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The outer mitochondrial membrane channel, VDAC, is modulated by a protein localized in the intermembrane space

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The mitochondrial outer membrane channel, VDAC, provides a pathway for the flux of metabolites between the cytoplasm and mitochondrion. VDAC is voltage-dependent and occupies states of differing conductivity and ion selectivity that are dependent on transmembrane potential. A protein, derived from preparations of mitochondria, has been shown to increase the voltage dependence of VDAC and is called the VDAC modulator. Both VDAC and the VDAC modulator have been extensively characterized by reconstitution into planar lipid bilayers. In order for the VDAC modulator to have physiological significance it must have physical access to VDAC in the cell. This constraint dictates that the modulator be an extrinsic outer mitochondrial membrane protein, occupy the mitochondrial intermembrane space, or be a cytoplasmic constituent. To address the question of subcellular localization, purified mitochondria were selectively lysed with digitonin or treated with trypsin while resuspended in hypo-osmotic or iso-osmotic medium. Marker enzymes and modulator activity were monitored during the various treatments. Results indicate that the integrity of the outer membrane was necessary to prevent modulator release or protection from trypsin digestion. Outer membrane lysis, under conditions where the inner membrane remained intact, resulted in modulator release or inactivation by trypsin. These results suggest an intermembrane space location for the VDAC modulator in the mitochondrion.

Introduction

The mitochondrial outer membrane contains an ionic channel, VDAC. Like many well-known membrane ion channels, VDAC is voltage-dependent, being highly conductive (open) and anion-selective in the absence of a transmembrane potential and exhibiting multiple lower conductance (closed) states and becoming cation selective in the presence of transmembrane potentials exceeding 20 mV. The functionally large diameter of this channel suggests that it provides the primary permeability pathway for metabolites and ions exchanging between the cytoplasm and mitochondrial compartments (see Refs. 1,2 for recent reviews).

While voltage-dependence of conductance states is likely to be a primary regulator of metabolite and ionic traffic across the outer membrane, it is not the only known possible type of regulatory control of molecular traffic through VDAC. Polyanionic compounds, such as dextran sulfate [3] and Konig's polyanion [4], increase the voltage-dependence of VDAC, causing the channels to close at lower potentials. NADH also has a specific and sensitive effect on the gating charge of VDAC, doubling it from 3 to 6 (Zizi, M., Forte, M., Blachly-Dyson, E. and Colombini, M., unpublished data). The existence of a soluble protein(s), in the mitochondrial fraction isolated from *Neurospora crassa*, that increases the voltage dependence of VDAC has been previously described [5,6]. The modulator protein(s), active in nanomolar quantities [5], encourages VDAC to enter closed states at smaller transmembrane voltages. Furthermore, modulator-treated VDAC will occupy states of minimal conductance and make the transition at a faster rate. In the absence of the modulator, VDAC will rapidly (in μs) return to the open state once the transmembrane potential is reduced to zero. But modulator-treated channels are likely to remain for some minutes in the closed state once the transmembrane potential has been eliminated. This suggests that the modulator increases the stability of the closed state.

While much of the description of channel response has involved biophysical studies of channels reconstituted in planar bilayers, evidence exists for regulation of outer membrane channel traffic in studies with

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whole mitochondria. Both dextran sulfate and the modulator preparations specifically decreased mitochondrial State-3 respiration and activity of mitochondrial adenylate kinase in situ [7]. These effects were seen only with intact mitochondria and disappeared when respiration was measured in modulator-treated mitoplasts. In a similar manner, Benz et al. [8] have shown that increasing concentrations of Konig's polyanion decreased the activity of intermembrane space kinases, but not when the outer membrane was permeabilized with detergents.

Modulator proteins have been found in the mitochondrial fractions of tissues of animal, plant and fungal species [9]. These proteins not only modulate the voltage-dependence of VDAC derived from the same tissues, but crossreact with VDAC from other species. VDAC is structurally and functionally conserved and the modulator activity appears to be also, suggesting an important regulatory function for the modulator. All of this evidence supports the hypothesis that VDAC serves not only as a primary pathway for metabolite movement from mitochondria to cytosol and visa versa, but that multiple subtle regulatory devices could serve to fine tune the permeability of the outer membrane and control the rate of cellular and respiratory processes.

To begin to understand a possible physiological role for this VDAC modulator, it is important to localize the modulator activity to a subcellular compartment. As the modulator activity is derived from sonication of a mitochondrial fraction, it is likely that the modulator activity is localized in the mitochondrion. However, the modulator could be found in the mitochondrial fraction as a cytoplasmic component loosely bound to the outer membrane. There are also two submitochondrial compartments, the intermembrane and matrix space in which the modulator could be localized. The question of subcellular localization was addressed with two types of experiment. Digitonin was used to selectively permeabilize the outer mitochondrial membrane. Appearance of the modulator extramitochondrially was compared with release of marker enzymes. Secondly, mitochondrial membranes resuspended in osmotic or hypo-osmotic medium were subjected to digestion with trypsin. Assays were conducted for outer membrane intactness, marker enzymes and modulator activity in various fractions. The results are best explained by localization of the VDAC modulator to the mitochondrial intermembrane space.

Materials and Methods

Preparation of mitochondria

Mitochondria were purified from a wall-less mutant of *Neurospora crassa* (ATCC #32360). Cells were maintained, cultured and harvested as described by Mannella [10]. Mitochondria were isolated by differential centrifugation [10] and subsequently purified on percoll gradients [11]. Self-generating Percoll gradients consisted of 28% percoll, 0.3 M sucrose, 10 mM Mops (pH 7.2), and 1 mM EDTA. Concentrated washed mitochondria (3 ml) were layered on 36 ml percoll gradients and centrifuged at $39000 \times g_{max}$ for 30 min. The mitochondrial band was removed and diluted 10-fold with a buffer consisting of 0.3 M sucrose, 10 mM Mops and 1 mM EDTA and centrifuged at $13000 \times g_{max}$ for 30 min. VDAC was prepared from purified mitochondria which were sonicated in a buffer of 10 mM Tris-HCl (pH 7.5) 10 mM KCl and centrifuged at $150000 \times g_{max}$ for 30 min. The membrane pellet which served as a source of VDAC, was resuspended in 1 mM KCl, 1 mM Hepes (pH 7.0), 15% DMSO, and stored at −70°C.

Measurement of the intactness of the outer membrane of purified mitochondria was assayed by measuring O_2 consumption (Yellow Springs oxygen electrode) stimulated by addition of reduced cytochrome *c* to mitochondria [12]. Mitochondria were resuspended in electrode buffer (3 ml) consisting of 0.3 M mannitol, 10 mM KH_2PO_4, 10 mM Mops (pH 7.2), 10 mM KCl, 5 mM $MgCl_2$, 0.1% BSA, 8 mM ascorbate and 30 μM cytochrome *c*. The cytochrome-*c*-stimulated O_2 consumption rate of intact mitochondria was corrected for that remaining after addition of 0.2 mM KCN. Mitochondria were osmotically burst by resuspension in distilled water followed 30 s later by an equal volume of 2× electrode buffer. The cytochrome-*c*-stimulated O_2 consumption rate of the 'burst' mitochondria was compared to that of the mitochondria suspended in osmotic medium and intactness was calculated as percentage of the mitochondrial population with intact outer membranes.

Mitochondrial treatments and enzyme assays

Digitonin was used to permeabilize mitochondrial membranes by modifications of the method of Schwaiger et al. [13] in which recrystallized digitonin in a buffer consisting of 0.3 M sucrose, 10 mM Mops (pH 7.2), 1 mM EDTA is incubated with purified mitochondria (0.5 mg protein) in a total volume of 200 μl. After 5 min the solution was diluted with 1.0 ml of 0.6 M sucrose, 10 mM Mops (pH 7.2), 50 mM KCl and 1 mM EDTA. The tubes were centrifuged for 8 min at $12000 \times g_{max}$ in a refrigerated Eppendorf centrifuge and the supernatant was removed. An equal volume of buffer was added to the pellet which was sonicated and vortexed to resuspend the membranes.

Mitochondrial outer membranes were osmotically lysed by reducing the osmoticum in which the mitochondria were resuspended. After resuspending the mitochondria in the medium of choice for 10 to 30 min on ice, a high sucrose buffer (0.6 M sucrose, 10 mM

Mops, 1 mM EDTA, 50 mM KCl (pH 7.2)) was added. Mitochondria were centrifuged at $12000 \times g_{max}$ for 10 min and the supernatant was removed. The mitochondrial pellet was resuspended in the same buffer. Enzyme assays were performed on both supernatant and pellet. The total activity for each treatment (supernatant plus pellet) was consistent in a given experiment, except in experiments where trypsin was used. In the experiments shown in Table II, mitochondria were centrifuged at $12000 \times g_{max}$ for 10 min after treatment, the mitochondrial pellet was resuspended, sonicated and centrifuged at $100000 \times g$ for 30 min. The supernatant was separated from the pellet. Both supernatants were reduced in volume (Amicon YM 10 membrane) to 1.5 ml. A volume of 100 μl was assayed for modulator activity. If no activity was detected, then a volume of 200 μl was assayed.

The trypsin used in the experiments as shown in Table II was reconstituted just before use. At the end of the trypsin treatment, soybean trypsin inhibitor (2 × by weight) was added. Both the activity of the trypsin preparation and the effectiveness of soybean trypsin inhibitor to subsequently inactivate trypsin was monitored by observing lysis of fibrin (Endoproteinase test kit, Boehringer-Mannheim, Indianapolis, IN, USA). The quantity of trypsin and incubation time required to inactivate the modulator activity was determined by incubation of mitochondria with trypsin, subsequent inactivation with soybean trypsin inhibitor and assay for remaining activity on reconstituted VDAC. As a control, the effect of added inactivated trypsin on the enhancement of voltage-dependence of VDAC by modulator preparations was tested and no change was noted.

Fumarase [14], adylenate kinase [15] and Succinate-DCPIP reductase [16] were assayed by standard methods with the modifications of: (1) increasing the concentration of NADP to 0.4 mM from 0.2 mM in the adenylate kinase assay, and (2) adding 0.2% Triton X-100 to the fumarase assay to provide rapid access of substrate to all membrane compartments. Preliminary tests showed no inhibitory effect of Triton on fumarase activity up to a concentration of 1.0%. Protein was measured by the method of Peterson [17] or using bicinchoninic acid (BCA protein assay, Pierce, Rockford, IL, USA).

Measurement of modulator activity

The presence or absence of modulator activity in a given fraction was ascertained by measuring changes in voltage-dependence of VDAC reconstituted in planar bilayers as described in Holden and Colombini [5]. In brief, planar bilayers were prepared by the folded monolayer method of Montal and Mueller [18] from a synthetic lipid, diphytanoyl phosphatidylcholine (DPPC) or a 1:1 mixture of DPPC and soybean phospholipids which were purified by the method of Kagawa and Racker [19]. VDAC, solubilized by 1% Triton X-100, was added to a salt solution (1 M KCl, 5 mM $CaCl_2$) bathing the bilayer and spontaneously inserted into the bilayer. Currents across membranes containing multiple VDAC channels were monitored under voltage clamp conditions as described in Schein et al. [20]. Currents were monitored first at 10 mV, a voltage at which channels were generally open, followed by 60 mV for 3 min during which time the closure of channels was monitored. VDAC has multiple lower conductance (or 'closed') states, but rarely becomes completely nonconductive (see, for examples, Refs. 1,3,4–6,8,9). During the 3-min time period at 60 mV a stable closed-channel conductance was reached. This procedure was repeated until a consistent response by the channels was measured. Two voltage-dependent parameters were measured; (1) The difference between the open and closed conductance as a % of total conductance, and (2) the apparent time constant (τ) for channel closure. Aliquots of fractions to be tested for modulator activity were added to the salt solution bathing the membranes and mixed gently for 30 s. The measurement of voltage-dependent parameters was measured again and repeated again until consistent responses were measured. Modulator activity was judged to be present in tested fractions when large changes in the voltage-dependent parameters were measured (see Fig. 1 and Ref. 5)

DPPC was acquired from Avanti (Birmingham, AL, USA) and soybean phospholipids (Type II-S phosphotidylcholine) from Sigma (St. Louis, MO, USA). All other chemicals were reagent grade.

Results and Discussion

Digitonin digestion of mitochondrial membrane compartments

Digitonin disrupts membranes by binding sterol components of biological membranes [21]. It has been used to differentially permeabilize outer mitochondrial membranes [22]. The release of modulator activity and marker enzymes from the *N. crassa* mitochondria was titrated with digitonin. Fig. 1 shows that increasing concentrations of digitonin resulted first in the release of adenylate kinase, located in the intermembrane space between the outer and inner membranes. Significant release of adenylate kinase is seen after 5 min of treatment with 0.3% digitonin. In contrast, fumarase, a soluble enzyme of the mitochondrial matrix space, was not released until treatment with 0.6% digitonin. Under conditions of this experiment no release of the inner membrane electron transport complex II, measured as Succinate-DCPIP reductase was seen at any of the concentrations used suggesting that at 0.8%

digitonin, the inner membranes had not been significantly solubilized.

Fractions from experiments which contained only adenylate kinase activity (0.3 to 0.4% digitonin) were pooled (Pool A = 0.6 mg protein from 5.0 mg of total mitochondrial protein). This fraction represented material released from only the intermembrane space. Fractions which showed good release of both intermembrane and matrix components, that is adenylate kinase and fumarase (0.6 to 0.8% digitonin), were also pooled (Pool B = 0.84 mg protein from 5.0 mg total mitochondrial protein). The total volume of these pools was nearly identical and both pools were reduced 12-fold in volume to 0.8 ml using an Amicon filtration (YM-10 filter) system. The resulting fractions (100 μl representing 82 μg protein (Pool A); 105 μg protein (Pool B)) were tested for modulator activity using the bilayer assay as described in Materials and Methods. Both fractions increased equally the voltage-dependence of multiple VDAC channels in a manner characteristic of the modulator activity [5,6], indicating the presence of the modulator activity in both pools. This suggests that the modulator activity resides in the space between the inner and outer membrane as maximal activity was found under conditions where no matrix enzyme was released.

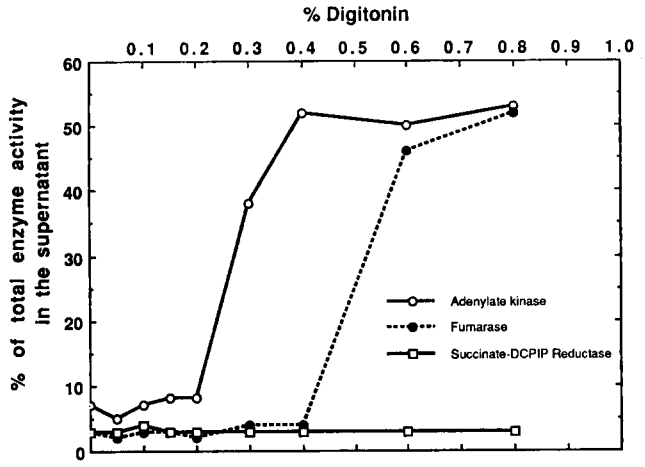

Fig. 1. Digitonin permeabilization of mitochondrial membrane compartments. Mitochondria, purified on percoll gradients, were treated (0.5 mg protein/treatment) with increasing concentrations of digitonin as described in Materials and Methods. The supernatant and mitochondrial fractions were assayed for enzyme activity and the % of total activity released to the supernatant was plotted as a function of % digitonin (w/v). In this experiment the mitochondria were 90% intact as determined by reduced cyt c assay. The activity (supernatant + pellet) for each digitonin treatment was consistent and averaged 913 nmol/min for fumarase and 219 nmol/min for adenylate kinase. Total succinate-DCPIP reductase activity decreased with increasing digitonin concentrations, but was negligible in the supernatant in all cases. This experiment was representative of three conducted on separate membrane preparations.

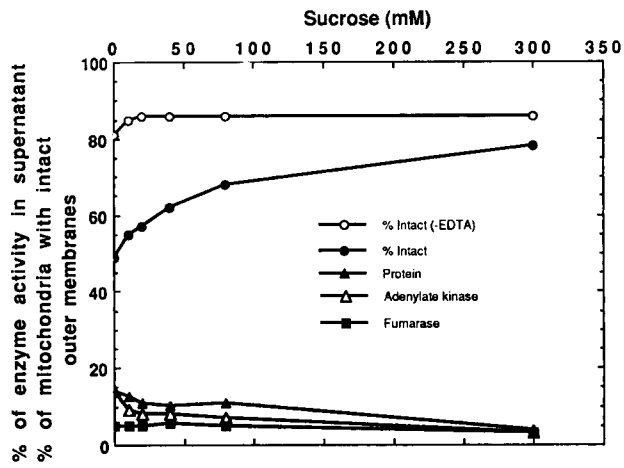

Fig. 2. Intactness of *N. crassa* outer mitochondrial membrane is maintained under hypo-osmotic conditions. Mitochondria were diluted into buffer (10 mM Mops, 1 mM EDTA) containing varying concentrations of sucrose. After a 30 min incubation on ice an equal volume of high sucrose buffer was added. An aliquot was removed to determine outer membrane intactness (●) and the remainder centrifuged to pellet the mitochondria. The supernatant was assayed for released enzymes and protein. A second experiment (○) shows result of similar experiment (intactness assay only) with a separate batch of mitochondria where EDTA was eliminated from the buffer.

Hypo-osmotic lysis of mitochondrial membranes

Experiments were undertaken to determine susceptibility to inactivation by trypsin of the modulator activity in situ. Due to the time required for inactivation of the modulator activity by trypsin, hypo-osmotic lysis, rather than digitonin solubilization, was used to permeabilize the mitochondrial outer membrane.

Osmotic conditions which resulted in the rupture of the outer mitochondrial membrane were first determined using an assay where the ability of reduced cytochrome *c* to stimulate oxygen consumption by mitochondria was monitored. Reduced cytochrome *c* should be able to stimulate oxygen consumption only under conditions where rupture of the outer membrane has occurred as cytochrome *c* is impermeant to intact mitochondria. Fig. 2 demonstrates that elimination of sucrose from the buffer in which mitochondria were resuspended did not result in a high percentage of outer membrane rupture. When sucrose was eliminated completely the percentage of intact mitochondria was still 50% (as compared to 78% for control mitochondria in 0.3 M sucrose). A separate experiment, shown also in Fig. 2, demonstrates that EDTA was required for even this limited rupture of the outer membrane. Elimination of 1 mM EDTA from the medium resulted in almost no outer membrane rupture. Table I shows that severe hypo-osmotic conditions were required for the complete rupture of the mitochondrial outer membrane of *N. crassa* as determined using the cytochrome-*c* stimulation assay. The

requirement for severe hypo-osmotic stress for rupture of the outer mitochondrial membranes of *N. crassa* has also been shown by Mannella [10]. In contrast to the digitonin experiments where adenylate kinase appeared in the supernatant with permeabilization of the outer membrane, the intermembrane space marker remained in the pellet. Thus, in these experiments the cytochrome-*c* assay was critical to determine integrity of the outer membrane.

Adenylate kinase was not significantly released from mitochondria by hypo-osmotic lysis (Table I) in contrast to digitonin permeabilization (Fig. 1). Subsequent sonication of iso-osmotically treated mitochondria resulted in the release of only about 50% of total adenylate kinase (Table II). This suggests that adenylate kinase in *N. crassa* mitochondria may be bound to either membrane bordering the intermembrane space. Digitonin may loosen this binding, while hypo-osmotic lysis does not. In potato mitochondria, adenylate kinase is so tightly bound to the outer face of the inner membrane that digitonin treatment (at the concentrations used in the experiments reported here) does not release adenylate kinase from the membrane [23]. Table II also shows that adenylate kinase is somewhat sensitive to trypsin under conditions which completely inactivate the modulator activity.

The matrix enzyme, fumarase, was not released by hypo-osmotic conditions, but 80% of fumarase activity was released by sonication (Table II). This suggests that hypo-osmotic lysis of the outer membrane did not disturb the integrity of the inner membrane, but that subsequent sonication significantly disintegrated the inner membrane. Fumarase was also demonstrated to be generally insensitive to trypsin under the conditions imposed here.

TABLE I

Severe hypo-osmotic conditions are required to completely rupture the outer mitochondrial membrane

Treatment	Intact outer membrane (%)	% Activity in supernatant	
		Fumarase	Adenylate kinase
Iso-osmotic			
Buffered 0.3 M sucrose	73	1.1	2.0
Hypo-osmotic			
10 mM Mops, 1 mM EDTA	47	2.8	4.0
Dilutions of hypo-osmotic treatment			
1 mM EDTA, 1:10 dilution	37	3.0	6.8
1 mM EDTA, 1:20 dilution	22	4.0	5.3
1 mM EDTA, 1:40 dilution	0	4.1	6.2

TABLE II

Susceptibility of the VDAC modulator to inactivation by trypsin under iso- and hypo-osmotic conditions

Resuspension of mitochondria in 1 mM EDTA (1:20 dilution) was used for the hypo-osmotic conditions. Treatment ± trypsin was for 2 h at room temperature.

Treatment	Adenylate kinase (μmol/min fraction	Fumarase (μmol/min fraction	Modulator activity
A. Activity released from mitochondria			
1 Iso-osmotic	0.12	0.6	–
2 Hypo-osmotic	0.22	1.1	+
3 Iso-osmotic + trypsin	0.02	1.1	–
4 Hypo-osmotic + trypsin	0.01	1.8	–
B. Activity released from mitochondria by sonication – post treatment			
1 Iso-osmotic	2.81	20.4	+
2 Hypo-osmotic	2.09	18.4	+
3 Iso-osmotic + trypsin	2.49	18.2	+
4 Hypo-osmotic + trypsin	1.85	17.1	–
C. Activity remaining in mitochondrial membrane pellet			
1 Iso-osmotic	1.88	4.5	
2 Hypo-osmotic	2.32	4.8	
3 Iso-osmotic + trypsin	0.24	2.7	
4 Hypo-osmotic + trypsin	0.14	2.0	
D. Total activity			
1 Iso-osmotic	4.81	25.5	
2 Hypo-osmotic	4.63	24.3	
3 Iso-osmotic + trypsin	2.75	22.0	
4 Hypo-osmotic + trypsin	2.00	20.9	

Accessibility of the modulator activity to inactivation by trypsin under hypo-osmotic conditions

Washing mitochondria with iso-osmotic medium did not result in the release of modulator activity (Table II, A1). Sonication was required to release the modulator activity (Table II, B1) from mitochondria in iso-osmotic medium. Not surprisingly, addition of trypsin to the iso-osmotic medium resulted in no modulator activity in the supernatant (Table II, A3). However, when the trypsin-containing medium was washed away and soybean trypsin inhibitor added, sonication of the mitochondria resulted in the release of modulator activity (Table II, B3). Thus, in the intact mitochondrion, the modulator activity is protected from inactivation by trypsin.

Exposure of mitochondria to severe hypo-osmotic conditions that damaged most of the outer membranes but released less than 5% of the fumarase activity, resulted in the release of the modulator activity (Table II, A2). Not all of the modulator was released as subsequent sonication released more modulator activ-

ity (Table II, B2). The addition of trypsin to the hypo-osmotic medium destroyed all modulator activity (Table II, A4). The trypsin was also effective at destroying the additional modulator activity that would have been released by sonication treatment after removal of trypsin and addition of trypsin inhibitor. While it is possible that hypo-osmotic treatment both released some modulator and made the rest accessible to trypsin, it is also possible that the presence of trypsin with the hypo-osmotic medium resulted in more effective breakage of the outer membrane. The inner membrane in this treatment appears to be intact as evidenced by the lack of fumarase release. Thus, selectively damaging the outer membrane by hypo-osmotic treatment resulted in both modulator release and trypsin accessibility to the modulator. These data again support an intermembrane space localization for the modulator activity in situ.

While both types of experiment support the intermembrane space as the cellular location of the modulator, the possibility that the modulator is also present in the cytosol or in the matrix space has not been eliminated by these experiments. The demonstration that an intact outer membranes confer protection from trypsin digestion eliminates the possibility that the modulator activity is exclusively a cytoplasmic protein bound to the external side of the outer membrane, though we have not eliminated the possibility that the cytosol also contains modulator activity. The experiments do demonstrate a mitochondrial localization for the modulator activity. This could include the matrix also, but the data in Table II are more consistent with the the idea that the modulator is localized in the intermembrane space. For example, the treatment of mitochondria with trypsin in hypo-osmotic medium resulted in the elimination of all modulator activity in the mitochondria (Table I, Table II A4, B4) and no significant release of fumarase, suggesting that the inner membrane was undamaged.

The presence of the modulator in the intermembrane space (and/or the cytosol) would permit physical interaction with VDAC and the possibility of a physiological function for the modulator. Therefore, the modulator effects on VDAC are less likely to be in vitro curiosities. VDAC is more sensitive to transmembrane voltage in the presence of the modulator, leading to reduced conductance of the channel under conditions where permeability would be greater in the absence of the modulator. Planar bilayer membrane studies of the modulator interaction with VDAC demonstrated the requirement for a negative membrane potential on the side of the membrane where the modulator is physically located [7]. Thus, a negative potential with respect to the intermembrane space is also a requirement for modulator activity in vivo. The existence of such a potential has not yet been demonstrated, but is possible through a Donnan equilibrium set up by charged molecules too large to traverse the channels.

Addition of modulator preparations to intact mitochondria have decreased State-3 respiration and adenylate kinase activity [7] suggesting that the modulator can effect a change in VDAC conductance on the cytoplasmic side of the membrane. This is not inconsistent with the report in this paper of an intermembrane space location for the modulator activity because in bilayer studies the modulator has been shown to interact with either surface of the channel [5] as long as the membrane potential is negative with respect to the side of the membrane where the modulator is located.

As VDAC is a major pathway across the outer membrane for respiratory substrates and other metabolites, the modulator may provide a primary control on mitochondrial and cytosolic activities, such as ATP-dependent activities. VDAC has been found in mitochondria from any organism investigated and is a highly conserved protein with respect to structure and function. VDAC modulator, derived from mitochondria of one species, has been shown to increase the voltage-dependence of VDAC isolated from very different species [9]. This implies a common and well-conserved role for the VDAC modulator.

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