to the *cis* side (final protein concentration: 2.5 μg/ml). Bottom record: voltages applied to the membrane (negative on the modulator-containing, *cis* side). Note: one pulse to positive potentials (break in the trace) was omitted for clarity. The modulator normally acts only when the modulator-containing side is made negative.

channel's voltage sensitivity and the rate of channel closure. Prior to modulator addition, channel closure is a very slow process at −40 mV in a multi-channel DPyPC membrane. The addition of 10 μl (final protein conc. about 2.5 μg/ml) of a modulator-containing sample to the aqueous phase bathing the membrane dramatically increased the rate of channel closure by about 10- to 15-fold and decreased total membrane conductance.

The activity assay is more qualitative than quantitative, since (1) the action of the modulator changes with time after addition to the assay system; and (2) the action depends on the control closure rate of VDAC channels which, in DPyPC membranes, varies from membrane to membrane for complex reasons.

Isolation of mitochondrial intermembrane space proteins

The VDAC modulator was first found in the soluble fraction of sonicated mitochondria from *N. crassa*. Later, it was determined that the VDAC modulator activity exists in the intermembrane space [47]. To take advantage of this finding, we selectively broke the outer membrane to release the intermembrane space proteins by using hypotonic buffers. Both marker enzymes and total proteins released were monitored under a series of hypotonic buffer solutions (MOPS (pH 7.2), 0.25 mM EDTA). Submillimolar concentrations of EDTA were used in the buffer solutions since it was found to increase the extent of outer membrane lysis under the given hypoosmotic conditions without significantly damaging the inner membrane [32]. Table 1 shows the enzymatic activities of the marker enzymes measured after suspension of mitochondria in the indicated buffer solutions. From these experiments, 15 mM MOPS (pH 7.2, 0.25 mM EDTA) seems to be the critical concentration at which most mitochondrial outer membranes were ruptured without significantly damaging the inner membrane. The ability of cytochrome *c* (normally unable to cross the outer membrane) to be reduced by enzymes in the inner membrane, was determined by measuring the activity of succinate : cytochrome *c* oxidoreductase (succinate dehydrogenase). This activity was increased substantially when mitochondria were suspended in the 15 mM MOPS, indicating the lysis of the outer membrane. Damage to the inner membrane was assessed by both malate dehydrogenase (a matrix enzyme) and total mitochondrial proteins released. The release of malate dehydrogenase and total proteins into the supernatant were greatly increased at 10 mM, indicating damage of the inner mitochondrial membrane (Table 1). Thus in subsequent purifications, we selected a hypotonic buffer solution containing 15 mM MOPS, 0.25 mM EDTA (pH 7.2) to rupture the outer membrane selectively, releasing the VDAC modulator activity.

Table 1
Release of succinate : cytochrome *c* oxidoreductase, malate dehydrogenase, and total mitochondrial proteins

MOPS conc. (mM)	Succinate : cyt *c* oxidoreductase (nmol/min/mg protein)	Malate dehydrogenase (μmol/min/mg protein)	Total protein (μg/ml)
0	653	73	277
5	447	75	181
10	499	64	170
15	430	20	52
20	115	17	62
25	87	17	40
35	40	15	41
50	nd	20	37
10	nd	12	33

The buffer solutions contained 0.25 mM EDTA and the pH was adjusted to 7.2 by NaOH.

Partial purification of the VDAC modulator

After obtaining a fraction enriched in intermembrane space proteins, we undertook the purification procedure, starting with an ammonium sulfate fractionation. The critical concentration range (CCR) of ammonium sulfate for the precipitation of the VDAC modulator (the two concentrations of ammonium sulfate between which approximately 80% of the modulator activity was found to precipitate) was determined to be 40–60%. The modulator was then redissolved in a low salt buffer (10 mM MOPS (pH 7.2)) containing proteinase inhibitors and 1 mM DTT.

Following ammonium sulfate precipitation, two separate purification plans were pursued. The first one employed preparative isoelectric focusing and gel filtration chromatography. The second plan was a combination of gel filtration chromatography and semi-preparative HPLC anion exchange chromatography. Both procedures had limited success in the purification of the VDAC modulator.

Prior to the preparative isoelectric focusing, the ammonium sulfate was removed by dialysis against 0.1% glycine and 1 mM DTT. Fig. 2 shows the pH gradient and the protein distribution after the isoelectric focusing (IEF). Most of the proteins were focused around pH 6 (Fig. 2) and removed from the sample. The modulator activity was found at a p*I* of 5.1. This fraction increased the closing rate and voltage dependence of VDAC channels as described previously (Fig. 1). Fractions around p*I* 5.1 (fractions 10 to 14) were pooled. Potassium chloride (final concentration, 1 M) buffered with 50 mM phosphate buffer was added to the sample to dissociate the ampholyte from the proteins. Then, the sample was concentrated and centrifuged prior to gel filtration. The supernatant was applied to the gel filtration column (Sephacryl S-200 HR) and run at 4°C. Fig. 3 shows the protein and activity profiles. The highest VDAC modulator activity

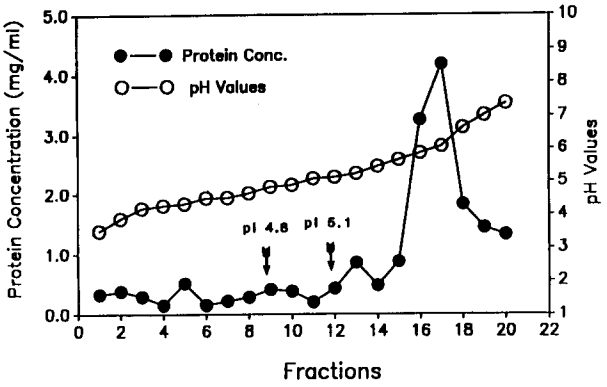

Fig. 2. Protein profile and pH gradient after preparative isoelectric focusing. Modulator-containing sample (total protein concentration: about 50 mg) was subjected to Rotofor (BioRad) isoelectric focusing for 4 h at 4°C. 1.5% Ampholyte (Biolyte from BioRad) from pH 4 to 6 was used. Two modulating activities were observed on reconstituted VDAC channels (as indicated by arrows): one at p*I* 4.8, another at p*I* 5.1. Most proteins were focused and some of them precipitated around pH 6. After clarification of the samples by centrifugation, protein concentration was estimated by UV absorbance (the actual amount of protein in fractions 16 and 17 was higher than indicated because of the removal of precipitated material).

was found in fractions corresponding to a molecular mass of 100 kDa. When analyzed by SDS-PAGE with silver staining, the active fractions showed three major bands, one at 80 kDa, one at about 54 kDa, and the last one at 30 kDa (Fig. 4, lanes 1, 2). Tubes containing most of the modulator activity were pooled and stored at −80°C for further purification. In the purification

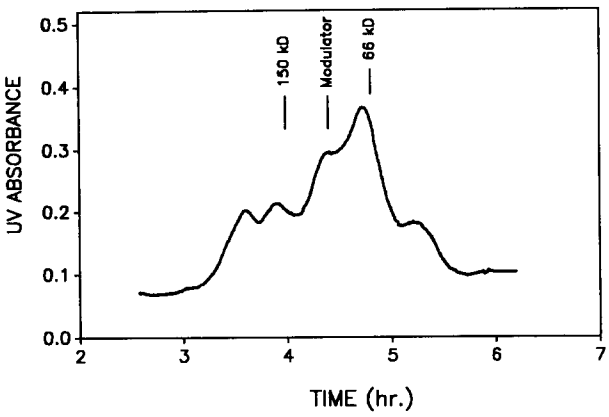

Fig. 3. Gel filtration of the modulator-containing fraction. A concentrated sample from preparative isoelectric focusing (0.5 ml containing about 4 mg of proteins) was subjected to chromatography on a 165-ml Sephacryl S-200 HR column (1.5×100 cm) at 4°C. The running buffer was 50 mM phosphate buffer, 1 mM DTT (pH 7.0). The protein profile was monitored on an ISCO UV absorbance monitor at 280 nm. Aliquots (50×100 μl) of various fractions were tested for modulator activity on reconstituted VDAC channels. The best activity was found at about 100 kDa. The elution positions of alcohol dehydrogenase (M_r 150000), and bovine serum albumin (M_r 67000) were determined by their absorbance profiles.

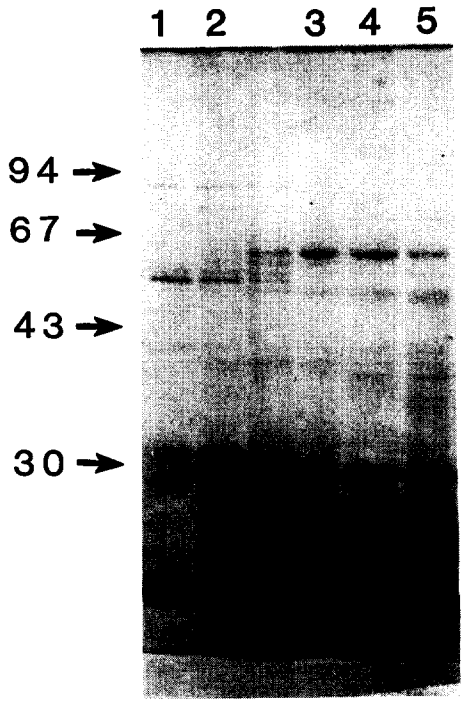

Fig. 4. SDS-polyacrylamide gel electrophoresis (10%) of the major fractions from gel filtration arranged in the order which they were collected. Lane 1 and 2 are fractions from the gel filtration column containing most of the modulator activity (total protein about 3 μg). Three major bands were observed with molecular masses of 80 kDa, 54 kDa, and 30 kDa. Lanes 3 to 5 contain 4–5 μg of protein from gel filtrations representing smaller molecular mass.

process, DTT was found to be important for maintaining the modulator activity.

Another purification process was also pursued. The modulator preparation, after ammonium sulfate precipitation, was applied to the gel filtration column (Sephacryl S-300 HR). Again, most of the modulator activity was recovered in a fraction corresponding to a molecular mass of about 100 kDa (Fig. 5). Active fractions were pooled together, concentrated, and further separated by semi-preparative HPLC-anion exchange column. Fig. 6 shows the protein elution profile from HPLC and the salt (KCl) gradient (dotted line) used to elute the proteins. The activity of the HPLC fractions were assayed on VDAC channels reconstituted into DPyPC planar membranes. The modulator activity was recovered in the fraction at 38 min and eluted at a salt concentration of 240 mM KCl. After SDS-PAGE and silver staining, two major bands were observed: one at 54 kDa and the other at 42 kDa (Fig. 7A, lane 6). When examined after native polyacrylamide gel electrophoresis under reducing conditions, only one major band was observed (Fig. 7B, lane 6).

Observation of a new, potent modulating activity

Two different modulating activities were observed when modulator-containing samples were purified by

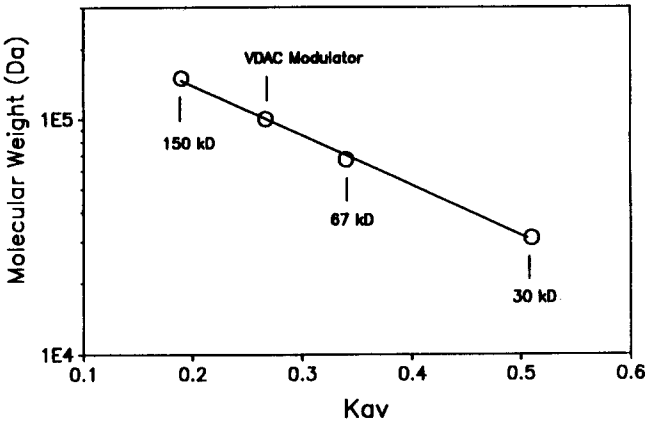

Fig. 5. Molecular mass estimation of the VDAC modulator determined by gel filtration chromatography. The running buffer contained 50 mM phosphate buffer (pH 7.0) and 1 mM DTT. Sample (1 ml, 40 mg of protein) was run on Sephacryl S-300 HR column (1.5×100 cm). The molecular mass standards ranged from 150 kDa to 31 kDa: alcohol dehydrogenase (M_r 150000), BSA (M_r 67000), carbonic anhydrase (M_r 30000) as indicated. The VDAC modulator activity was found at about 100 kDa with $K_{av} = 0.27$.

isoelectric focusing (Fig. 2, arrows). In addition to the normal activity (the activity reported to date) at p*I* 5.1, the fraction at p*I* 4.8 dramatically decreased the total membrane current of reconstituted VDAC channels, inducing most of the channels to enter a totally closed state (Fig. 8). In Fig. 8, the voltage dependence of VDAC channels showed some asymmetry prior to the addition of VDAC modulator (a typical observation for VDAC reconstituted into DPyPC membranes). The rate of channel closure is much faster at positive potentials than at negative potentials. The gating process responding to negative potentials was chosen to assay

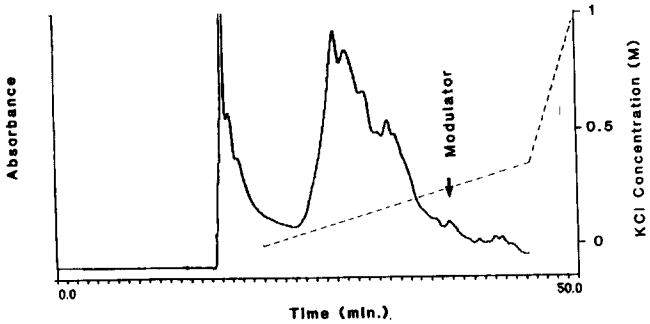

Fig. 6. The separation profile after high performance liquid chromatography (HPLC). The modulator sample (total protein concentration about 10 mg from gel filtration as in Fig. 4.5) was run on a Hydropore strong anion exchange column (Rainin). Proteins eluted were monitored by a UV monitor at 280 nm. VDAC modulator activity was recovered at a salt (KCl) concentration of 240 mM as indicated. The salt gradient used to elute proteins bound to the column is indicated by the dashed line.

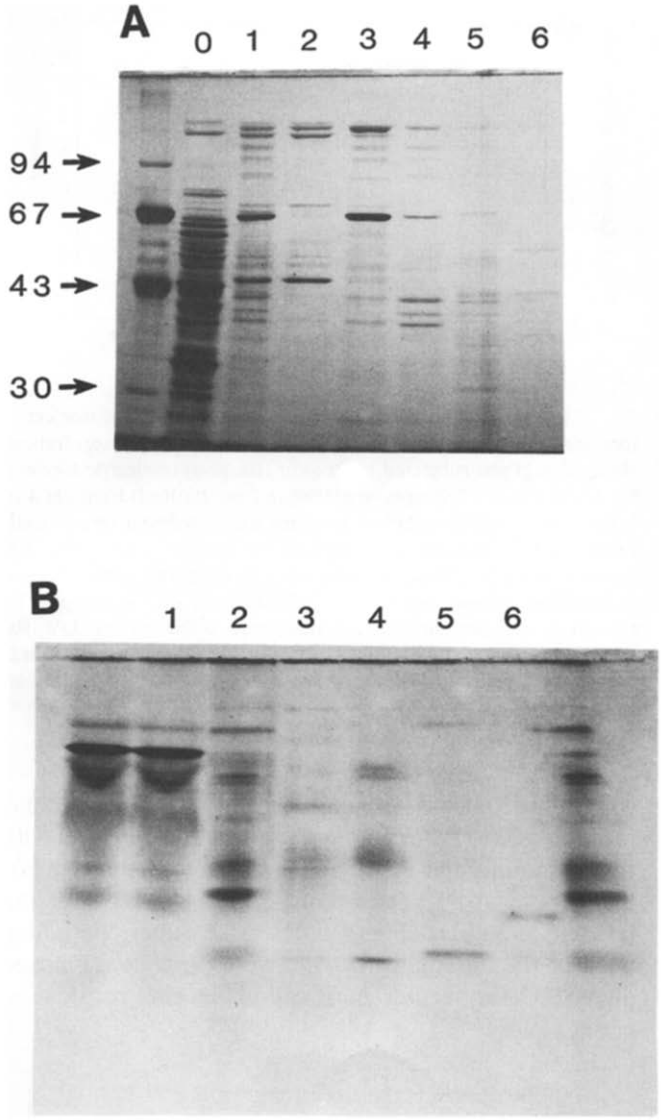

Fig. 7. Polyacrylamide gel electrophoresis (10%) of the VDAC modulator fraction from the HPLC anion exchange column. (A) SDS-PAGE of the HPLC separated fractions (50 μl) stained with silver. Lane 0 contained proteins not bound to the column. Lanes 1 to 6 are fractions eluted by the KCl gradient (low to high). The highest VDAC modulator activity was found in fraction 6 (lane 6) which showed two major bands at 54 kDa and 43 kDa. (B) Native-PAGE of the same samples (60 μl) as above. Lanes 1 to 6 are six fractions eluted from the HPLC column (as in Fig. 4.6). Lane 6, (sample containing modulator activity) showed one major band which moved a little faster than BSA (marker not shown).

the modulator activity. After the addition of the modulator to the *cis* side, not only was the rate of channel closure increased (only to negative potentials), but also the total membrane conductance began to decrease with time at both positive and negative potentials. To determine whether the reduction in conductance is due to channel closure (or blockage) as opposed to irreversible channel damage (e.g., proteinase activity) or loss of channels from the membrane, pronase (5

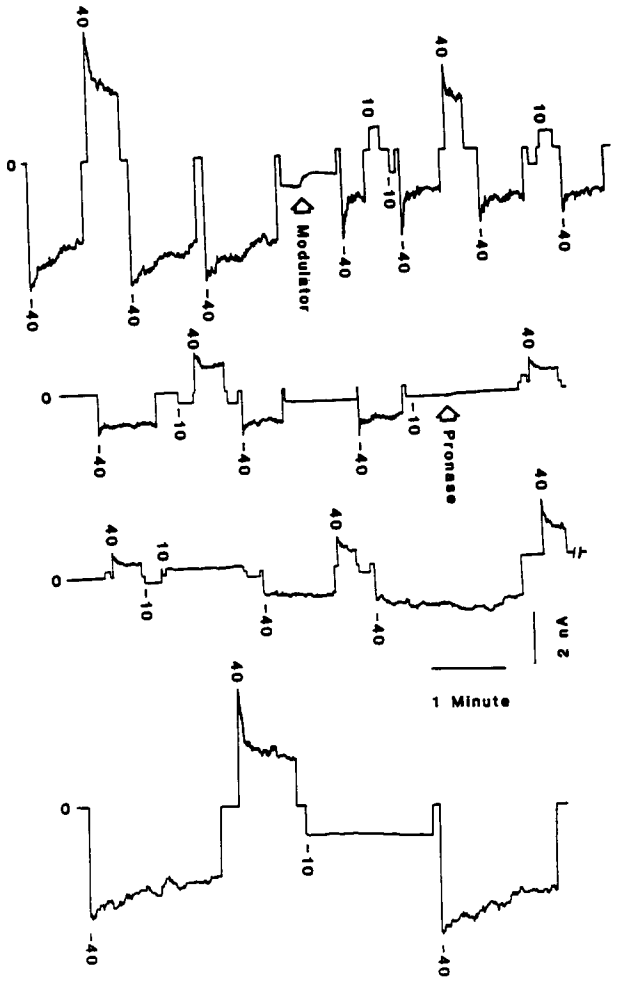

Fig. 8. The new modulating activity of the fraction collected at pI = 4.8 on reconstituted VDAC channels and the effect of pronase. The fraction at pI = 4.8 (final protein concentration 2 μg/ml) was added to the *cis* side of a bilayer membrane containing about 30 channels isolated from *N. crassa*. The record shows the current response to the indicated voltage steps. The voltage was always returned to zero prior to the next voltage step. Pronase (5 mg/ml) was added to the same side as the modulator fraction. The break before the channels return to normal is about 15 min.

mg/ml) was added to the same side as the modulator (*cis* side). Immediately after the addition, VDAC channels lost the increased voltage dependence (first pulse of −40 mV after pronase addition, Fig. 8) and the conductance began to increase. In about 10 min, the membrane conductance returned to its original level and the channels' properties returned to normal, nondistinguishable from the channels prior to the addition of the sample from the fraction at pI = 4.8 (Fig. 8). Pronase at 10 × higher concentration (50 mg/ml) has no effect on the gating behavior of VDAC channels reconstituted into planar phospholipid membranes. In some experiments, the total membrane conductance dropped as much as 50-fold after the addition of this modulating fraction (pI 4.8).

Determination of the optimum pH for interaction between VDAC and its modulator

The modulator activity was examined as a function of pH on multichannel DPyPC bilayer membranes. It is known that the voltage-dependent properties of VDAC channels change little in the pH range from 5 to 9 [35]. Aqueous solutions (1 M KCl, 5 mM $CaCl_2$) were buffered with 25 mM potassium phosphate from pH 5.0 to 6.0, with 25 mM MOPS at pH 7.0, and 25 mM Tris-HCl at pH 8.0 and pH 9.0. Control experiments showed no effect on VDAC channels with different buffer systems alone. Prior to the experiments, the modulator was treated in a specific pH buffer for 3–5 min. It was found that the modulator has the best activity on VDAC channels from pH 7.0 to 9.0, but is much less active at pH 5.0. Multichannel neutral DPyPC membranes (100–150 channels) were used to avoid the stochastic property of single channels and the effect of charges on the phospholipids on the local pH at the membrane surface.

Failure to reverse the binding of the modulator to VDAC channels by perfusion

We used a perfusion system to examine the nature of interactions between VDAC and the modulator. VDAC channels were reconstituted into a planar phospholipid membrane. Then, a sample of a VDAC modulator-containing fraction was added to the cis compartment (medium: 1 M KCl, 5 mM $CaCl_2$, 10 mM MOPS (pH 7.2)). The binding of modulator to VDAC channels was monitored by the increased rate of channel closure at −40 mV applied potential (Fig. 9). Fresh buffer was perfused into the modulator-containing chamber (chamber volume = 4 ml) at a rate of 5.5 ml/min for a total perfusion volume of 25 ml. Control experiments using a water-soluble colored substance

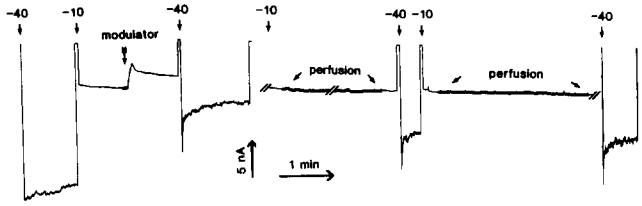

Fig. 9. Perfusion fails to wash out the modulator. An aliquot of modulator-containing sample was applied to the aqueous phase (total volume = 4 ml) on the *cis* side of the membrane as in Fig. 1. The modulator was washed out by perfusing in fresh solution at a rate of 4.5 ml/min for a total of 25 ml while simultaneously keeping the volume of solution in the chamber constant by sipping out from the surface excess solution under constant stirring. After testing the voltage dependence of the channels, a second perfusion was performed at double the rate for a second 25 ml of perfused solution. The numbers above the arrows indicate the point of application of the indicated voltage (in mV). The voltage was returned to zero prior to the application of the next potential. The break in the record after the second perfusion was for a 10-min time span.

(Bromophenol blue) showed that the perfusion protocol results in total replacement of the solution in the chamber. No reversal of the modulator's effect on VDAC was observed after perfusion and following a second perfusion of 25 ml (Fig. 9). 10 min after the second perfusion the rate and extent of VDAC closure remained unchanged, indicating no diminution in the action of the modulator.

The action of the VDAC modulator does not require the presence of divalent ions

Calcium or magnesium ions at 1–5 mM levels are routinely used in the reconstitution assays for VDAC channels and to monitor VDAC modulator activity. While it had already been shown that it is not necessary to add divalent ions in order to reconstitute and study VDAC, no experiments had been done in such a way as to maintain free levels of calcium at or below 10^{-7} M. In addition, initial indications pointed to a requirement for divalent ions to observe the action of the modulator. This raised the possibility that the VDAC modulator might only act at elevated cytoplasmic calcium ion levels.

In order to determine whether divalent ions might be required to stabilize the VDAC-modulator complex, sufficient chelator was added after complex formation in an attempt to reverse the action of the modulator. An aliquot of a modulator-containing fraction was added to the *cis* side of a DPyPC membrane containing many VDAC channels (medium: 1.0 M KCl, 1 mM $CaCl_2$, 30 mM MOPS (pH 7.2)). After obtaining the standard modulator effect, 0.5 M EDTA (pH 7.2) (5 mM final conc.) was added to each of the *cis* and *trans* compartments. This should have reduced the free $[Ca^{2+}]$ to $7 \cdot 10^{-9}$ M. No change in the modulator effect was observed after 45 min.

In order to test the possibility that divalent ions might be necessary to form the VDAC-modulator complex, the chelator was added to both compartments prior to addition of the modulator-containing fraction to the *cis* side. The modulator was capable of acting on VDAC and producing an increased voltage dependence that was quite similar to that observed in the presence of Ca^{2+}.

Since EDTA has an affinity for Mg^{2+} that is similar to that for Ca^{2+} and since no Mg^{2+} was added to the solution, the free concentration of Mg^{2+} in the presence of the chelator must have been very low indeed. Thus Mg^{2+} is also not needed for the modulator action on VDAC.

4. Discussion

In the present study, we described the characterization and partial purification of the VDAC modulator from calf liver mitochondria. In addition, we also described a new modulator activity that dramatically decreases the total membrane conductance, inducing VDAC channels to close completely.

Purification was performed by a combination of preparative isoelectric focusing, gel filtration, and ion exchange HPLC. The fraction eluted from the anion exchange HPLC column has good activity and shows two major bands of 54 kDa and 42 kDa on SDS-PAGE with silver staining. Samples obtained after isoelectric focusing and gel filtration chromatography showed a few major bands. Comparing the results from the two purification procedures, the 54 kDa protein was found in the active fractions from both processes, suggesting that the 54 kDa protein might be the source of VDAC modulator activity. Under reduced native conditions, the activity of the VDAC modulator was observed at a molecular mass of about 100 kDa by gel filtration. A dimer is possible for the native VDAC modulator.

An estimate of the degree of purification achieved by the procedures described here can be made. The assay used to detect the VDAC modulator is only semi-quantitative, but by determining the amount of protein that must be added to observe an effect one can obtain some estimate of 'purity' and 'specific activity'. Active samples from the ion exchange HPLC column resulted in clear modulator effects on VDAC channels when 800 ng of protein (assayed by the micro BCA system of Pierce) were added to 4 ml of solution bathing the membrane. If all the protein were VDAC modulator (100 kDa) this would represent 8 pmol. This compares favorably with the total amount of VDAC added to the chamber, approximately 0.3 pmol (only a very small fraction actually inserts into the planar membrane). If the modulator were all in solution (none bound to VDAC) then the concentration would be 2 nM. As compared to the amount of protein added from the ammonium sulfate fraction (essentially an extract of the proteins in the intermembrane space) to observe an effect on VDAC, the subsequent steps increased the specific activity a few hundred fold. This would correspond to a purification over total mitochondrial protein of several thousand fold.

We have observed two isoelectric forms of the VDAC modulator. One has $pI = 4.8$, the other has pI 5.1. The fraction at pI 4.8 has a much stronger effect on VDAC channels (almost totally close VDAC channels) compared to the one described previously [28–30]. There are two possibilities to explain the observations. One is that two modulating proteins coexist, each acting on VDAC channels in different ways. These two proteins may work together, increasing the voltage dependence of VDAC channels, and inducing channel closure totally if necessary. Another possibility is that the VDAC modulator exists in two forms resulting from the action of some protein modification process,

such as phosphorylation. The modified form of the modulator may have more negative charges, perhaps resulting in much stronger effects on VDAC. The relative abundance of these two forms could be regulated by enzymatic activity, such as the levels of protein kinases and phosphatases, in response to cellular requirements.

VDAC channels are found in the mitochondrial outer membrane of all eukaryotes. Both the structure and electrophysiological properties are very conserved in VDACs from organisms studied so far [3,16]. A relatively small protein (31 kDa) forms a large aqueous pore (about 3 nm in diameter) [12,16,39] in outer mitochondrial membrane, providing a major permeability pathway for negatively-charged metabolites. Recently, there is immunofluorescence and electrophysiological evidence for the existence of VDAC channels in the plasma membranes of human skeletal muscle and human B lymphocytes [6,7,36–38]. The existence of VDAC channels in the plasma membranes may require a different regulatory mechanism compared to the channels found in the mitochondrial outer membrane. VDAC's high conductance requires a mechanism by which the channel may be totally closed. The new modulating activity we have described greatly decreased the channel's conductance and induced the channels to enter a totally-closed state with very little, if any, current passing through the pore. Therefore, the discovery of this potent intracellular VDAC modulator, together with the potential across plasma membrane, may provide a way for the cell to tolerate the existence of VDAC channels in the plasma membrane.

In this study, we used proteins from the intermembrane space of mitochondria as the starting material for further purification. One subcellular location of the VDAC modulator is the mitochondrial intermembrane space [47]. However, since VDAC channels were reported to be found in the plasma membrane, it is possible that the VDAC modulator or a similar modulating protein may also be present in the cytoplasmic compartment. The exact subcellular location needs to be investigated in the future by immunocytochemical techniques with antibodies against the purified VDAC modulator.

A change in medium pH could result in charge changes both on the modulator and VDAC channels. These charge changes may affect the electrostatic interactions between the VDAC channel and its modulator. Although there is no strong pH dependence of modulator activity from pH 7 to pH 9 in the reconstituted system, the pH does play a role from 5 to 6. It indicates that electrostatic interaction could be one of the forces between VDAC and its modulator.

The interaction between the VDAC channel and its modulator seems to be very stable because attempts to wash out the modulator were unsuccessful. This is surprising because we can extract modulator from mitochondria simply by tearing the outer membrane with a hypotonic solution. In addition, VDAC channels solubilized from the mitochondrial membranes obtained after hypotonic lysis behave normally. Their voltage dependence is unaffected by the addition of pronase, indicating that they do not have modulator bound to them. It may be that the detergent dissociates VDAC from its modulator. The detergent may even inactivate the very sensitive modulator protein. In addition, in isolating mitochondrial membranes as a source of VDAC, it is a standard procedure not to use proteinase inhibitors. Thus, the modulator bound to VDAC may inadvertently be destroyed. Finally, the VDAC reconstitution methods select for VDAC that inserts into the membrane. This selection may exclude VDAC molecules bound to the VDAC modulator.

VDAC channels can exist in different conformation states with different selectivities and permeabilities. In the open state, the channel is anion-selective. In the closed states, VDAC not only has a smaller pore size and pore volume [9–11], but also has a reversed selectivity. The closed state of VDAC channels could hinder the permeation of negatively-charged metabolites (such as ATP and ADP) across outer membrane [13,15]. VDAC channels have also been identified as a reversible binding sites for hexokinase [17,18], glycerol kinase [17], and creatine kinase [40]. The bound hexokinase may have preferred access to intramitochondrial ATP [41,42], although this is not universally accepted [43]. Therefore, it is possible that metabolic 'channeling' of ATP may depend on VDAC's conformational states. There are also indications that VDAC is a component of a mitochondrial benzodiazepine receptor complex [44]. It would be interesting to find out how the gating of VDAC affects the binding of kinases and the function of the peripheral benzodiazepine receptor in the intact cell. The isolation and partial purification of one VDAC modulating protein, which can dramatically affect the gating behavior of VDAC channels, should continue to contribute to our understanding of the regulation of mitochondrial metabolism.

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