Plant Physiol. (1993) 101: 579–587

Purification and Characterization of the Voltage-Dependent Anion-Selective Channel Protein from Wheat Mitochondrial Membranes[1]

Amnon Blumenthal, Kathy Kahn, Oded Beja, Esra Galun, Marco Colombini, and Adina Breiman*

Department of Plant Genetics (A.B., E.G.) and Department of Membrane Research and Biophysics (O.B.), The Weizmann Institute of Science, Rehovot 76100, Israel; Department of Field and Vegetable Crops, Faculty of Agriculture, The Hebrew University, Rehovot 76100, Israel (K.K.); Laboratories of Cell Biology, Department of Zoology, University of Maryland, College Park, Maryland 20742 (M.C.); and Department of Botany, Tel Aviv University, Tel Aviv 69978, Israel (A. Breiman)

An approximately 29-kD protein was purified from the membrane fraction of wheat (*Triticum aestivum* cv Dganit) mitochondria by the utilization of standard liquid chromatography techniques. The protein, designated MmP29 for mitochondrial membrane protein having a molecular mass of approximately 29 kD, exhibited cationic properties in a buffering solution, adjusted to pH 7.5. This positive charge enabled its passage through a diethylaminoethyl column, without interaction with the positively charged matrix. Subsequently, this protein was separated from the remaining polypeptides by a preferential elution from a hydroxylapatite/celite mixed column. Reconstituted liposomes containing this protein were characterized as being permeable to 8-aminonaphthalene 1,3,6-trisulfonic acid disodium salt (M_r 445) but nonpermeable to dextran fluorescein (M_r 40,000). Additionally, MmP29 was inserted into planar phospholipid membranes, and anion-selective, voltage-dependent channels were demonstrated. All of the MmP29 properties mentioned highly resemble voltage-dependent, anion-selective channel (VDAC) proteins, suggesting that MmP29 is the mitochondrial outer membrane VDAC protein of wheat.

A major protein of the outer membrane of mitochondria that forms channels with selective permeability for hydrophilic, small to medium size metabolites has been identified in all studied eukaryotic organisms (Schein et al., 1976; Colombini, 1979; Zalman et al., 1980; Mannella et al., 1983; Benz, 1985; Colombini, 1989). Some investigators referred to this mitochondrial protein as "porin" (Mihara and Sato, 1985; Kleene et al., 1987). Evidence for the existence of these channels was based on EM (Parsons et al., 1965; Mannella, 1982; Mannella and Frank, 1984) and x-ray diffraction studies (Mannella and Bonner, 1975; Mannella, 1981). Additionally, this mitochondrial protein behaves as a VDAC (Schein et al., 1976; Colombini, 1979). The properties of VDACs were demonstrated by electric channel conductance studies (Schein et al., 1976; Colombini, 1980; Freitag et al., 1982; Roos et al., 1982). Furthermore, VDACs could be successfully integrated within liposomal membranes (Zalman et al., 1980; Lindén et al., 1982). These VDAC-embedded liposomes displayed in vitro pore formation characteristics by exhibiting permeability for small hydrophilic compounds. Liposome-swelling and leakage studies were used to follow VDAC properties (Zalman et al., 1980; Lindén et al., 1982). From these studies it was surmised that the exclusion limit for uncharged aqueous solutes lies between 2000 and 6000 D.

Despite the relative comprehensive understanding of fungal and mammalian VDACs (Mihara and Sato, 1985; Kleene et al., 1987; Benz et al., 1989; De Pinto et al., 1989; Blachly-Dyson et al., 1990), there is little information concerning plant mitochondrial VDACs. Evidence for the existence of pores in the outer membrane of plant mitochondria was reported (Parsons et al., 1965), but plant mitochondrial VDAC was partially characterized only in 1980 (Zalman et al., 1980), when a molecular size-dependent leakage was found through channels formed in the phospholipid membrane of vesicles reconstituted with an enriched preparation of the major outer mitochondrial protein of mung bean (*Phaseolus aureus*). A further characterization of mitochondrial membrane preparation from corn (*Zea mays*) seedlings exhibiting VDAC properties was reported by Smack and Colombini (1985).

VDACs may have an especially vital role in plants due to some unique metabolic processes in their mitochondria. These processes include several alternative respiration pathways, sophisticated matrix dehydrogenases, glycine oxidation, matrix-located malic enzyme, and the existence of inner membrane carriers for oxaloacetate, aspartate, and glutamate (Douce and Neuburger, 1989).

We report the experimental methods that enabled the purification of a plant (*Triticum aestivum*) mitochondrial protein exhibiting VDAC characteristics and designated as MmP29. We also describe the utilization of a fluorescence technique for testing the leakage caused by the channel-forming properties of this purified protein while it is inte-

[1] This study was supported by the Dr. J. Cohn Center for Biomembrane Research and by the German Israeli Foundation Grant to A. Breiman.

* Corresponding author; fax 972-3-6409380.

Abbreviations: ANTS, 8-aminonaphthalene 1,3,6-trisulfonic acid disodium salt; HTP, hydroxylapatite; nS, nanosiemens; VDAC, voltage-dependent anion-selective channel.

grated within the liposomal bilayer. This highly sensitive fluorescence methodology enabled us to carry out reconstitution experiments despite the minute amounts of MmP29 (10 ng) initially available. Moreover, the spectrofluorimetric techniques served as a quality control method, suitable for detecting nonspecific leakage from the liposomes. This experimental tool furnished a more advanced approach over the previously used radioactive methods, resulting in a better way to build up a reliable vesicle reconstitution protocol.

MATERIALS AND METHODS

Chemicals

Analytical grade reagents were utilized in all experimental procedures. Percoll was obtained from Pharmacia; octyl glucoside was obtained from Chemex or Sigma; hydroxylapatite was obtained from Bio-Rad; celite 535 was obtained from Fluka; DE52 was obtained from Whatman; asolectin (45% 1-α phosphatidylcholine) and dicetylphosphate were obtained from Sigma; Sepharose 4B was obtained from Pharmacia; ANTS, dextran fluorescein, and anti-fluorescein antibody were obtained from Molecular Probes.

Mass Production of Aseptic Wheat Etiolated Seedlings

A total of 400 g of *Triticum aestivum* cv Dganit seeds were layered on eight rectangular gauze pieces, each fastened between two stainless steel screens. The entrapped seeds were surface sterilized to minimize fungal (mold) spore germination and subsequent mycelia development. This was accomplished by bathing the entrapped seeds in a 3% sodium hypochloride solution for 30 min under continuous stirring. The sodium hypochloride solution was rinsed extensively by spraying the devices with a jet of distilled and filter-sterilized water. Subsequently, each of the eight screen "sandwiches," containing the disinfected seeds, was transferred aseptically onto a fine plastic screen (2-mm mesh). The latter were put on top of presterilized wetted vermiculite in 30- × 40-cm plastic containers. After 4 d the seedlings exhibiting an average of 4-cm-long coleoptiles and 8-cm-long roots were removed from the containers. Coleoptiles (200 g) were collected manually (with gloves), leaving 100 g of roots to be harvested by electrical hair clippers. The germinated seeds were kept entrapped within the sandwiches, by which means a massive contribution of undesired proteases to the extraction buffer was prevented.

Isolation of Wheat Mitochondria

Wheat (*T. aestivum*) mitochondria were initially isolated from etiolated seedlings and later mainly from wheat germ, because the wheat germ resulted in larger mitochondrial yields.

Isolation of Mitochondria from Seedlings

Organelles (including mitochondria, which are highly contaminated with proplastides and to a minor extent with peroxisomes) were isolated from 100 g of roots and 200 g of coleoptiles (including the enclosed first leaf). This initial step was carried out by scaling up protocols for organelle isolation according to the method of Boutry et al. (1984). The highly enriched mitochondrial fraction, having 99% intactness (as determined by utilizing the Cyt *c* oxidation reaction), was separated from the rest of the organelles by a self-generating Percoll gradient, according to the method of Douce et al. (1987). The mitochondrial pellets from roots and coleoptiles contained approximately 5 and 10 mg of protein, respectively, as determined by the Bradford (1976) method.

Isolation of Mitochondria from Wheat Germ

An enriched mitochondrial pellet containing approximately 100 mg of protein, as determined by the Bradford (1976) method, was isolated from 100 g of wheat germ (*T. aestivum*) by slightly modifying the procedures of Peiffer et al. (1990). In our modified version, the mitochondria-enriched loose pellet, found on top of a tight pellet being generated after the first 13,000*g* centrifugation step, was resuspended in the supernatant left after the same 13,000*g* centrifugation step, resulting in doubling the mitochondrial yield from the quantity achieved by the original Peiffer et al. (1990) protocol.

Protein Purification Procedures

The procedures were based on those of De Pinto et al. (1989) with some modifications. Mitochondria (100 mg of protein) were resuspended in 150 mL of a hypotonic solution (10 mM Tris, 1 mM EDTA, pH 7.5), ruptured during 20 min at −20°C, thawed, homogenized in a hand homogenizer, and centrifuged (30 min, 120,000*g*). This procedure was repeated, resulting in the isolation of an enriched membrane preparation (25 mg of protein). This total amount of membrane proteins was solubilized by resuspending the pellet in 150 mL of a solution containing 2% octyl glucoside, 10 mM Tris, and 1 mM EDTA (pH 8.0). Proteins were released into the solution by gently shaking the suspension for 60 min at 2°C. Nonsolubilized material was pelleted (30 min, 120,000*g*). The supernatant, containing 145 mL of solubilized membrane proteins, was loaded onto a column consisting of 35 g of DE52 matrix, preequilibrated with the same solubilization buffer except for its pH value, which was adjusted to 7.5. Subsequently, an additional volume of 90 mL of the same equilibration buffer was applied for washing out any nonbound protein molecules still entrapped within the column.

Proteins that did not interact with the column started to elute after loading 35 mL of the solubilized membrane proteins. Subsequently, the resulting pool of 200 mL of protein solution was loaded onto a 14-g HTP/14-g celite column that was preequilibrated with the same buffer as the previous DE52 column. Immediately after the column was loaded, the buffer composition was supplemented with 25 mM KCl and 5 mM KH_2PO_4, and the column was washed with 150 mL of this buffer to induce the release of only moderately bound protein molecules. Aliquots were used to analyze the protein profiles by one-dimensional SDS-12.5% PAGE (Laemmli, 1970). Quantitative protein analysis was based on densitometric measurements taken with a Molecular Dynamics 300A Computing Densitometer equipped with a 633-nm laser beam.

Reconstitution of Liposomes

Two aliquots of 600 μL, one containing 1.2 μg of MmP29 (the putative VDAC protein), shown in Figure 1C, and the other containing 1.2 μg of a nonrelated 38-kD protein released from the HTP/celite column by a further increase of KCl and KH_2PO_4 concentrations (not shown), were dialyzed for 24 h (three changes) against 200 mL of 1 mM Hepes, 0.1 mM $MgCl_2$ (pH 7.4) in the presence of 150 μg of soybean asolectin (45% 1-α phosphatidylcholine) and 1.9 μg of dicetylphosphate, the latter for imposing a slight negative charge over the liposomal membrane. The evolving liposomes were disrupted, and subsequently new vesicles were reconstituted by utilizing the "dried reconstituted vesicles" technique (Kirby and Gregoriadis, 1984). During this reconstitution stage a mixture of two fluorophores, titrated to pH 7.4, were entrapped within the liposomes. The smaller fluorophore, ANTS (M_r 445 and negatively charged), should diffuse through VDAC channels, whereas the other fluorophore, dextran fluorescein (M_r 40,000), is not expected to diffuse through these channels. At the completion of the reconstitution step the entrapped fluorophore concentration was 50 mM for ANTS and 0.1 mM for dextran fluorescein. The separation of liposomes from the free fluorescent dyes was performed according to the method of Zalman et al. (1980), who utilized this method for the separation of vesicles from the free radioactive sucrose-dextran mixture. To minimize the disruption of vesicles during this separation step, it was essential to reach isoosmolarity with the entrapment mixture (approximately 350 mOsM) by the addition of NaCl to the elution buffer. Osmotic measurements were conducted with a Knauer digital osmometer.

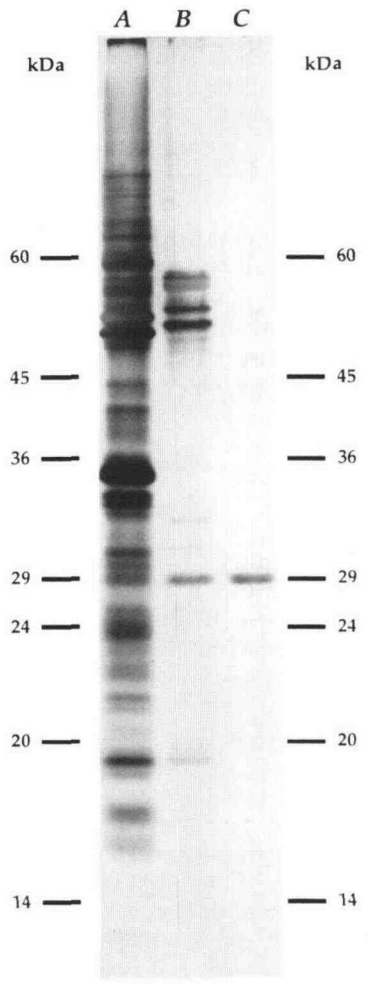

Figure 1. Three major steps in the purification of VDAC from etiolated wheat seedlings as analyzed by SDS-PAGE. Detergent-solubilized mitochondrial membrane proteins (lane A) were loaded on a DE52 column equilibrated to pH 7.5. Proteins not interacting with the column matrix (lane B) were loaded on an HTP/celite column. As a result of subsequent inclusion of 25 mM KCl and 5 mM KH_2PO_4 in the equilibration buffer, a single 29-kD protein (MmP29), characterized later as the putative wheat mitochondrial VDAC, was eluted (lane C). Protein bands were silver stained (Blum et al., 1987). The protein band shown in lane C is equivalent to 500 ng.

Determination of Liposomal Membrane Permeability

Spectrofluorimetric techniques were applied to monitor VDAC-induced membrane permeability. These fluorescence measurements were carried out in a Perkin-Elmer LS-5B spectrofluorimeter, enabling evaluation of differences in membrane permeability between liposomes reconstituted with either the putative VDAC protein or with the 38-kD protein. The fluorescence properties of the two fluorophores utilized in this study are as follows: dextran fluorescein, 485 nm excitation and 515 nm emission; ANTS, 370 nm excitation and 515 nm emission.

Liposomal fractions taken for the fluorescence measurements should have been practically free of nonentrapped fluorophores. There was a possibility that dextran fluorescein and/or ANTS might be present free in the solution surrounding the liposomes due to adhesion of the fluorophores to the vesicles, leakage through unstabilized or damaged liposomal membranes, or inadequate Sepharose 4B gel filtration separation of the free fluorophores from the vesicles after the entrapment event took place. A reliable quantitative comparison between vesicles reconstituted with either the putative VDAC protein or with the 38-kD protein could be achieved only under conditions avoiding these possibilities. To ascertain the fulfillment of this requirement, two assays were conducted, using the vesicles reconstituted with the 38-kD protein. The first test was carried out to determine the percentage of free dextran fluorescein by utilizing anti-fluorescein antibodies that are cross-reactive against antibody-accessible dextran fluorescein present outside the liposomes. According to the manufacturer's protocol, antigen-antibody complex formed by interaction of 1.5 pmol of fluorescein with 20 μL or more (one manufacturer unit) of this antibody results in 90% quenching of the fluorescein emission. The second test was carried out to determine the percentage of free ANTS, present as a result of adhesion to the vesicles, by mixing "empty" liposomes with 50 mM ANTS solution, followed by passage of this mixture through a Sepharose 4B column.

Based on calculations undertaken after performing these assays, it was found that, by following Sepharose 4B column

Table I. *Purification of VDAC from wheat germ*

Purification Step	Volume	Total Protein	VDAC	Purification	Yield
	mL	*μg*	*μg*	*-fold*[a]	%
Solubilized mitochondrial membranes	145	20,000	300	1	100[b]
DE52 pass through	200	3,000	270	6	90
HTP/celite elution	40	120	120	66	40

[a] The actual purification fold starting from crude cell extract is much higher and has not been determined in this study. [b] Assuming that all of the VDAC was solubilized by the detergent solution.

and elution volumes as described by Zalman et al. (1980), the second 0.5-mL liposomal fraction eluted from the column included low quantities (<10%) of free dextran fluorescein and an undetectable level of adhered ANTS. Moreover, this fraction is the most liposome-enriched one, resulting in cumulative high emission values of the entrapped fluorophores. These low quantities of free dextran fluorescein and undetectable level of ANTS indicated little or no dextran fluorescein and ANTS adhesion, adequate Sepharose 4B separation, and liposomal membrane intactness. Based on these data, we decided to utilize exclusively the second 0.5-mL fractions corresponding to liposomes including approximately 0.8 μg of either MmP29 or 38-kD protein. Data were recorded and later averaged from three independent experiments, assaying membrane permeability of liposomes that were reconstituted with proteins from three separate mitochondrial isolations and purifications.

Generation of Planar Lipid Bilayers and Assay of the Purified MmP29

Planar phospholipid membranes were made by the monolayer method of Montal and Mueller (1972), as previously described (Colombini, 1987). The membranes were made using soybean phospholipids supplemented with 20% (w/v) cholesterol. The aqueous phases were either 1 M KCl, 5 mM $CaCl_2$, or 1 mM Mes (pH 5.8) on both sides of the membrane or 1 M KCl on one side and 0.1 M KCl on the other side (same Ca and Mes). The latter was used for selectivity measurements. Calomel electrodes were used, the voltage was clamped, and the current through the membrane was measured.

MmP29, purified from wheat germ to be inserted into the planar membrane, was treated as follows: 9 μg of protein, solubilized in 4.5 mL of the HTP/celite elution buffer, were dialyzed for 36 h against 4 L of 1 mM KCl, 1 mM Hepes, 0.02% sodium azide (pH 7.0) in the presence of 200 μg of asolectin, and 40 μg cholesterol to remove the octyl glucoside detergent. The liposomes containing MmP29 were pelleted (30 min, 120,000*g*). These vesicles were resuspended in 400 μL of the dialyzing solution, followed by the addition of DMSO to a final concentration of 15%. The liposomal suspension just prepared was aliquoted and frozen at −20°C. Aliquots of 10 to 20 μL, including 225 to 450 ng of protein, to which Triton X-100 was added just before the assays to a final concentration of 1%, were utilized in the various electrical conductance experiments. In experiments in which a 10-fold gradient of KCl was utilized, aliquots were added to the 1 M KCl aqueous phase. Thawed aliquots were kept on ice before utilization. The electrophysiological assays were carried out at room temperature.

RESULTS

Protein Purification

Mitochondrial membranes prepared during this study were derived from two different sources—seedlings and wheat germ. A complex mixture of proteins resulted from solubilizing the mitochondrial membranes from either source with the octyl glucoside detergent. The mixture included a 29-kD protein at about 1.5% abundance. (Fig. 1A). This heterogenic protein blend was applied atop the anion exchanger DE52, resulting in retardation of the majority of these proteins within the column. Only 7 to 10 positively charged different major proteins, including the 29-kD protein at approximately 9% abundance, passed through the column under the applied conditions (Fig. 1B). The majority of the 29-kD protein-detergent micelles did not interact with the *o*-DEAE cellulose matrix, resulting in a high 29-kD protein yield (90%) at this purification step (Table I).

Subsequently, all of these proteins (Fig. 1B) were bound to the HTP/celite column. Only MmP29, the putative VDAC protein, was released within the last 40 mL (out of 150 mL) by washing the column with 25 mM KCl, 5 mM KH_2PO_4 elution buffer (Fig. 1C). As opposed to the remarkably high 29-kD protein yield at the DE52 purification step, MmP29 eluted only partially (approximately 45%) from the HTP/celite column at these KCl and KH_2PO_4 salt concentrations (Table I). The remaining 55% of the 29-kD protein eluted only when higher salt concentrations were applied to the column, accompanied by the majority of the other proteins (not shown). Variations in protein profiles between the two different sources—seedlings and wheat germ—throughout the purification procedure were insignificant.

VDAC Pore-Forming Properties as Determined from the Reconstituted Vesicles

Liposomes entrapping ANTS and dextran fluorescein were reconstituted with either the 38-kD protein or with MmP29. Vesicles were separated from the free fluorophores by means of gel filtration. The entire volumes of the second fractions

eluted from columns were measured for the fluorescence emission of ANTS and dextran fluorescein. No significant differences in dextran fluorescein-retaining properties could be found between the two populations of vesicles, as demonstrated in Figure 2. However, liposomes reconstituted with MmP29 included only 22% of entrapped ANTS compared to ANTS retained in liposomes reconstituted with the 38-kD protein. This drastic reduction in ANTS content, which accompanied the vesicles reconstituted with MmP29, clearly indicated an extensive leakage of this relatively small compound (M_r 445) through well-defined membrane channels, which were not wide enough to enable the diffusion of dextran fluorescein. These results strongly suggested that MmP29 is the wheat mitochondrial VDAC.

Measurements of Channel-Forming Activity as Determined from the Planar Membrane Assays

Channels with properties essentially indistinguishable from those of VDAC channels from other sources such as *Paramecium aurelia* (Schein et al., 1976), *Neurospora crassa* (Freitag et al., 1982), *Rattus norvegicus* liver (Colombini, 1983), *Zea mays* root (Smack and Colombini, 1985), or *Saccharomyces cerevisiae* (Forte et al., 1987) were observed when MmP29 was reconstituted into planar phospholipid membranes. Like other VDAC channels, MmP29 inserted spontaneously when 10 to 20 μL of a Triton X-100-solubilized sample (1% v/v) was added to the aqueous solution (about 4 mL) on one side of the planar membrane.

Figure 3A shows a recording of current flow through a membrane in response to voltage applied in the form of a triangular wave. The current flowing through the membrane was essentially zero until one channel inserted (indicated by arrow). As the voltage continued to increase, the current through the channel formed a straight line with a slope that yielded a conductance value of 4.1 nS. This conductance is typical for a single VDAC channel (Table II). Just beyond the peak of the applied voltage (41 mV) as the voltage was being reduced, the channel closes (downward deflection) to a low-conducting, "closed" state. The slope of the current record was greatly diminished but not zero, indicating a low-conducting state. The channel reopened briefly (transient upward spike), and afterward it reopened (upward deflection) for an extended period (constant slope). The channel did not close again until the applied voltage was well into the negative range (upward deflection toward zero current). As was the case for closure at the elevated positive potentials, closure at negative potentials resulted in a low-conducting state rather than zero conductance. The channel reopened (downward deflection) as the applied voltage returned toward zero. Thus, as is the case for VDACs from other sources such as *R. norvegicus* (Colombini, 1979), *Z. mays* (Smack and Colombini, 1985), or *S. cerevisiae* (Forte et al., 1987), MmP29 produces a channel that closes at both positive and negative potentials.

Experiments performed as shown in Figure 3A except with multichannel membranes yielded quantitative estimates concerning the voltage dependence of MmP29. The steepness of the voltage dependence was estimated by fitting the data to a two-state model as previously described (Schein et al., 1976). The results yielded a value consistent with three charges on the channels' voltage sensor moving through the entire potential difference. The location of the switching region (the voltage at which half the channels are closed and half open) was between 20 and 25 mV for closure at both positive and negative potentials at a concentration of 1 M KCl on each side of the membrane. The switching region location is very similar to those of other VDACs studied so far (Table II). These estimates were made on recordings in which the voltage was being decreased in absolute values (channel opening) to avoid the kinetic delays inherent in the much slower closure process, also found in other VDAC channels being tested (Schein et al., 1976; Colombini, 1979; Smack and Colombini, 1985; Colombini et al., 1987).

The experiments shown in Figure 3, B and C, were performed in the presence of a 10-fold gradient of KCl to assess the ion selectivity. In Figure 3B, the membrane before channel insertion (far left) was highly impermeable to ions as shown by the lack of change in the current recording (current level remained essentially at zero) following the application of a −20-mV voltage step. Soon after the applied voltage was

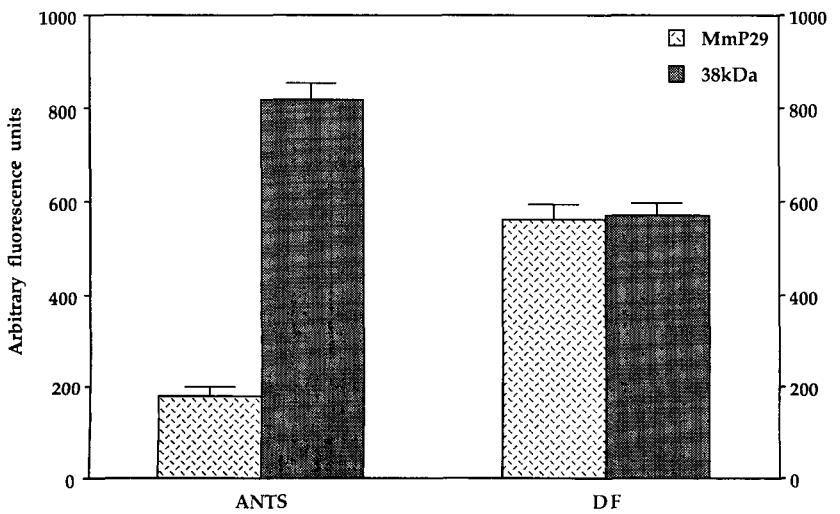

Figure 2. Fluorescence of ANTS and dextran fluorescein (DF) emitted from vesicles reconstituted with either MmP29 or a 38-kD protein. Both reconstitution assays included 150 μg of soybean asolectin, 1.9 μg of dicetylphosphate, and 1.2 μg of protein in 200 μL of Hepes buffering solution. During the reconstitution procedure 50 mM ANTS and 0.1 mM dextran fluorescein (DF) were entrapped within liposomes. Liposomes were separated from free dyes by loading the suspensions on Sepharose 4B gel filtration columns. (For detailed techniques refer to "Materials and Methods"). Bars represents SE.

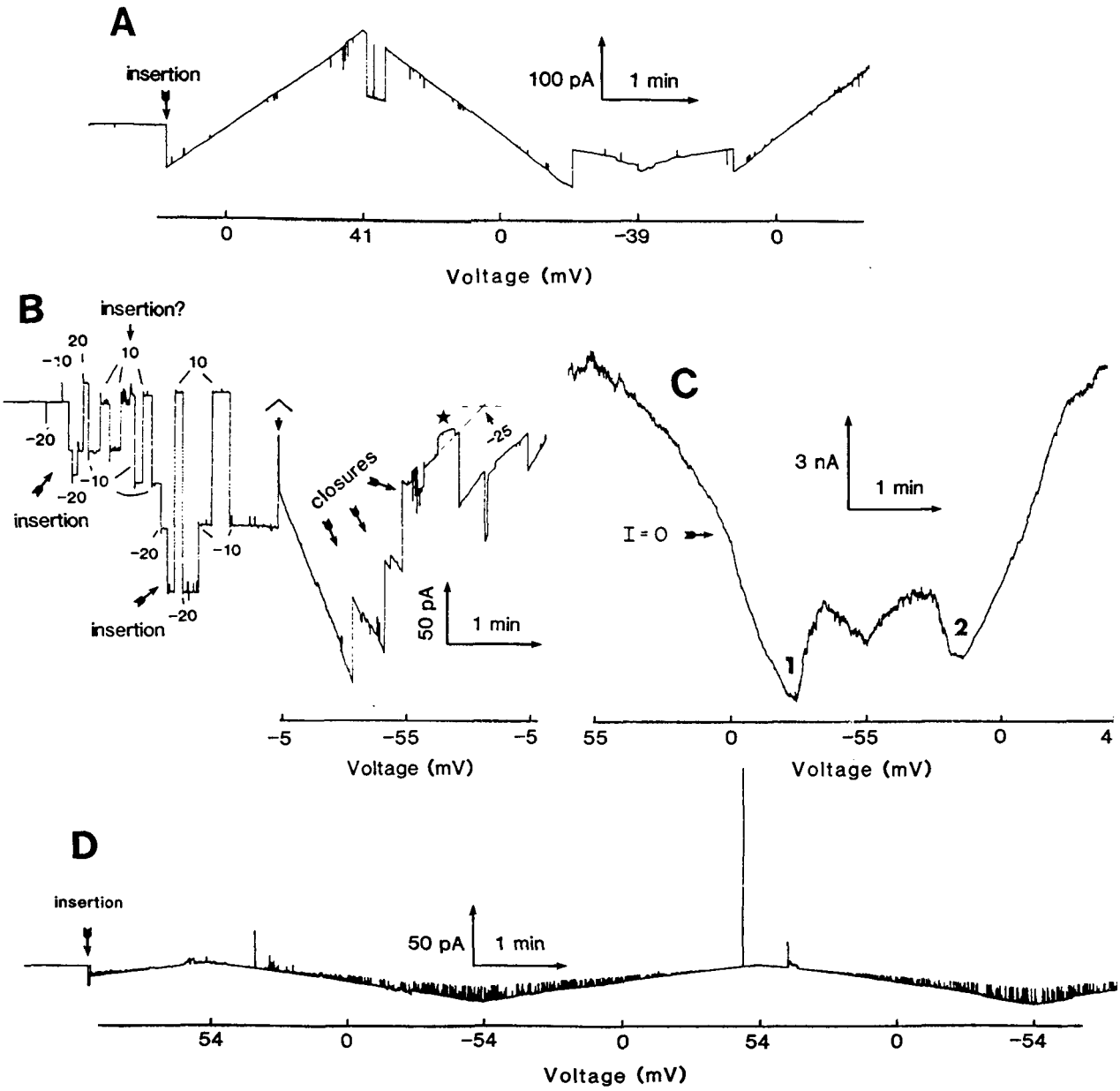

Figure 3. Examples of conductances observed after reconstitution of MmP29, purified from wheat germ, into planar phospholipid membranes. The membranes were made in the presence of solutions containing the indicated amount of KCl in addition to 5 mM $CaCl_2$, 1 mM Mes, pH 5.8. A, Recording of current flowing through a single channel inserted in a membrane (medium, 1 M KCl on both sides) in response to a triangular voltage wave. B, Multiple channels inserted into a membrane (medium, 1 M KCl versus 0.1 M KCl). On the left side the indicated voltage (in mV) was applied as steps. At the point indicated by the caret, a triangular voltage wave was applied, resulting in channel closure as indicated. Extrapolation of the slope of the closed-channel current (dotted line) intersected with the zero-current line at 25 mV. C, Same conditions as in B except that the membrane contained many channels. D, Same conditions as in B except that the membrane contained one conducting unit.

switched to −10 mV, a spontaneous downward step occurred. This is the insertion of a single channel. The voltage was then switched to the values indicated to find the voltage that would bring the current to zero, the reversal potential. From the current levels at the different voltages we calculate the reversal potential for the first channel inserted to be 10.8 mV positive on the high-salt side. This value illustrates again that the characteristics of these channels are the same as those of VDACs from other sources (Table II). This reversal potential value shows a weak selectivity for Cl^- over K^+ (about 2:1 if this value is fitted to the Goldman equation). After two more insertions, a triangular wave was applied. As the potential

Table II. *Properties of VDAC from various sources*

The various values were taken from Colombini (1989) except for the data corresponding to *T. aestivum* found during the present investigation.

Organisms	Single-Channel Conductance[a]	Selectivity at High-Conducting "Open" State (1 vs. 0.1 M KCl)	Voltage Dependence (V_0)
	nS	$P(Cl^-)/P(K^+)$	*mV*
Protist (*P. aurelia*)	0.55	1.9	20
Fungi			
N. crassa	4.5	1.9	23
S. cerevisiae	4.5	1.8	22
Mammal (*R. norvegicus*)	4.5; 4.4; 4.2	1.7; 1.8	31; 24; 21
Plants			
Z. mays	3.9	1.7	20
T. aestivum	4.1	2.0	~22

[a] All single-channel conductance measurements were recorded at 1 M KCl on both sides of the membrane, except for VDAC from *Paramecium*, for which single-channel conductance measurements were recorded at 0.1 M KCl.

became more negative, three large upward steps in the current record occurred (up toward zero current), marked "closures" in Figure 3B, corresponding to the closure of each of the three channels. Soon after the third channel closed, the voltage wave hit its minimum and began to increase again. The slope of the current during this increasing voltage was extrapolated (dotted line) to the zero current level (horizontal dotted line). The intersection point corresponds to the reversal potential for the closed channels and was equal to −25 mV. This demonstrates that, like VDAC from fungal (Ludwig et al., 1988), protist (Ludwig et al., 1989), and mammalian (Colombini, 1983) sources, the selectivity of the closed states of these plant mitochondrial channels is for cations. Note that a different closed state (indicated with an asterisk), whose conductance is still lower (lower slope), is achieved for a short time. The selectivity switch upon channel closure is believed to be important in making the closed state of VDAC essentially impermeable to organic ions such as ATP (Liu and Colombini, 1992).

The behavior of the large population of channels illustrated in Figure 3C shows that MmP29-containing samples can insert many channels into a membrane whose properties are very similar to phospholipid membranes previously utilized to reconstitute VDAC from different organisms. This behavior also illustrates that the characteristics of these channels are the same as those of VDAC from other sources. Despite the low frequency of the triangular voltage wave used, a clear hysteresis is observed in the record. The channels close at higher potentials, either positive or negative, than those at which they reopen (the deeper trough on the left marked "1," as compared to the trough on the right side of the figure marked "2," shows closing and reopening of channels at negative potentials). This is due to the large difference in the rates of closure as compared to reopening, the latter being much faster. The channels tend to be delayed in closing, and therefore, they close at more negative potentials. Similar hysteresis can be demonstrated for positive potentials.

In examining the conductances induced in planar membranes by MmP29, other than conductances attributed to membrane instability, we could recognize most conductances as having behaviors characteristic of VDAC. However, there was one notable exception found in 1 of 10 assays (Fig. 3D). This record shows a conductance that is much more selective for Cl^- than for K^+ (observed in the presence of a salt gradient) and displayed a very different gating behavior. Noting that the straight current line at the far left is at zero current (before the insertion of the single channel), the reversal potential of the conductance is at a rather high positive potential (approximately 48 mV), indicating rather high selectivity for Cl^- over K^+. The nature and source of this observation is unknown.

DISCUSSION

Intensive research has been carried out during the last 15 years that has resulted in a comprehensive characterization of the fungal mitochondrial VDAC (Forte et al., 1987; Blachly-Dyson et al., 1990; Kiebler et al., 1990). In studies involving mainly *S. cerevisiae* and *N. crassa*, the VDAC proteins were purified (Freitag et al., 1982; Mihara and Sato, 1985; Forte et al., 1987), and their electrophysiological properties were revealed (Colombini, 1979; Freitag et al., 1982). These investigations have been accompanied by liposome-leakage assays (Liu and Colombini, 1992). Additionally, great progress has been made in deducing the amino acid sequence of these VDACs by the isolation of their respective cDNAs (Mihara and Sato, 1985; Kleene et al., 1987). By transfecting the VDAC cDNA into a VDAC-deleted yeast strain and regaining complementation of the VDAC activity, Blachly-Dyson et al. (1990) verified that the previously purified 29-kD protein is indeed the cause of the VDAC activity. By imposing site-directed mutagenesis on the yeast VDAC cDNA and by transfecting the modified genes into a VDAC-deleted yeast strain, they (Blachly-Dyson et al., 1990) determined the protein regions responsible for the unique VDAC properties. Moreover, a three-dimensional structure model of

the protein molecule was developed based on the results gained from these experiments (Blachly-Dyson et al., 1990). Finally, a major contribution has been made to the understanding of the VDAC targeting and import into mitochondria by the isolation of several important receptors that are related to these processes (Kiebler et al., 1990).

In parallel to the studies of fungal VDAC, somewhat less intensive work was conducted on the mammalian VDAC. The electrophysiological properties of these VDACs were found to be almost identical with those of fungal organisms (Colombini, 1979; Colombini, 1983). The respective mammalian protein was purified (Colombini, 1983; De Pinto et al., 1989), and an import experiment of an in vitro translated precursor into mitochondria was performed (Ono and Tuboi, 1987). Moreover, a set of studies was carried out involving VDAC's compartmentation among mitochondria of specific organs and between morphologically different types of mitochondria within the same organ. Interesting findings were revealed concerning the involvement of VDAC in mitochondria of highly metabolic organs (brain or tumor tissues). VDAC exhibited receptor properties for a specific hexokinase, possibly capable of elevating metabolism by acting as a sink for ATP molecules diffusing throughout the mitochondrial outer membrane channels (Nakashima et al., 1986).

In comparison to the vast efforts made to gain a better understanding of VDACs from fungal and mammalian sources, very limited efforts were invested to extend our knowledge of VDACs in plants. Zalman et al. (1980) demonstrated pore activity in their liposomal preparations but were unable to determine unequivocally which of the outer membrane mitochondrial proteins isolated from mung bean hypocotyls is responsible for this activity. Smack and Colombini (1985) demonstrated a VDAC activity originating from corn mitochondrial membranes but did not extend this research to isolation of the maize VDAC protein.

In this study we succeeded for the first time in purifying a plant VDAC to homogeneity and revealed several of its properties by utilizing in vitro electrophysiological tools and a highly sensitive in vitro fluorescence liposome-leakage method. However, because MmP29 was purified from total mitochondrial membranes, its localization on the outer membrane is not unequivocally demonstrated.

The purified VDAC protein, MmP29, displayed an estimated abundance of 0.3 to approximately 0.4%, out of the total mitochondrial proteins, which correlated very well to VDAC abundance found in fungal (Forte et al., 1987) and mammalian (Roos et al., 1982) mitochondria (0.2–0.4%). This value was calculated by multiplying the estimated abundance of 25% for the membrane proteins out of the total mitochondrial proteins (or 20% for the detergent-solubilized part) by 0.015, corresponding to about 1.5% abundance of the VDAC protein within the detergent-solubilized part (Fig. 1A), assuming that all of the 29-kD protein found within the detergent-solubilized membrane corresponded to MmP29. The protein demonstrated cationic properties (positive charge) at pH 7.5, as was demonstrated previously for *N. crassa* (Freitag et al., 1982), *R. norvegicus* (Lindén et al., 1982), and *S. cerevisiae* VDAC (Forte et al., 1987).

MmP29 channels were characterized by a selective permeability to small (M_r 445) molecules and impermeability to large (M_r 40,000) molecules. Vesicles reconstituted with MmP29 retained only 22% of ANTS as compared to ANTS retained within vesicles reconstituted with 38-kD protein (Fig. 2). The mathematical treatment involved in obtaining this value required a small correction to normalize for the same emission values of dextran fluorescein. In comparison, liposomes reconstituted with the mung bean mitochondrial outer membrane particles retained only 19% of [^{14}C]sucrose relative to vesicles reconstituted without proteins, as calculated from the work by Zalman et al. (1980). The permeability behavior of the mung bean mitochondrial outer membrane pore-forming protein did not differ significantly from that of the wheat MmP29 (19 and 22%, respectively, of the small probe retained). Moreover, both channels shared impermeability to the large probe tested ([^{3}H]dextran or dextran fluorescein). These findings strongly suggest that both channels belong to the mitochondrial outer membrane VDAC family.

MmP29 channels reconstituted in planar phospholipid bilayers demonstrated electrophysiological properties that were very similar to those of other VDACs studied so far. For a comprehensive summary and comparison of the electrophysiological properties of MmP29 channels to other VDACs, refer to Table II. The present investigation of wheat mitochondrial VDAC may open the way to more research on plant VDAC channels.

ACKNOWLEDGMENTS

We thank Professor Walter Neupert, Institut für Physiologische Chemie, Physikalische Biochemie und Zellbiologie der Ludwig-Maximilians-Universität, Munich, Germany, and Dr. Ruppert Pfaller, Department of Biology, University of California, San Diego, La Jolla, CA, for their guidance at the start of this project. We are grateful to Professor Haim Garty, Professor Carlos Gitler, Professor Meir Wilchek, Dr. Diana Bach, and Dr. Talia Miron (all from the Weizmann Institute of Sceince, Rehovot, Israel), for useful suggestions, and we thank Tehila Zelig and Abigail S. Hackam for technical assistance. An EMBO grant enabled A. Blumenthal to visit the laboratory of Professor Walter Neupert.

Received June 23, 1992; accepted October 8, 1992.

LITERATURE CITED

Benz R (1985) Porin from bacterial and mitochondrial outer membranes. Crit Rev Biochem **19:** 145–190

Benz R, Schmid A, Dihanich M (1989) Pores from mitochondrial outer membranes of yeast and a porin-deficient yeast mutant: a comparison. J Bioenerg Biomembr **21:** 439–450

Blachly-Dyson E, Peng S, Colombini M, Forte M (1990) Selectivity changes in site-directed mutants of the VDAC ion channel: structural implications. Science **247:** 1233–1236

Blum H, Beier H, Gross HJ (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis **8:** 93–99

Boutry M, Faber A-M, Charbonnier M, Briquet M (1984) Microanalysis of plant mitochondrial protein synthesis products: detection of variant polypeptides associated with cytoplasmatic male sterility. Plant Mol Biol **3:** 445–452

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Colombini M (1979) A candidate for the permeability pathway of the outer mitochondrial membrane. Nature **279:** 643–645

Colombini M (1980) Structure and mode of action of a voltage-dependent anion-selective channel (VDAC) located in the outer mitochondrial membrane. Ann NY Acad Sci **341:** 552–563

Colombini M (1983) Purification of VDAC (voltage-dependent anion-selective channel) from rat liver mitochondria. J Membr Biol **74:** 115–121

Colombini M (1987) Characterization of channels isolated from plant mitochondria. Methods Enzymol **148:** 465–475

Colombini M (1989) Voltage gating in the mitochondrial channel, VDAC. J Membr Biol **111:** 103–111

Colombini M, Yeung Cl, Tung J, König T (1987) The mitochondrial outer membrane channel, VDAC, is regulated by a synthetic polyanion. Biochim Biophys Acta **905:** 279–286

De Pinto V, Benz R, Palmieri F (1989) Interaction of non-classical detergents with the mitochondrial porin. Eur J Biochem **183:** 179–187

Douce R, Bourguignon J, Brouquisse R, Neuburger M (1987) Isolation of plant mitochondria: general principles and criteria of integrity. Methods Enzymol **148:** 403–415

Douce R, Neuburger M (1989) The uniqueness of plant mitochondria. Annu Rev Plant Physiol **40:** 371–414

Forte M, Adelsberger-Mangan D, Colombini M (1987) Purification and characterization of the voltage-dependent anion channel from the outer mitochondrial membrane of yeast. J Membr Biol **99:** 65–72

Freitag H, Neupert W, Benz R (1982) Purification and characterization of a pore protein of the outer mitochondrial membrane from *Neurospora crassa*. Eur J Biochem **123:** 629–636

Kiebler M, Pfaller R, Söllner T, Griffiths G, Horstmann H, Pfanner N, Neupert W (1990) Identification of a mitochondrial receptor complex required for recognition and membrane insertion of precursor proteins. Nature **348:** 610–616

Kirby C, Gregoriadis G (1984) Dehydration-rehydration vesicles: a simple method for high yield drug entrapment in liposomes. Biotechnology **2:** 979–984

Kleene R, Pfanner N, Pfaller R, Link TA, Sebald W, Neupert W, Tropschug M (1987) Mitochondrial porin of *Neurospora crassa*: cDNA cloning, *in vitro* expression and import into mitochondria. EMBO J **6:** 2627–2633

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Lindén M, Gellerfors P, Nelson BD (1982) Purification of a protein having pore forming activity from the rat liver mitochondrial outer membrane. Biochem J **208:** 77–82

Liu MY, Colombini M (1992) Regulation of mitochondrial respiration by controlling the permeability of the outer membrane through the mitochondrial channel, VDAC. Biochim Biophys Acta **1098:** 255–260

Ludwig O, Benz R, Schultz IE (1989) Porin of *Paramecium* mitochondria: isolation, characterization and ion selectivity of the closed state. Biochim Biophys Acta **978:** 319–327

Ludwig O, Krause J, Hay R, Benz R (1988) Purification and characterization of the pore forming protein of yeast mitochondrial outer membrane. Eur Biophys Biophys J **15:** 269–276

Mannella CA (1981) Structure of the outer mitochondrial membrane. Analysis of x-ray diffraction from the plant membrane. Biochim Biophys Acta **645:** 33–40

Mannella CA (1982) Structure of the outer mitochondrial membrane: ordered arrays of porelike subunits in outer-membrane fractions from *Neurospora crassa* mitochondria. J Cell Biol **94:** 680–687

Mannella CA, Bonner WD (1975) X-ray diffraction from oriented outer mitochondrial membranes. Detection of in-plane subunit structure. Biochim Biophys Acta **413:** 226–233

Manella CA, Colombini M, Frank, J (1983) Structure and functional evidence for multiple channel complexes in the outer membrane of *Neurospora crassa* mitochondria. Proc Natl Acad Sci USA **80:** 2243–2247

Mannella CA, Frank J (1984) Electron microscopic stains as probes of the surface charge of mitochondrial outer membrane channels. Biophys J **45:** 139–141

Mihara K, Sato R (1985) Molecular cloning and sequencing of cDNA of yeast porin, an outer mitochondrial membrane protein: a search for targeting signal in the primary structure. EMBO J **4:** 769–774

Montal M, Mueller P (1972) Formation of bimolecular membranes from lipid monolayers and a study of their electrical properties. Proc Natl Acad Sci USA **67:** 3561–3566

Nakashima RA, Mangan PS, Colombini M, Pedersen PL (1986) Hexokinase receptor complex in hepatoma mitochondria: evidence from N,N'-dicyclohexylcarbodiimide-labeling studies for the involvement of the pore-forming protein VDAC. Biochemistry **25:** 1015–1021

Ono H, Tuboi S (1987) Integration of porin synthesized *in vitro* into outer mitochondrial membranes. Eur J Biochem **168:** 509–514

Parsons DF, Bonner WD, Verboon JG (1965) Electron microscopy of isolated plant mitochondria and plastids using both thin-section and negative staining techniques. Can J Bot **43:** 647–655

Peiffer WE, Ingle RT, Ferguson-Miller S (1990) Structurally unique plant cytochrome c oxidase isolated from wheat germ, a rich source of plant mitochondrial enzymes. Biochemistry **29:** 8696–8701

Roos N, Benz R, Brdizka D (1982) Identification and characterization of the pore-forming protein in the outer membrane of rat liver mitochondria. Biochim Biophys Acta **686:** 204–214

Schein SJ, Colombini M, Finkelstein A (1976) Reconstitution in planar lipid bilayers of a voltage-dependent anion-selective channel obtained from *Paramecium* mitochondria. J Membr Biol **30:** 99–120

Smack DF, Colombini M (1985) Voltage-dependent channels found in the membrane fraction of corn mitochondria. Plant Physiol **79:** 1094–1097

Zalman LS, Nikaido H, Kagawa Y (1980) Mitochondrial outer membrane contains a protein producing nonspecific diffusion channels. J Biol Chem **255:** 1771–1774