Functional Differences among Wheat Voltage-dependent Anion Channel (VDAC) Isoforms Expressed in Yeast

INDICATION FOR THE PRESENCE OF A NOVEL VDAC-MODULATING PROTEIN*

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VDAC is a voltage-gated anion channel located in the mitochondrial outer membrane, presumably participating in controlling aerobic metabolism. Three distinct wheat vdac cDNAs were expressed in a vdac-minus yeast strain and successfully complemented its defective phenotype. The growth curves of these transformants were different. The wheat channel isoforms were functionally characterized following purification from yeast mitochondria and reconstitution into soybean phospholipid planar membranes. All three isoforms yielded voltage-dependent anion channels with electrophysiological parameters comparable to known VDACs. Isoform-related functional features (specific conductance levels, kinetics, and gating behaviors) are reported for the first time in VDACs. The presence (or absence) of protease inhibitors during the purification procedure, and the use of Pronase on reconstituted channels, strongly suggest that some of the unique wheat VDAC properties are due to co-purification of a yeast channel-modulating protein. Its effects, different from the reported functional interactions of the channel with hexo- or creatine kinases, could not be mimicked by the protein termed VDAC modulator, indicating the presence of a novel VDAC modulator. In addition to strengthening VDAC presumed role in metabolism, the functional diversity of the channels (as shown here in two different systems) implies a highly dynamic outer membrane permeability. Our results are consistent with VDAC functioning as a heteromer including one pore protein and other modulating subunits.

Mitochondria are organelles with a complex structure delimited by two membranes. Since its discovery 20 years ago (1), the major outer membrane protein, mitochondrial porin (more descriptively termed VDAC1 because it forms large voltage-dependent anion channels in reconstituted systems) was presumed to play an important role as a controlled gateway for channeling adenine nucleotides and metabolites to and from mitochondria (2). Under the influence of a transmembrane voltage, the reconstituted VDAC channels undergo transitions (or gating) from a high conducting anion-selective “open” state to lower current-conducting states. These lower states are referred to as “closed,” as they are cation-selective (3) and impermeant to nucleotide phosphates (4). VDAC is also remarkable in that the channel (a monomer; Ref. 5) is able to close at both positive and negative values of the voltage (above 20 mV) possessing two symmetrical gating mechanisms. Cellular factors, such as the VDAC-modulating protein (6) and NADH (7), were found to modulate the voltage-gating mechanisms of VDAC and inhibit respiration in intact mitochondria (8, 9).

Multiple vdac genes were found in human (10), potato (11), mouse (12), and wheat (13), showing that VDAC belongs to a small multigene family. Although very dissimilar primary sequences have been reported for VDAC proteins cloned from various organisms, their predicted secondary structure (an amphipathic ß-barrel) (13, 14) and their basic functional characteristics are very conserved (14). Two human VDAC channels were successfully expressed in a VDAC-minus yeast strain (10), complementing the phenotypic defect caused by the disruption of the endogenous vdac gene (15). Differential binding of the two human gene products with hexokinase was reported in that study (10), demonstrating that this expression system enables the compared study of the properties of different VDAC isoforms from the same species. Two potato isoforms expressed in a yeast VDAC-minus strain did not complement its phenotypic defects (11), raising questions regarding the properties and functions of plant VDAC isoforms. To address these apparently different results obtained with the human and plant vdac genes, three previously cloned wheat vdac cDNAs (13) were expressed in a yeast VDAC-minus strain, and their individual properties were characterized in the expression system and after reconstitution into planar phospholipid membranes.

EXPERIMENTAL PROCEDURES

Expression of Wheat VDAC Isoforms in Yeast—The yeast system used for the expression of the three different wheat vdac cDNAs has been described previously (15, 16). Briefly, each isoform was designed by PCR amplification, so that it could replace the yeast coding sequence residing on a M13 vector between the NcoI and NsiI sites, thus enabling correct expression through the yeast promoter. Specific primers for each cDNA were constructed, and the necessary changes were introduced into the primer sequences. Tvdac1 was directly amplified with the 5′ primer (TCACGGCGGCCATGGGCGG) and the 3′ primer (CCCGAATGCATCCGAAACCGAGG) from poly(A)+ RNA extracted from wheat embryos imibed for 24 h, using the RNA as a template for reverse transcription PCR. Tvdac2 has a DraIII site at position 572. This site resides within the overlapping sequence of the two subclones representing the cDNA sequence of Tvdac2 (13). Restriction digest of these two subclones with DraIII was followed by ligation and PCR amplification with the 5′ primer (GGCCATGCGGCCCGGACGATTCG) and the 3′ primer (GATCGATCCTAAGCCGCGG). This

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1 The abbreviations used are: VDAC, voltage-dependent anion channel; PCR, polymerase chain reaction; S, siemen(s).

2 E. Blachly-Dyson, personal communication.

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resulted in the generation of a full-length cDNA representing the Tavdac2 sequence. As the Tavdac2 sequence was missing the first three amino acids (including the first ATG codon), the 5' primer included the corresponding missing sequences from Tavdac1, along with the NcoI site. Tavdac3 was directly amplified from the full-length cDNA clone with the 5' primer (CGCCATGGCTCCGGGCCT) and the 3' primer (GGGTCCCAGCTATGCATCTC). All PCR reactions were initiated by 3 min at 94°C. This was followed by 30 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 55°C, and 1 min of extension at 72°C. Reaction was completed after a 10-min final extension at 72°C. Template DNA was separated from primers by partitioning them between DynaWax (Finnzymes, Oy, Denmark). The resulting PCR fragments from each clone were blunt-end ligated into the EcoRV restriction site of pBLKS, and introduced by transformation into Escherichia coli XL1-blue cells. Each full-length wheat vdac clone was restriction digested with NcoI and NsiI, ligated into the M13 vector, and introduced by transformation into E. coli XL1-blue cells. To verify that each transformant contained the correct vdac cDNA, PCR amplification was conducted on each transformant by using the specific primers designed. In each case, only the correct transformed clones were able to amplify a DNA fragment of the expected size, whereas no amplification products were observed in the plasmid containing the yeast vdac gene. Next, each clone in the M13 vector was digested by the restriction enzymes BamHI and HindIII (which releases a fragment spanning the yeast upstream and downstream vdac sequences plus the coding sequence), and the isolated fragment was ligated into a yeast shuttle vector linearized by the same restriction enzymes. In the case of Tavdac3, as the coding sequence of the cDNA contains a HindIII restriction site, the M13 vector was partially digested with HindIII. The larger linearized fragment was isolated, cut with BamHI, and ligated into the yeast shuttle vector. To further verify that each construct contains the correct vdac cDNA, sequencing at the 5' insertion site in each case was performed by a primer specific for a sequence in the yeast shuttle vector that flanks the 5' insertion site (TATAGCCAGCAGACG). Finally, each construct was introduced into a yeast strain lacking the endogenous yeast vdac gene by transformation.

Purification of VDAC Proteins from Yeast, and Functional Reconstitution into Planar Phospholipid Membranes—Wheat VDAC proteins expressed in Saccharomyces cerevisiae were purified to homogeneity according to the procedure of Pinto et al. (17, 18). To improve VDAC yields during purification, fresh aliquots of an antiproteolytic mixture (mixture composition: 10 μg/ml leupeptin, 1 μg/ml pepstatin, 6 μg/ml chymostatin, 80 μg/ml benzamidine, 2 μg/ml aprotinin, 0.2 mg/ml phenylmethylsulfonyl fluoride) were added to all the buffers, starting at the cell homogenization step. The VDAC proteins were purified by collecting the flow-through of a Triton X-100-solubilized mitochondrial outer...
Functional Differences between Wheat VDAC Isoforms

Planar phospholipid membranes were built according to the method of Montal and Mueller (19), as modified by Schein et al. (1). Membranes were made out of repurified asolectin (1% w/v) and cholesterol (0.2% w/v) dissolved in n-hexane. The two-sided (cis-trans) chamber was connected with the recording equipment through glass tubing. The preparation was voltage-clamped by a custom-made high impedance amplifier with built-in digitized triangular holding voltage waves, and the current across the bilayer was monitored. The current signal was displayed on a strip chart recorder, and simultaneously acquired at 500 Hz (TL2 AD board and Axotape software, Axon Instruments, Foster City, CA) after filtering at 50–100 Hz. Cis refers to the side where the voltage was held or changed, whereas trans was the side of the membrane kept at ground potential. Whenever needed, cis or trans sides were perfused 5 min (or eight chamber volumes) while stirring to change the buffer solutions. The digitized single-channel data were analyzed as reported (20) and transferred to Origin 4.0 (Microcal Software Inc., Northampton, MA) for further processing.

Purification of Sheep VDAC Modulator—The VDAC modulator was purified from fresh sheep livers. Livers were collected at a slaughter-house and processed essentially according to Liu et al. (21). At the end of the procedure, the protein-containing solution was gel-filtered on a Sepharyl S-300 HR column. The collected fractions were assayed for the modulating activity on VDAC channels from Neurospora crassa reconstituted into diphytanoyl phosphatidylcholine planar membranes (Avanti Biochemicals, Birmingham, AL). Membranes built with this neutral lipid are well suited for the screening of the modulator, because VDAC voltage-dependent closure in absence of any modulating protein is very poor within this environment; this choice of lipid thus gives an improved resolution. The active fractions were then pooled and stored at −80 °C. This procedure resulted in a semi-pure modulator preparation. The modulating activity of this preparation was several thousand-fold higher in comparison to the activity of a crude mitochondrial protein preparation.

Other Methods—Discontinued protein separation by SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (22). Transfer of proteins to nitrocellulose membranes (Schleicher & Schuell) and immunoblot detection by the alkaline phosphatase method were performed according to established protocols (23).

Materials—The yeast expression system was a gift of Dr. M. Forte, Vollum Institute, Portland, OR. Antibodies against VDAC from potato (anti-POM36) (which cross-react against the three wheat VDAC isoforms expressed in E. coli, data not shown), were a gift of Dr. U. Schmitz, Institut fu¨r Genbiologische Forschung, Berlin, Germany. Reverse transcriptase (Superscript II) was purchased from Life Technologies, Inc. Taq DNA polymerase was purchased from Promega Corp., Madison, WI. All other molecular biology reagents, including restriction enzymes, were purchased from New England Biolabs, Beverly, MA. Protease inhibitors, Pronase (protease type XIV, bacterial), other biochemical and immunological reagents were purchased from Sigma. Celite 535 and hydroxylapatite “high resolution” were purchased from Fluka Chemie AG, Buchs, Switzerland.

RESULTS

Wheat VDACs Can Complement the Phenotypic Defects of a Yeast VDAC-minus Strain—Wheat vdac cDNAs were constructed to contain the promoter and the 5′-untranslated region of the yeast vdac gene (see “Experimental Procedures”) and were introduced by transformation into the yeast strain M22–2 (15). This strain, whose endogenous vdac gene was disrupted, cannot grow at 37 °C in the presence of glycerol as a sole carbon source. The transformed yeast strains containing each of the wheat vdac cDNAs were able to grow well, even at the selective temperature of 37 °C on minimal medium plates containing glycerol as a sole carbon source (Fig. 1A). When the complemented yeast cells were grown on glycerol by shaking at 37 °C, it was consistently recorded that the yeast containing the Tavdac1 and Tavdac2 cDNAs had the same growth kinetics as wild type yeast VDAC (Fig. 1B). The yeast containing the Tavdac3 cDNA displayed a 50% decrease in the doubling time at the logarithmic growth phase in comparison to the yeast VDAC, while reaching a similar growth maximum (Fig. 1B).

The Three Wheat Isoforms Are Sensitive to Endogenous Yeast Proteases—When mitochondrial membranes were purified from each strain, separated on SDS-polyacrylamide gels, blotted, and decorated with potato VDAC antibodies (anti-POM36), at first no protein was detected. This result was unexpected, as complementation of the yeast defective phenotype is supposed to be due to correct synthesis and targeting of each isoform into the mitochondrial outer membrane. The relative transcription levels of the wheat vdac isoforms analyzed by Northern blot were found to be similar to those of yeast vdac (data not shown). As it has been recently reported that VDACs bound to the mitochondrial membranes may be exposed to proteolytic digest (24), we investigated the possibility that the wheat...
The different conductance levels and their ionic selectivity are presented. The "open" level is the high conducting state; the "closed" levels represent different lower conducting states that are not permeable to nucleotide phosphates and metabolites. Each experiment represents one planar phospholipid membrane with one reconstituted channel. The number of experiments for which these channel states were reliably measured are given in column 4. Note that for TAVDAC2, the fully open channel conductance was not recorded during all experiments (in 8 experiments out of 15). In the case of TAVDAC3, besides an isoform-specific state (0.6 nS), the low conducting substates are more stable and thus more consistently seen. These experiments were performed in symmetrical 1 M KCl solutions, 10 mM HEPES, 5 mM CaCl₂, pH 7.2. The ion selectivity experiments were performed in the presence of a 10-fold activity gradient for KCl (0.604 vs. 0.078 M), same pH, using molal activity coefficients (38). The permeability ratios were calculated according to the Goldman equation. The measurement of the open state reversal potential for TAVDAC2 was technically difficult which may explain its difference. The number of experiments is given in column 7. For both sets of results, data are mean ± S.E. ND, not determined.

| Channel isoform | States | Conductance levels (nS) | No. of exp. | Reversal potential of open state (mV) | Selectivity (Cl⁻:K⁺) | No. of exp. |
|----------------|--------|-------------------------|-------------|-------------------------------------|----------------------|-------------|
| TAVDAC1        | Open   | 3.8 ± 0.1               | 14          | −11 ± 0.5                           | 1.5                  | 3           |
|                | Closed 1| 1.6 ± 0.1               | 14          | +24 ± 1                             | 0.4                  |             |
|                | Closed 2| 1.4 ± 0.1               | 1           | +44                                 | 0.1                  |             |
|                | Closed 3| 0.3 ± 0.03              | 3           | ND                                  | ND                   |             |
| TAVDAC2        | Open   | 4.0 ± 0.1               | 8           | −8 ± 2                              | 1.3                  | 4           |
|                | Closed 1| 2.1 ± 0.4               | 15          | +23 ± 2                             | 0.4                  |             |
|                | Closed 2| 1.4 ± 0.1               | 6           | ND                                  | ND                   |             |
|                | Closed 3| 0.3 ± 0.03              | 1           | ND                                  | ND                   |             |
| TAVDAC3        | Open   | 3.96 ± 0.05             | 23          | −12 ± 1                             | 1.6                  | 3           |
|                | Closed 1| 2.3 ± 0.1               | 23          | +24 ± 1                             | 0.4                  |             |
|                | Closed 2| 1.2 ± 0.1               | 8           | +39 ± 5                             | 0.2                  |             |
|                | Closed 3| 0.6 ± 0.03              | 5           | ND                                  | ND                   |             |
|                | Closed 4| 0.28 ± 0.02             | 5           | ND                                  | ND                   |             |

### Table II

**Functional comparison between TAVDAC1 and the corresponding native mitochondrial VDAC MmP29**

The values for MmP29 were taken from Blumenthal et al. (26). The values for TAVDAC1 are the mean ± S.E. of 14 experiments. "n" and $V_0$ are as in Fig. 4. Conditions were in both cases: 1 M KCl, 5 mM CaCl₂. The pH was 5.8 (MES-buffered) for (26) and 7.2 (HEPES-buffered) for this study.

| Channel type | Conductance open state (nS) | Reversal potential of open state (mV) | Reversal potential of closed state (mV) | $V_0$ (mV) | n |
|--------------|------------------------------|--------------------------------------|----------------------------------------|------------|---|
| MmP29        | 3.8 ± 0.1                    | −10.8                                | +25                                    | −22        | 3 |
| TAVDAC1      | 4.1                          | −11 ± 0.5                            | +24                                    | 25 ± 2     | 3.4 ± 0.2 |

**The Three Wheat VDACs Display Specific Characteristics**

The three wheat VDAC isoforms present several differences in their conductance levels (Table I) and their kinetics (Fig. 2). In addition to the two frequently described conductance states, termed open and closed, the wheat VDACs can dwell in less conductive states reaching around 1 nS, 600 pS, and even 300 pS in 1 M KCl (Table I). Each isoform displayed specifically some preferred (i.e. stabilized) levels. TAVDAC1 displayed slow kinetics and very rare deeper closures (4 experiments out of 14). TAVDAC2 was more stabilized around its 2-nS state and fully opened only for very brief periods of time. This isoform was fast flickering most of the time and its transitions were poorly resolved at the filter settings used. This could explain the slightly different reversal potential value, and hence ion selectivity, measured for the open state. TAVDAC3 exhibited very stable open and closed states with frequent and time-resolvable sojourns in very low conducting states. A 600-pS state, seemingly specific for this isoform, was recorded in one third of the experiments (Table I).

VDAC behavior in planar phospholipid membranes made of asolectin is symmetrical (14, 20). However, TAVDAC2, TAVDAC3, and, in some experiments, TAVDAC1 displayed a strong asymmetry in the voltage dependence curves between their two gating mechanisms (Figs. 3 and 4). Whereas one of the voltage gating processes was identical for the three isoforms, the other gating process yielded n values of 4.7 and 5.5 elementary charges for TAVDAC2 and TAVDAC3, respectively. The midpoint potential was around 10 mV in both cases. As a consequence, for these two isoforms, there was a net decrease in the free energy difference ($\Delta G$ values in Fig. 4) between open and closed states when this gate was probed relative to the other channel gate. This decreased difference indicates that the VDAC proteins are degraded by yeast proteases during the purification procedure (25). Addition of a mixture of different protease inhibitors (see “Experimental Procedures”) facilitated the detection of all three isoforms by anti-POM36 (Fig. 1C). It was concluded that each of the wheat vdac cDNAs complemented the yeast vdac-minus strain, being responsible for its ability to grow under the selective conditions.

**Basic Channel Properties of the Wheat VDACs Are Similar to Known VDACs**—When wheat VDACs purified from yeast mitochondrial membranes were added to asolectin planar phospholipid membranes, channel activity was always recorded (Fig. 2). All three wheat isoforms exhibited two main conducting states (Table I): a main anion-selective high conducting state of about 4 nS, and a main cation-selective lower conducting state around 2 nS (values obtained in 1 M KCl). Quite often the channels inserted in the 2-nS closed state and subsequently switched to the 4-nS open state. All three isoforms were found to share similar voltage-gating parameters for at least one voltage gating process. The gating charges or “n” values (the functional equivalent charges that have to move across the energy barrier to induce the channel open-closed transition) were around 3.5 elementary units. The midpoint potential ($V_0$) values (the transmembrane potential for which the probability for the channel to be found in the open state equals the probability of finding it in the closed state) were around 25 mV (Fig. 4). All these channel properties compare well with other reported VDAC channels (14).
closed state reached from one gating mechanism is not thermodynamically the same as the closed state reached from the other gating process. As seen in Fig. 2, fast open-closed transitions (or flickers) characterized one gating mechanism (one polarity of the voltage ramp). The current obtained in response to linear voltage ramps remained linear. When the potential polarity was reversed and the other gating mechanism was probed, the current responded gradually to the change in voltage, without sudden state transitions, presenting a curved shape reminiscent of single channel rectification (27). This behavior was always sided, as fast flickers and rectification always happened at opposed polarities of the probing voltage ramp. This asymmetrical behavior easily differentiated each of the two channel gating processes, enabling us to define the direction of the channel insertion into the membrane.

The direction of insertion was always identical. It was not dependent on the voltage polarity applied to the membrane during insertion (i.e. ruling out a dipolar orientation of the protein prior insertion), but was solely dependent on the side of the chamber to which the channels were added (Fig. 5 and Table III). The “rectifying” ramp was always trans relative to the side of addition, while the “fast flickering” ramp was cis relative to the side of addition. Table III shows that this behavior was identical to all three isoforms.

TAVDAC1 Displays Two Functional Channel Populations—When TAVDAC2 and TAVDAC3 were reconstituted, only asymmetrical channels could be recorded (Fig. 2, middle and lower panels). However, TAVDAC1 reconstituted channels fell into two populations: symmetrical channels with slow kinetics (Fig. 2, upper panel) or asymmetric fast flickering and rectifying channels (Fig. 6, upper panel). These results were obtained with three independent mitochondrial preparations ruling out preparative singularities. The existence of two TAVDAC1 populations suggested that the novel behavior of the reconstituted wheat VDAC isoforms could be extrinsic to the VDAC polypeptide. As a previously isolated effector of VDAC is a protein (the VDAC modulator), we applied Pronase to the reconstituted wheat VDAC channels (VDAC is resistant to Pronase; Ref. 21), purified the control yeast VDAC in absence or presence of the antiproteolytic protection, and examined the effect of the known VDAC modulator on TAVDAC1 channel behavior.

**FIG. 3. Probability versus voltage plots.** One gating mechanism (right portion of the curves) is nearly identical for the three isoforms. The other gating process (left part of the curves) is different. These plots were generated with the data collected from 25–30 voltage ramps like those shown in Fig. 2. Each complete ramp lasted around 5.5 min. All the data came in each case from a single channel. Conditions were as detailed in Fig. 2.

**FIG. 4. Wheat VDAC gating parameters.** The calculated gating charge ($n$), the midpoint potential or switching region ($V_0$, voltage for which probability of the channel being open equals probability of being closed), and the free energy differences between the states are plotted for both gating processes of each isoform. These are the values for the voltage-dependent gating between the open and the first (main) closed state. Data are mean ± S.E. (#, number of experiments).
ishes Rectification—VDAC data accumulated so far indicate that a positively-charged voltage-sensing domain (or gate) moves out of the channel barreled structure in response to the voltage (20, 28). The asymmetry displayed by the wheat VDACs allowed us to test addition of Pronase knowing which gating mechanism we are probing and where the voltage sensor should be. According to our data, rectification happens only when VDAC voltage sensor is attracted toward the side to which the channel was added, i.e. when this side of the chamber was maintained negative. When Pronase (5 μg/ml final concentration) was added to the side where the TAVDAC1 channel had been added (cis and trans do not matter here), rectification was abolished immediately (Fig. 6, lower panel). This occurred even when the chamber was perfused and Pronase washed away 15 s after its addition to the chamber. The channel current became linear in response to the voltage ramp, and VDAC maximal conductive state and typical voltage gating were gained back. However, the fast state transitions displayed by the second gating process were still present (Fig. 6). Identical results were obtained with the TAVDAC3 isoform. When Pronase was added to the chamber side opposite to the side of channel addition, rectification was not abolished and the fast state transitions could be inconsistently suppressed (data not shown).

Yeast VDAC, Isolated in the Presence of Protease Inhibitors, Displays Asymmetric Fast Flickering and Rectifying Channels—The wheat VDAC characteristics described here (Figs. 2–4) were never reported for yeast VDAC reconstituted into planar phospholipid membranes (14). A critical experiment, to determine the origin of the wheat channels unique characteristics, was to purify our control yeast VDAC in the presence or absence of the same protease inhibitors (Fig. 7). Slow symmetrical channels were always recorded when the yeast VDAC was isolated in absence of protease inhibitors (4 out of 4 experiments) (upper panel). When isolated in the presence of the protease inhibitor mixture, yeast VDAC displayed two channel populations in planar phospholipid membranes: slow symmetrical channels or asymmetrical fast flickering and rectifying channels (lower panel). Yeast VDAC being highly resistant to proteases (29), it was concluded that the alteration in the isolation procedure resulted in some protein co-purification. To further elucidate the nature of this channel-modulating protein, we compared its effects to those of the already known VDAC modulator.

The VDAC Modulator Cannot Convert TAVDAC1 from a Slow and Symmetrical Channel Type into an Asymmetrical Fast Flickering and Rectification Type—The VDAC modulator acts on the channel gating only when added to the negative side of the membrane, and is known to bind VDAC very tightly (21). This protein favors VDAC closure by increasing the channel gating charge (n) and lowering the voltage switching region.

![Graph showing channel asymmetry](Image)

**Figure 5.** Channel asymmetry allows an easy determination of the direction of one VDAC channel into the phospholipid membrane. The arrow defines the direction as used in Table III, last column.

**Table III**

| Channel isoform | Side of addition | Polarity of holding V | Side of rectification | Direction |
|-----------------|------------------|----------------------|-----------------------|-----------|
| TAVDAC1         | Trans            | +                    | Cis                   | →         |
|                 | Cis              | +                    | Trans                 | →         |
|                 | Cis              | –                    | Trans                 | →         |
| TAVDAC2         | Trans            | –                    | Cis                   | →         |
|                 | Trans            | +                    | Cis                   | →         |
|                 | Trans            | +                    | Cis                   | →         |
|                 | Trans            | +                    | Cis                   | →         |
|                 | Cis              | +                    | Trans                 | →         |
|                 | Cis              | +                    | Trans                 | →         |
|                 | Cis              | +                    | Cis                   | →         |
|                 | Cis              | –                    | Trans                 | →         |
| TAVDAC3         | Trans            | +                    | Trans                 | EXC ←     |
|                 | Trans            | +                    | Cis                   | →         |
|                 | Cis              | +                    | Trans                 | →         |
|                 | Cis              | +                    | Trans                 | →         |
|                 | Cis              | +                    | Trans                 | →         |
|                 | Cis              | –                    | Trans                 | →         |
|                 | Cis              | –                    | Trans                 | →         |
|                 | Cis              | –                    | Trans                 | →         |
|                 | Cis              | –                    | Trans                 | →         |

The results of individual reconstitution experiments with the three wheat VDAC isoforms are presented. The VDAC proteins were added to one side of the planar phospholipid membrane under a constant voltage (10 mV); after insertion, triangular voltage ramps were used to probe the channel-gating mechanisms. The asymmetry displayed at the single-channel level depended only on the side of addition of the VDAC molecule relative to the bilayer membrane. Rectification happens only when the positively charged voltage sensor of the channel is attracted toward the side of the chamber that the protein was reconstituted from. Twice with TAVDAC3, the insertion went opposite (EXC); in these cases, rectification was abolished immediately (Fig. 6, lower panel).
It could thus in principle account for the observed gating values we report (see Fig. 4). As only one paper deals with the effect of the modulator at a single channel level (6), and only voltage steps were used in this study, the possibility exists that the modulating protein could induce rectification by a very fast and voltage-dependent block of the channel during our voltage ramps. When added to the reconstituted TAVDAC1 of the slow and symmetrical (non-rectifying) type, the modulator could induce further closure and faster state transitions during negative voltage ramps (Fig. 8), but could never bring about channel rectification and very fast kinetics.

DISCUSSION

Functional Properties of the Wheat VDAC Isoforms Are Similar to Other VDACs—Our work presents the first successful complementation of a yeast vdac minus strain by three individual vdac cDNAs originating from plants (Fig. 1). In this study we used the same yeast strain previously used successfully to complement two human VDAC isoforms (10). As the yeast, human, and plant VDAC proteins vary in their predicted amino acid sequences (13), our complementation results point to the conservation of the functional VDAC physiology. Differences between the yeast strain used in this study, as compared to the yeast strain used for expression of potato VDAC proteins (11), might account for the different complementation abilities observed for these two gene families of plant VDACs. It is, however, difficult to speculate on the nature of these discrepancies, as the potato VDAC proteins were shown to be correctly translated and targeted to the yeast mitochondrial membrane (11).

The three wheat VDACs, which are differentially expressed within plant tissues (13), display phenotypic differences in the transformed yeast. The complementation by the Tavdac3 cDNA gives rise to a slowly growing yeast phenotype (Fig. 1B). As the transcription of all VDAC isoforms in yeast is facilitated by the same promoter, it is unlikely that differences in transcription rates account for our observation. After purification of mitochondrial membranes in the presence of protease inhibitors, TAVDAC3 gave a strong protein signal on Western blots (Fig. 1C). Moreover, its sensitivity to proteolytic digestion could be further reduced by using a different protease inhibitor mixture (data not shown). Therefore, low protein levels of this isoform in the mitochondrial outer membrane cannot explain these growth results. It is possible that some endogenous properties of the TAVDAC3 sequence accounts for the altered phenotype observed in yeast.

The channel properties recorded for the three wheat isoforms (Table I) are consistent with VDAC (14). Comparing basic channel characteristics of TAVDAC1 to those previously documented for the wheat mitochondrial VDAC MmP29 (26) (Table II), we found that TAVDAC1 functional data (conductances, gating parameters, and symmetry of the voltage gating mechanisms), monitored at a single-channel level, were superimposable for native and recombinant molecules. This is the first known comparison between native and recombinant VDACs.

Unique Properties of the Wheat VDAC Isoforms—Features unique to the wheat isoforms were observed. The three isoforms could give asymmetric channels. When one of their two gating mechanisms was probed, all three isoforms tended to dwell in various closed states and exhibited fast flickering transitions (Fig. 2 and Table I). When the other gating mechanism was probed, raised n values and lowered \( V_0 \) (Fig. 4)
and rectification was never recorded. Conditions were as in Fig. 2. DAC1 displayed two channel populations: in most cases slow TAVDAC3 was consistently more stable than TAVDAC2. TAVmetrical fast flickering and rectifying channel behavior (Fig. 2). TAVDAC2 and TAVDAC3 always displayed an asym-

terminal. The molecular nature of current rectification in ion channels may vary between channel types (27, 30, 31) and is not yet fully understood; voltage-dependent block by magnesium ions and/or polyamines and the presence of charged residues located at the mouth (entrance) of a channel have been reported. Our solutions contained neither Mg^{2+} nor polyamines, suggesting an intrinsic or extrinsic protein domain as the cause.

**Intrinsic or Extrinsic Modulation of Channel Activity**—The kinetic and conductance differences observed between the iso-

forms could be either intrinsic to the VDAC polypeptides, or extrinsic as co-purification of interacting proteins is sometimes hard to avoid. In the first hypothesis, VDACs (from yeast and wheat alike) could be cleaved during unprotected purification protocols, yielding channel activities of the slow type. Addition of the protease inhibitors would then protect the protein giving fast and rectifying channels. In our study, the presence of antiproteases did not alter VDAC migration during SDS-poly-

acrylamide gel electrophoresis. However, the gel resolution cannot exclude short N- or C-terminal cleavage of the molecule. A C-terminal deletion is ruled out. Numerous residues of the yeast sequence (up to the last one) have been site-mutated, and calculated functional effects could be recorded (15, 28) on slow channels. This attests to an intact molecule. A N-terminal cleavage hypothesis would contradict two recent reports (32, 33) indicating that N-terminal deletions of VDAC (Saccharomyces and Neurospora) lead to altered faster kinetics, the non-
deleted controls being slow. An additional argument against a N-terminal cleavage is that the original cloning and identifica-
tion of yeast VDAC was done without protease inhibitors (29, 34). In that study, microsequencing the N-terminal portion of the yeast protein led to the isolation of yeast VDAC cDNA. Moreover, VDAC within the mitochondrial outer membrane is highly resistant to proteases including trypsin (29).

The alternative hypothesis (an extrinsic protein interacting with VDAC and affecting its gating behavior) then becomes more likely. The two channel populations seen for TAVDAC1 could result from a lower binding affinity of this isoform for such factor. As the first VDAC insertion into membranes is reported random (20), the non-random channel insertion in this work could result from a bound intervening factor biasing the channel affinity for the phospholipid membrane toward one direction, therefore losing the expected randomness. Moreover, the current rectification at one polarity of the transmembrane potential could also be due to such cofactor causing a gradual (instead of a sudden) gating of the channel. Our use of protease inhibitors during the channel purification procedure could protect this supramolecular assembly.

We therefore investigated further this possibility. VDAC voltage sensor is a positively charged domain of the polypeptide moving across the membrane energy barrier according to the polarity of the electrical field. The rectification phenomenon occurs for one gate (one side of the membrane) only when this side is made relatively negative (i.e. attracting the voltage sensor). A protein termed the VDAC modulator is known to facilitate channel closure by interacting with the voltage sensor from the negative side of the membrane (21). As this interaction is very stable (21), the altered gating we measured could be attributed to such regulatory element bound to VDAC. By adding Pronase to the appropriate chamber side at the negative polarity of the transmembrane voltage, such intervening protein could be cleaved leaving VDAC intact (reconstituted VDAC is supposedly resistant to Pronase). We show that Pronase treatment to the correct side had the predicted effect; rectifi-

**Fig. 8. The extrinsic protein modifying VDAC function is not the VDAC modulator.** The upper panel shows the control current trace from one TAVDAC1 channel undergoing open-closed transitions as a function of the transmembrane voltage. In the lower panel, the modulator protein preparation was added to the chamber (5 ng/ml final). When the side of addition was made negative, as expected, this modulating protein induced channel closure evidenced by some flicker-

and a lowered single-channel conductance. The closure lasted much longer, and the channel did not reopen fully until 0 mV was reached (see differences in slope with control trace after the arrow; the control trace is overlaid as dotted points). This experiment was performed three times, and rectification was never recorded. Conditions were as in Fig. 2.

biased the channels toward a more closed state. The current response to very slow linear voltage ramps was bent (Fig. 2). This phenomenon is termed rectification (27). The asymmetry between the two gating mechanisms exhibited by the wheat isoforms (Fig. 4) enabled us to show that the channel insertions into the reconstituted membrane is determined by the side of the membrane to which the channels were added (Table II). Autocatalytic directional insertion of VDAC channels into plan-

lar phospholipid membranes has been reported previously (20). By using mutated yeast VDACs exhibiting asymmetrical gating parameters, the authors showed that the first channel molecule inserted into planar phospholipid membranes in a random direction, but could force the subsequent channel in-

sertions to be oriented in the same direction. As our results show that the first channel insertion is not random, the direc-
tional insertion we reveal here is not conceptually the same as the autocatalytic phenomenon. These data are not mutually exclusive, as will be discussed further.

Our study demonstrates (for the first time) differences in ion channel behavior between VDAC isoforms. When reconstituted into planar phospholipid membranes, each isoform could be recognized by its pattern of activity at a single channel level (Fig. 2). TAVDAC2 and TAVDAC3 always displayed an asym-

metrical fast flickering and rectifying channel behavior (Fig. 2). TAVDAC3 was consistently more stable than TAVDAC2. TAV-

dac1 displayed two channel populations: in most cases slow
cation was immediately abolished, restoring the fully open conductance level and the slow pattern of voltage gating (Fig. 6). This observation shows that the Pronase treatment does not impair the channel voltage-sensing properties and is compatible with the selective cleavage of an extrinsic protein. The fast flickering behavior of the other gating mechanism remained, however, unaffected. Giving Pronase to the chamber side opposite to where the channel was added, thus probing this other gate, did not abolish rectification (data not shown). From this side, Pronase could markedly slow down the channel kinetics but not consistently, thereby not excluding some contribution of the VDAC polypeptide itself to these faster transitions.

A further test of our hypothesis was to isolate the channel from our yeast control strain in the presence, or the absence, of the same antiproteolytic mixture we used for yeast-expressed wheat channels. Solely yeast VDAC purified in the presence of the protease inhibitor mixture led to the recording of asymmetrical fast flickering and rectifying channels (Fig. 7). Results were once again consistent with the antiprotease agents protecting a supramolecular assembly of the VDAC channel.

The VDAC modulator, isolated from sheep liver in our case, affected the reconstituted wheat channels as expected but was unable to reproduce the fast kinetics and the rectification we are reporting. Our data are thus consistent with the involvement of another extrinsic protein modulator in VDAC gating. Although the nature of this other modulating factor is still elusive, its effects do not fit the recently reported in vitro interactions between VDAC and the mitochondrial creatine kinase (35), indicating that our co-purifying protein is not the mitochondrial enzyme.

Other proteins, in addition to the known VDAC modulator (6) and peripheral kinases, are likely to tightly interact with the VDAC monomer and modulate its functions. A probable view of VDAC could be a multimeric assembly comprising one pore-forming polypeptide associated with modulating effectors. While the molecular nature of VDAC behavior in vivo and its complexity are yet unknown, differences in purification methods, as exemplified by our results, may explain some discrepancies in VDAC recordings reported by various groups (36, 37).

As purification of VDAC does not yield all the elements modulating the channel, the functional diversity of this channel may lay hidden when using cell-free systems to monitor VDAC properties. Our results may provide an explanation for the presence of numerous VDAC genes in all species studied so far and add evidence to the claim that the mitochondrial outer membrane permeability has to be highly dynamic to adapt to the cellular needs at any given time.

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REFERENCES

1. Schein, S. J., Colombini, M., and Finkelstein, A. (1976) J. Membr. Biol. 30, 99–120
2. Colombini, M. (1979) Nature 279, 643–645
3. Benz, R., Kottke, M., and Brdiczka, D. (1990) Biochim. Biophys. Acta 1022, 311–318
4. Rostovtseva, T., and Colombini, M. (1990) Biophys. J. 70, A1
5. Thomas, L., Koczis, E., Colombini, M., Erbe, E., Trus, B. L., and Steven, A. C. (1991) J. Struct. Biol. 106, 161–171
6. Holden, M., and Colombini, M. (1988) FEBS Lett. 241, 105–109
7. Zizi, M., Forte, M., Blachly-Dyson, E., and Colombini, M. (1994) J. Biol. Chem. 269, 1614–1616
8. Liu, M., and Colombini, M. (1992) Biochim. Biophys. Acta 1098, 255–260
9. Lee, A.-C., Zizi, M., and Colombini, M. (1994) J. Biol. Chem. 269, 30974–30980
10. Blachly-Dyson, E., Zamboninzi, E. B., Yu, W. H., Adams, V., McCabe, E. R. B., Adelman, J., Colombini, M., and Forte, M. (1995) J. Biol. Chem. 268, 1835–1841
11. Heins, L., Mentzel, H., Schmid, A., Benz, R., and Schmitz, U. K. (1994) J. Biol. Chem. 269, 26402–26410
12. Sampson, M. J., Lovell, R. S., and Craigie, W. J. (1996) Genomics 33, 283–288
13. Elkeles, A., Devos, K. M., Graur, D., Zizi, M., and Breiman, A. (1995) Plant. Mol. Biol. 29, 109–124
14. Colombini, M. (1994) Curr. Top. Membr. 42, 73–101
15. Blachly-Dyson, E., Peng, S., Colombini, M., and Forte, M. (1989) J. Bioenerg. Biomembr. 21, 471–483
16. Blachly-Dyson, E., Peng, S., Colombini, M., and Forte, M. (1990) Science 247, 1233–1236
17. de Pinto, V., Ludwig, O., Krause, J., Benz, R., and Palmieri, F. (1987) Biochim. Biophys. Acta 984, 109–119
18. de Pinto, V., Prezioso, G., and Palmieri, F. (1987) Biochim. Biophys. Acta 905, 499–502
19. Montal, M., and Mueller, P. (1962) Proc. Natl. Acad. Sci. U.S.A. 59, 5561–5568
20. Zizi, M., Thomas, L., Blachly-Dyson, E., Forte, M., and Colombini, M. (1995) J. Membr. Biol. 144, 121–129
21. Liu, M., Torgrimson, A., and Colombini, M. (1994) Biochim. Biophys. Acta 1185, 203–212
22. Laemmli, U. K. (1970) Nature 227, 680–685
23. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
24. Smith, M. D., Petrait, M., Boucher, P. D., Barton, K. N., Carter, L., Reddy, G., Blachly-Dyson, E., Forte, M., Price, J., Verner, K., and McAuley, R. B. (1995) J. Biol. Chem. 270, 28331–28336
25. Jones, E. W. (1991) Methods Enzymol. 194, 428–453
26. Blumenthal, A., Kahn, K., Beja, O., Galun, E., Colombini, M., and Breiman, A. (1993) Plant Physiol. 101, 579–587
27. Hille, B. (1992) Ions Channels of Excitable Membranes, pp. 472–503, Sinauer Associates Inc., Sunderland, MA
28. Thomas, L., Blachly-Dyson, E., Colombini, M., and Forte, M. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 5446–5449
29. Mihara, K., Bloke, G., and Sato, R. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 7102–7106
30. Ficker, E., Tagliasaleta, M., Wible, B. A., Henley, C. M., and Brown, A. M. (1994) Science 266, 1068–1072
31. Bociek, R., and Mayer, M. L. (1995) Neuron 15, 453–462
32. Koppel, D. A., Kinnally, K. W., and Mannella, C. A. (1996) Biochim. Biophys. J. 70, A2
33. Popp, B., Court, D. A., Benz, R., Neupert, W., and Lill, R. (1996) J. Biol. Chem. 271, 13583–13589
34. Mihara, K., and Sato, R. (1985) EMBO J. 4, 769–774
35. Brdiczka, D., Kaldis, P., and Wallimann, T. (1994) J. Biol. Chem. 269, 27640–27644
36. Wunder, U. R., and Colombini, M. (1991) J. Membr. Biol. 123, 83–91
37. Mirzabekov, T., Ballarin, C., Minollini, M., Zotta, P., and Sorgato, M. C. (1993) J. Membr. Biol. 132, 129–143
38. Robinson, R. A., and Stokes, R. H. (1965) Electrolyte Solutions: The Measurement and Interpretation of Conductance, Chemical Potential and Diffusion in Solutions of Simple Electrolytes, 2nd Ed. (revised), Butterworth, London