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The Topology of VDAC as Probed by Biotin Modification*

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The outer membrane of mitochondria contains channels called VDAC (mitochondrial porin), which are formed by a single 30-kDa protein. Cysteine residues introduced by site-directed mutagenesis at sites throughout *Neurospora crassa* VDAC (naturally devoid of cysteine) were specifically biotinylated prior to reconstitution into planar phospholipid membranes. From previous studies, binding of streptavidin to single biotinylated sites results in one of two effects: reduced single-channel conductance without blockage of voltage gating (type 1) or locking of the channels in a closed conformation (type 2). All sites react with streptavidin only from one side of the membrane. Here, we extend this approach to VDAC molecules containing two cysteines and determine the location of each biotinylated residue with respect to the other within the membrane. When a combination of a type 1 and a type 2 site was used, each site could be observed to react with streptavidin. Two sets of sites located on opposite surfaces of the membrane were identified, thereby establishing the transmembrane topology of VDAC. A revised folding pattern for VDAC, consisting of 1 α helix and 13 β strands, is proposed by combining these results with previously obtained information on which sites are lining the aqueous pore.

VDAC channels are found in the mitochondrial outer membrane of cells from all eukaryotic kingdoms (1). They are the major pathways by which metabolites, including ATP, pass through this outer membrane (2, 3) and, therefore, are likely to play important roles in the regulation of mitochondrial functions.

Each VDAC channel consisting of a single 30-kDa polypeptide (4, 5) forms an aqueous pore ~3 nm in diameter (6, 7). Detailed information on the voltage-gating properties of VDAC and the associated conformational rearrangements have been obtained from studies of channels reconstituted into planar phospholipid membranes. These studies have demonstrated that, in response to transmembrane potentials of 30 mV or above, part of the channel wall, which serves as the voltage sensor, moves out of the membrane resulting in channel closure (8–10). These closed channels are essentially impermeable to ATP and have a reduced permeability to organic anions (2, 11). However, they are still permeable to small non-electrolytes (up to 1.8 nm in diameter) and to small monovalent cations (12). VDAC can close in response to transmembrane potentials of both polarities, due to the presence of two different gating processes.

The transmembrane topology of VDAC molecules has yet to be firmly established. Theoretical considerations predicted a "β-barrel" structure (13, 14). "Sided" β strands that would be appropriate to separate an apolar from a polar environment, were tested by the use of site-directed mutations (15). If engineered charge changes in proposed transmembrane segments affected channel selectivity, the associated protein segment was proposed to be a transmembrane strand. A lack of effect resulted in assignment of the segment to the membrane surface. In these experiments, the observed selectivity changes were in the expected direction, their magnitude correlated with the magnitude of the charge change, and the effects were additive when two or three mutations were present simultaneously. The results were consistent with a model in which a single, N-terminal α helix and 12 β strands form transmembrane domains that are organized into a barrel-like, water-filled channel in the open conformation. Other β-barrel structures have been proposed that are largely based on the structures of bacterial porins. Some are supported by studies in which proteases and/or peptide-specific antibodies were used to localize sites on VDAC in intact mitochondria (16, 17). There is disagreement both within and among studies over the reliability and interpretation of the data. As a result, there is much disagreement on the folding pattern for VDAC in membranes.

In this study, we probed the topology of VDAC by using a variation of an approach pioneered by Qiu *et al.* (18) for colicin channels. Previous research (10) applied this method to VDAC by introducing single cysteine residues into VDAC from *Neurospora crassa*, a protein naturally devoid of cysteine residues. The purified protein was biotinylated with a cysteine-specific reagent and reconstituted into planar phospholipid membranes. Streptavidin, a soluble, 60-kDa protein that binds very tightly to biotin, was then used to probe the accessibility of the biotinylated cysteine residue. Here we introduced a pair of cysteine residues in order to determine if the chosen sites were on the same or the opposite side of the membrane. By using a common reference site, we were able to identify two sets of sites located on opposite surfaces of the membrane and thereby establish the transmembrane topology of the VDAC channel in planar membranes. A revised folding pattern for VDAC, which consists of 1 α helix and 13 β strands, is proposed by combining these results with previously obtained information on which sites are lining the aqueous pore and which are facing the bulk aqueous compartments.

EXPERIMENTAL PROCEDURES

Selection of Experimental System and Residues to Be Mutated—The experimental approach requires that a pair of cysteine residues be introduced into a VDAC molecule and that both residues be biotinylated without significantly changing the structure and function of the molecule. Previous work (10) introduced only single cysteine residues into

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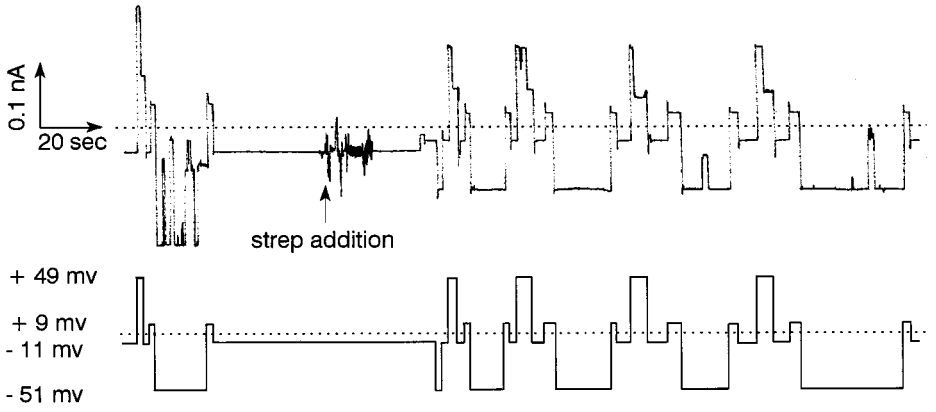

FIG. 1. **Type 1 effect: streptavidin binding reduced the conductance but did not stop the gating processes.** This is an experiment with a single biotinylated D156C channel. Illustrated are the current trace (*upper panel*), the corresponding applied voltages (*lower panel*), and the zero current level (*dotted line*). Prior to streptavidin addition, the channel was open at low potentials (+9 or −11 mV) and closed at high potentials (+49 or −51 mV). After binding streptavidin, the conductance was reduced but voltage-gating still occurred. However, closure at negative potential (−51 mV) was interfered with.

VDAC channels encoded by the *N. crassa* genome. This version of VDAC lacks cysteine residues and is tolerant of the insertion of cysteine residues. The sites chosen for mutation were likely water-accessible, based on the polarity of the amino acid being replaced and the current working model of the structure of the channel. Experimentally, the mutant channels were biotinylated with little change in function and thus presumably in structure.

Sixteen pairs of cysteine residues were introduced into VDAC (one pair at a time) into positions that had been characterized as single mutants. Most of the mutants shared a common cysteine introduced at position 53. This served as a common reference point. However, other pairs were used to confirm the results and ensure self-consistency.

Mutants are labeled as follows: the number is the location of the amino acid in the primary sequence and the leading and tailing letters represent the amino acid in the wild-type and mutant, respectively. The numbering includes the N-terminal methionine, which is probably removed in the mature protein.

Introduction of Cystine Residues by Site-directed Mutagenesis—The wild-type VDAC gene from *N. crassa* was subjected to site-directed mutagenesis and introduced into a yeast strain, M22-2, which lacks the chromosomal copy of the gene coding for YVDAC1, the only gene product that forms classical VDAC channels (19). VDAC proteins were isolated from the mitochondrial membranes as described previously (15).

Biotinylation of Mutant VDAC Proteins—Cysteine was performed as described previously (18). About 2 μg of mutant VDAC protein in a 100-μl volume were biotinylated immediately after isolation from mitochondrial membranes. The reaction contained 125 mM of sodium phosphate, 6 mM EDTA, pH7.0, and 150 μg of 3-(*N*-maleimidylpropionyl)biocytin (Molecular Probes, M1602) (3 mg/ml dissolved in 3:2 dimethyl sulfoxide:dimethylformamide). Reactions were carried out at room temperature for 1 h and stopped by adding 16 μl of β-mercaptoethanol. Reaction by-products were removed by dialysis against 1 liter of 1% Triton X-100, 50 mM KCl, 10 mM HEPES, 1 mM EDTA, pH 7.0 (24 h at 4 °C).

Electrophysiological Studies—VDAC proteins were reconstituted into planar phospholipid membranes (soybean phospholipids) by adding detergent-solubilized biotinylated VDAC sample (1–5 μl) to the cis side of the chamber (5 ml). The membranes were made as described previously (20). The aqueous phases were 1.0 M KCl, 1 mM $CaCl_2$, 5 mM MES,[1] pH5.8. Calomel electrodes were used and results corrected for electrode asymmetry. The experiments were performed under voltage clamp conditions. The sign of the applied voltage always refers to cis side (trans is virtual ground). Generally, 40 μg of streptavidin or neutravidin (Pierce) was added to 5 ml solution on one side or both sides of the membrane, as appropriate.

Estimation of the Percentage of Reconstituted Channels Affected by Streptavidin—Single channels were studied in order to obtain the percentage conductance decrease upon addition of streptavidin. This percentage decrease was compared with that observed in a membrane containing many channels (typically 25–50). By dividing the latter by the former and multiplying by 100, one obtains the percentage of channels in the membrane affected by streptavidin. For each mutant, the results obtained from different multi-channel experiments were multiplied by a weighting factor (to compensate for the different numbers of channels present in different experiments) and then averaged. The weighting factor was: (no. of channels present in the individual experiment)/(average no. of channels present in the experimental set to be pooled). The same weighting factor was used to determine the weighted standard deviation. In this case, the squares of the differences between the values and the weighted means were multiplied by the weighting factor.

[1] The abbreviation used is: MES, 2-(*N*-morpholino)ethanesulfonic acid.

RESULTS

Cysteine residues were introduced into two sites on *N. crassa* VDAC at positions that had been previous characterized as single mutations (10). Following purification and biotin modification, biotinylated channels were reconstituted into planar membranes and their electrophysiological properties examined prior and subsequent to streptavidin addition.

Mutants containing single biotinylated cysteine residues were affected by streptavidin binding in one of two ways depending on the location of the cysteine site. At type 1 sites, channels could still undergo voltage-dependent closure but the single-channel conductance was reduced. As illustrated in Fig. 1, channels containing a biotin-modified cysteine at position 156 (D156C) closed readily when either positive of negative potentials of 50 mV were applied. After streptavidin addition, the single-channel conductance was reduced but voltage gating still occurred. Channel closure in response to negative potentials was less likely but was possible. Cysteine residues at sites Asn-38, Lys-112, Thr-135, Asp-156, Ser-211, Arg-240, and Asp-264 were previously determined to be type 1 sites.

At a second set of sites, type 2 sites, channels could not gate and were locked in a low-conducting, closed, state. As shown in Fig. 2, biotinylation of cysteine at position 183 (T183C) resulted in channels being locked into a low-conducting state following streptavidin addition. No voltage gating was ever observed. Cysteine residues at positions Thr-53, Ser-7, His-23, Thr-69, Ala-79, Thr-183, Ser-190, and Glu-282 were previously determined to be type 2 sites. In these studies, no type 2 sites were identified that became exposed to added streptavidin at both positive and negative potentials.

Some Double Mutants Were Affected by Streptavidin Only When It Was Added to One Particular Side of the Membrane—Site-directed mutagenesis was used to generate VDAC molecules containing cysteine mutations at two separate sites. If both biotinylated cysteines are on the same side of the membrane, streptavidin addition should only affect the channels when added to the side where the biotinylated cysteines are exposed. Fig. 3 illustrates an experiment in which channels contain biotinylated cysteines at positions 53 and 79 (T53C/A79C). Trans addition of streptavidin had no detectable effect on channel function or conductance. Addition of streptavidin to the cis side of the membrane resulted in a pronounced reduction in conductance. Other pairs of biotinylated cysteines (T53C/T135C, T53C/T156C, T53C/R240C, T53C/D264C, T53C/H23C, H23/S7C, and T69C/S190C) resulted in channels that

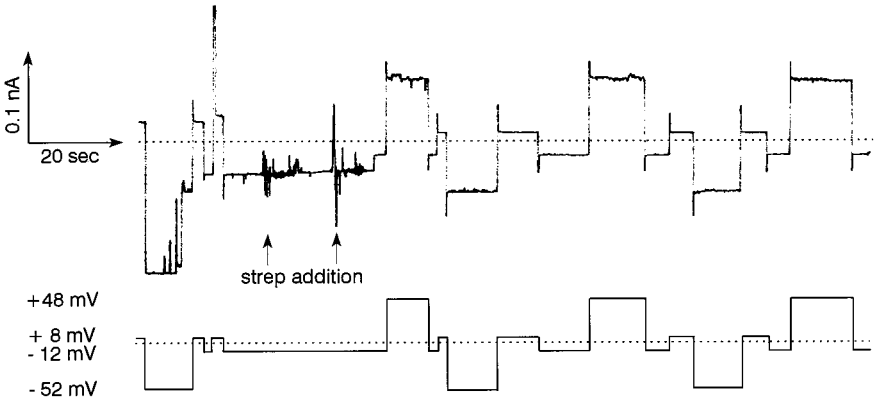

FIG. 2. **Type 2 effect: streptavidin binding trapped the channel in a closed state.** Streptavidin was added to a single biotinylated T183C channel while the channel was in the open state under a weak negative potential (−12 mV). The conductance dropped as the channel was trapped closed. Irrespective of the applied voltage, the conductance remained essentially unchanged (the channel remained closed).

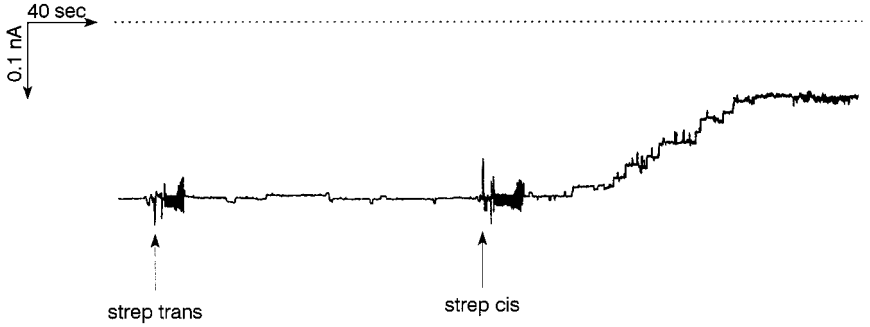

FIG. 3. **Biotinylated T53C/A79C mutant channels (~10) were only affected by streptavidin from the cis side.** The voltage was held at −7mV. Streptavidin added to the trans side had no effect, but addition to the cis side resulted in the channels being trapped closed seen from the steep reduction in current.

were affected in similar ways, *i.e.* streptavidin added to only one particular side of the membrane (not necessarily the cis side) caused an alteration in channel function. These pairs form the set called group 1 (Table II). One straightforward conclusion would be that Ser-7, His-23, Thr-53, Thr-135, Thr-156, Arg-240, and Asp-264 all face the same side of the membrane in at least one conformation of the channel.

Experiments were devised to reveal streptavidin binding at each of the two sites so as to eliminate the possibility that only one of the two sites was actually biotinylated. Pair of sites were chosen that included both a type 1 and a type 2 site. The different effects of streptavidin binding to these sites allowed each site to be distinguished. Fig. 4 shows an experiment with a single biotinylated T53C/D264C channel; Asp-264 represents a type 1 site and Thr-53 a type 2 site. Streptavidin was only added to the cis side of the membrane. The type 1 effect was observed soon after streptavidin addition (Fig. 4*A*), *i.e.* channels can still respond to voltage changes yet the conductance is reduced. In all similar experiments, a type 1 effect was always observed first. Apparently streptavidin binds more readily to the biotin attached to type 1 sites (here, position 264). Previous studies have shown that these sites are exposed in open channels and type 2 sites are probably not. As shown in Fig. 4*A*, the application of 40 mV resulted in good closure at positive potentials but not at negative potentials. Higher voltages, 60 mV, were needed to observe closure at negative potentials (Fig. 4*B*). This closure delivers site 53 to the surface, allowing binding to cis-added streptavidin (10). After the first observed closure at negative potentials, the channel could be easily reopened, but after the second (indicated by the *arrow*), the channel never reopened, consistent with previous findings that streptavidin binding at type 2 sites like Thr-53 locks channels in the closed conformation. Thus, two distinct actions of streptavidin can be observed on the same molecule and from the same side of the membrane, indicating that both sites contain biotin-modified cysteines. Similar results were observed with molecules containing modified cysteines at T53C/T135C, T53C/D156C, T53C/R240C, and T53C/D264C (group 1A, Table II). In all of these cases, a type 1 site was paired with a type 2 site and effects consistent with streptavidin binding to each site were observed.

Some Double Mutants Were Affected by Streptavidin Added to Either Side of the Membrane—On a membrane containing multiple biotinylated T53C/S190C channels, streptavidin was first added to the trans side of the membrane (Fig. 5), and some reduction in current was observed. After the current had become stable, streptavidin was added to the cis side of the membrane and a further reduction in current was observed. Similar results were observed with the following pairs of mutations: T53C/N38C, T53C/K112C, T53C/S211C, T69C/R240C, T53C/T183C, T53C/T69C, T53C/S190C, and T53C/E282C. These collectively form the second group of double mutants (Table II). These are interpreted as pairs of sites located on opposite sides of the membrane. However, the result could also be due to channels inserting in opposite directions. By examining single channels containing a mutation at both a type 1 and a type 2 site, one can demonstrate that both sites are biotinylated on a single molecule and located on opposite sides of the membrane.

Fig. 6 illustrates an experiment with a single biotinylated T53C/N38C channel. The addition of streptavidin to the cis side of the membrane did not stop the gating process, but it did reduce the conductance of the channel. This was the type 1 effect expected from the binding of streptavidin to biotin at position 38. Trans addition of streptavidin completely stopped the gating processes. This is the type 2 effect expected from the binding of streptavidin at position 53. Note the rapid, unobstructed, binding of streptavidin to the type 2 site in this case (in contrast to Fig. 4). Therefore, both type 1 and type 2 effects were observed on the same molecule but from opposite sides of the membrane.

Similar results were observed with biotinylated group 2A mutants: T53C/K112C, T53C/S211C, and T69C/R240C (Table II).

Demonstration of Localization on Opposite Sides of the Membrane in Pairs of Type 2 Sites—For channels containing

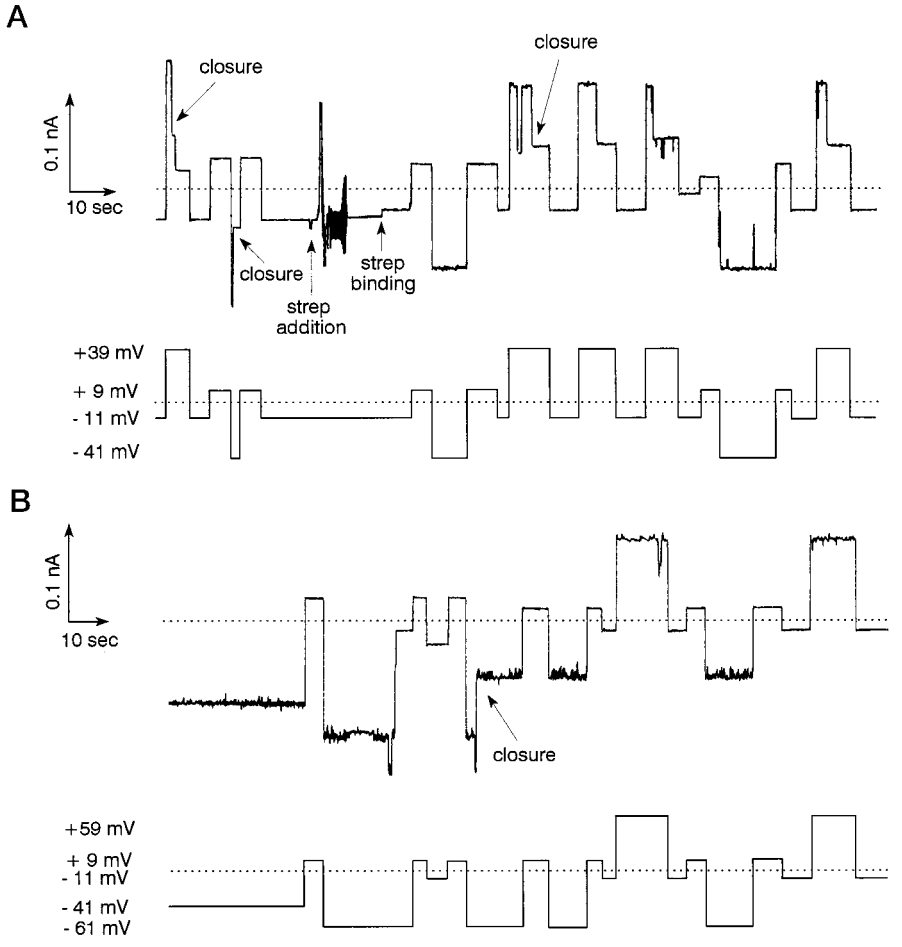

FIG. 4. **Both type 1 and type 2 effects were sequentially observed when streptavidin was added to one side of a membrane containing one biotinylated T53C/D264C VDAC channel.** The experiment begins in *panel A* and continues in *B*. Streptavidin was only added to the cis side. A type 1 effect was first observed (*panel A*). The overall conductance was reduced, but voltage-gating was still evident. The closure at negative potentials was interfered with but could be elicited at greater voltages (−61 mV, *panel B*). After the second closure at negative potentials, the channels could not reopen (type 2 effect).

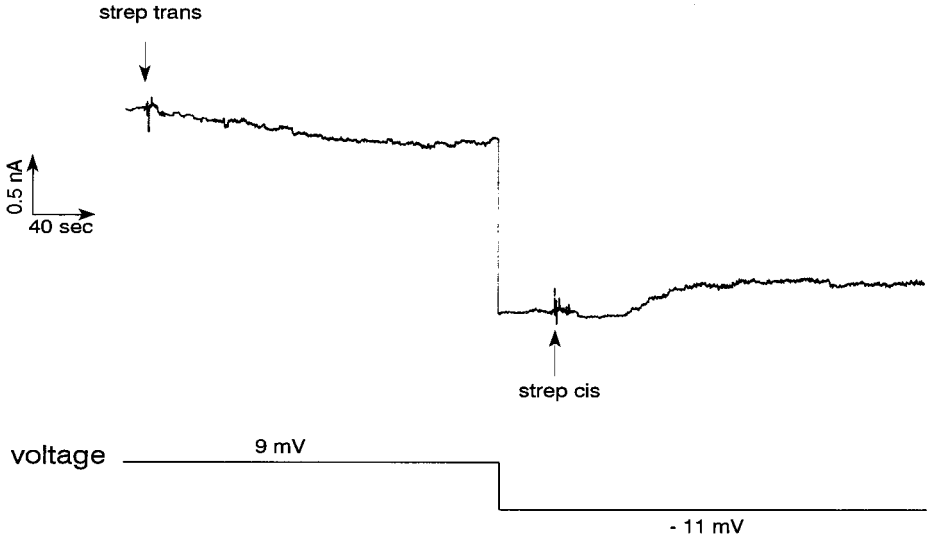

FIG. 5. **Biotinylated T53C/S190C channels were affected by streptavidin addition to either side of the membrane.** Streptavidin was first added to the trans side of the multi-channel membrane, resulting in a reduction in current. After the reduction stopped, streptavidin was added to the cis side, resulting in a further reduction in current.

the following pairs of mutations, T53C/T69C, T53C/T183C, T53C/S190C, and T53C/E282C, both of the cysteine residues were introduced at type 2 sites. Streptavidin binding at such sites locks the channels in a closed conformation, and hence the tactics used above cannot be used to verify that both sites are binding streptavidin. However, by taking advantage of incomplete biotinylation, one can show that the results are consistent with two sites located on opposite sides of the membrane.

Streptavidin added to multi-channel membranes affected these mutants from either side of the membrane. A partial effect was observed when streptavidin was added to one side, and further inhibition occurred when subsequently added to the other side. If both sites are on the same side of the membrane, the only way one could have this result is if channels inserted in a mixed orientation (hypothesis 1). Alternatively, the sites could be located on opposite sides of the membrane but the biotinylation might be incomplete, resulting in only a partial effect from streptavidin addition to one side of the membrane (hypothesis 2).

These two hypotheses make different experimental predictions. In the first, the degree of effect of streptavidin addition to

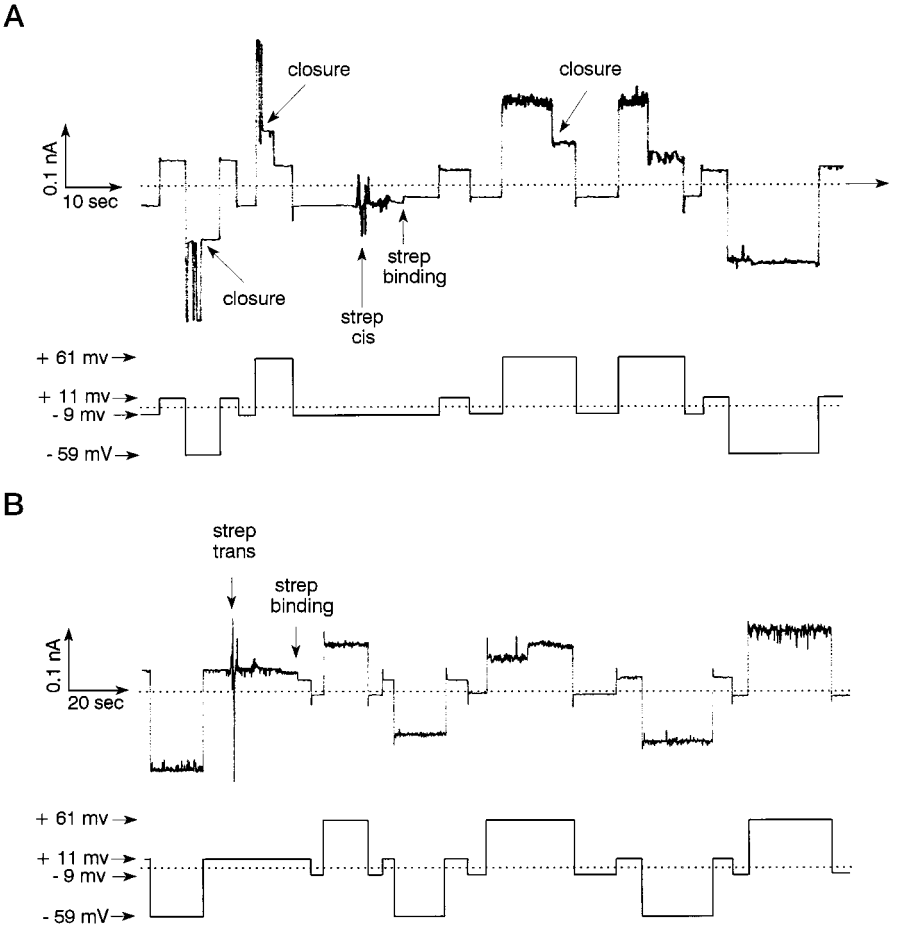

FIG. 6. **Both type 1 and type 2 effects were observed on a single biotinylated T53C/N38C channel but only if streptavidin was added to both sides of the membrane.** The experiment begins in *panel A* and continues in *B*. Streptavidin added to the cis side (*panel A*) reduced the conductance of the channel, but voltage gating was still evident (type 1 effect). Addition of streptavidin to the trans side (*panel B*) resulted in further current reduction. Voltage gating no longer occurred (type 2 effect).

one side is independent of which side streptavidin was added to first. In the second, the degree of effect of streptavidin addition will depend on which side it was added first and also on the degree of biotinylation. There would be three different populations of channels in the membrane. One population (I) has only the cis side cysteine biotinylated. Another population (II) has only the trans side cysteine biotinylated. The third population (III) has both cysteines biotinylated. The first two populations of channels can only bind to streptavidin when added to the correct side. The third population can bind streptavidin irrespective of the side to which it is added.

To distinguish between these two hypotheses, experiments were performed in two ways: 1) streptavidin was added first to the cis side and the drop in current was quantitated (effect *a*) and then to the trans side (effect *b*); 2) streptavidin was first added to the trans side (effect *c*) and then to the cis side (effect *d*).

If hypothesis 1 is correct, *i.e.* both sites are on the same side but channels insert in both directions, there should be no difference between *a* and *d* or *b* and *c*. However, if hypothesis 2 is correct, *i.e.* the sites are on opposite sides but the biotinylation is incomplete, "*a*" should reflect streptavidin binding to both population I and III. Effect "*b*" should reflect binding to population II only. Effect "*c*" should reflect streptavidin binding to population II and III, and effect "*d*" should reflect binding to population I only. In theory, the probability that both cis and trans cysteine residues were biotinylated (population II) should equal to the product of the probabilities that only the cis or trans side was biotinylated.

Thus, $a = a \cdot c + d$, and $c = a \cdot c + b$.

Table I summarizes the experimental observations and shows agreement with hypothesis 2 and sharp disagreement with the predictions of hypothesis 1. Thus, the cysteine residues in these pairs were on opposite sides of the membrane.

Summary of the Effects of Streptavidin on All Pairs of Mutations (Table II)—All the mutants can be divided into two groups, according to the effects of streptavidin on these channels. The biotinylated group 1 mutants were affected by streptavidin only from one side of the membrane, whereas biotinylated group 2 mutants were affected by streptavidin from either side. Each group of mutants was subdivided into A and B subgroups. For the mutants in the A subgroup, one of the cysteine residues was introduced into a type 1 site and the other in a type 2 site. For the mutants in subgroup B, both cysteines were introduced into type 2 sites.

DISCUSSION

The introduction of cysteine residues into a VDAC channel generates sites that can be specifically biotinylated, allowing the location of modified sites to be identified following interaction with streptavidin. Molecules containing introduced cysteines function normally following incorporation into planar phospholipid bilayers before and after biotin modification and the binding of streptavidin to the tethered biotin results in clearly measurable changes in the properties of the channel. Therefore, the binding of streptavidin yields unambiguous information on the location of individual residues both with respect to the membrane and to other sites in the molecule within the overall topological organization of the VDAC channel. Localization is precise to one amino acid residue. Unlike small molecular weight reagents that might penetrate into protein crevices, cross the membrane through the pore or, by solubility/diffusion, through the lipid membrane, streptavidin is large (60 kDa) and highly water-soluble and

TABLE I

The percentages of channels affected by streptavidin in experiments with membranes containing multiple channels

The values are means ± S.D. of two to five experiments. The number of channels averaged ranged from 16 to 175.

Mutants	Cis first *a*	Trans later *b*	Trans first *c*	Cis later *d*	$(a \cdot c + d)$	$(a \cdot c + b)$
T53C/T69C	68 ± 9	21 ± 2	79 ± 5	16 ± 8	70	75
T53C/T183C	92 ± 6	3 ± 3	19 ± 0	81 ± 1	98	20
T53C/S190C	84 ± 3	5 ± 3	53 ± 4	42 ± 9	88	51
T53C/E282C	77 ± 9	6 ± 3	40 ± 10	40 ± 13	71	37

TABLE II

The effective side of streptavidin addition on membranes containing multiple channels (+, effective; −, not effective)

Mutant	Cis side	Trans side
Group 1		
A		
T53C/T135C	+	−
T53C/D156C	+	−
T53C/R240C	+	−
T53C/D264C	+	−
B		
T53C/H23C	+	−
T53C/A79C	+	−
H23C/S7C	+	−
T69C/S190C	−	+
Group 2		
A		
T53C/N38C	+	+
T53C/K112C	+	+
T53C/S211C	+	+
T69C/R240C	+	+
B		
T53C/T69C	+	+
T53C/T183C	+	+
T53C/S190C	+	+
T53C/E282C	+	+

thus cannot penetrate membranes to any meaningful extent. In addition, the arm connecting the cysteine sulfur to the biotin results in a 0.7-nm molecular connector, which joins the surface of the streptavidin to the surface of the channel once the biotin modified cysteines have bound to streptavidin. Thus, these structural constraints permit little error in localizing the site of interest to one surface of the membrane or individual sites with respect to each other. Although biotinylation influences the direction of channel insertion (10), the location of residues with respect to a common reference point (here Thr-53) can be used to precisely constrain models of the transmembrane topology of the VDAC channel.

In group 1 mutants, biotin-modified cysteines were accessible to streptavidin only from one side of the membrane and not from the other side. In the case of group 1A mutants, both type 1 and type 2 effects in single channels were observed on streptavidin addition to the same side of the membrane demonstrating that both cysteine residues were biotin-modified. Therefore, these results are consistent with models in which Ser-7, His-23, Ala-79, Thr-135, Asp-156, Arg-240, and Asp-264 are on the same side of the membrane as Thr-53. The results also show that positions Thr-69 and Ser-190 are on the same side of the membrane, but not the same side as Thr-53.

Although the data clearly show that biotin-modified cysteines at Ser-7 and His-23 are accessible to added streptavidin from the same side of the membrane, the model shown in Fig. 7 would suggest that these sites should be on opposite sides of the membrane. Previous studies have demonstrated that this N-terminal α helix is a mobile domain that may partition preferentially to one side of the membrane (21). The model shown in Fig. 7 is necessarily a static depiction of the transmembrane topology of the VDAC channel whereas the actual structure in a membrane is likely dynamic. Thus, the accessibility of these residues from the same side of the membrane is likely to represent dynamic fluctuations in the topology of the molecule that occur even in the absence of applied voltage, as has been observed previously (10).

Biotinylated group 2 mutants were accessible to streptavidin from both sides of the membrane, indicating that each site within each pair resides on opposite sides of the membrane. Using combinations of type 1 and type 2 mutations, it was possible to demonstrate the effect of streptavidin binding to each biotin-modified cysteine in single channels but from opposite sides of the membrane. This excludes the possibility that the dual effect of streptavidin was due to molecules inserted in opposite direction. Additional evidence is supplied by the dependence of the magnitude of the streptavidin effect on the sequence of addition of streptavidin in group 2B mutants. The results followed the predicted mathematical pattern expected from two sites located on opposite sides of the membrane. Therefore, we conclude that Asn-38, Thr-69, Lys-112, Thr-183, Ser-190, Ser-211, and Glu-282 are located on the opposite side of the membrane from Thr-53. The results also showed that Arg-240 was located on the opposite side of membrane to Thr-69 and thus must be on the same side as Thr-53.

The self-consistency of this approach is supported by the analysis of channels containing cysteine residues at position Thr-53, Arg-240, Thr-69, and Ser-190. According to the results discussed so far, Thr-53 and Arg-240 should be on one side of the membrane, whereas Thr-69 and Ser-190 should be on the opposite side. Mutants T53C/R240C and T69C/S190C were found to be accessible to streptavidin only from one side of the membrane, whereas mutants T53C/T69C, T53C/S190C, and S190C/R240C were found to be accessible to streptavidin from either side of the membrane. These results agree exactly with each other and support the validity of the approach.

The sites examined in this work include both type 1 and type 2 sites. Previous work showed that type 1 sites are static and permanently located on the VDAC surface facing the bulk phase. However, type 2 sites are located on mobile domains and are likely inaccessible to streptavidin in the channel's open conformation. Thus, type 2 sites become accessible only after conformational rearrangements associated with voltage-dependent closure and therefore come to the surface from an unknown depth within the protein or become exposed during this process. Nevertheless, as all sites are only accessible to one membrane surface (*i.e.* no single site binds streptavidin in channels closed at both positive and negative potentials), it is reasonable to define them as being on or closest to one or the other surface of the membrane.

These results require a major change in the transmembrane folding pattern for VDAC from *N .crassa*. The current working folding pattern is based on that deduced for VDAC1 from the yeast, *Saccharomyces cerevisiae* (22). The results presented here agree with this folding pattern if a new transmembrane β strand is inserted between position 135 and 144. This causes the C-terminal half of the molecule to be flipped, so that now the N and C termini are oriented on the same side of the membrane (Fig. 7). We still believe that the basic folding patter is conserved in VDAC from all known species and the insertion

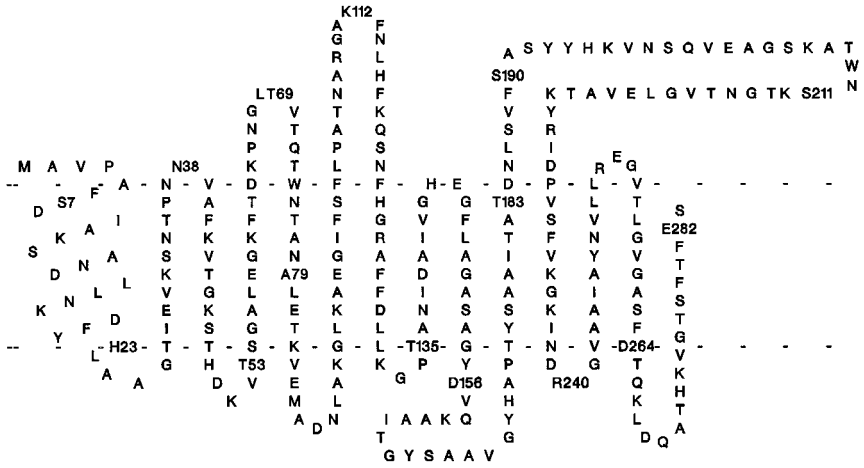

FIG. 7. **The modified folding pattern for VDAC from *N. crassa* in the open state.** Starting from the *left side*, the figure illustrates 1 α helix and 13 β strands spanning the hydrophobic part of the membrane (*dashed lines*). Amino acids are indicated by the *single-letter code*, and those mutated to cysteine are *numbered*. The newly added transmembrane strand includes position 135.

of the new strand does not contradict previous work on yeast VDAC1 and human VDAC1.[2] Importantly, this folding pattern is consistent with previous electrophysiological data on engineered point mutations in the yeast VDAC1 (15).

Previous studies aimed at probing the transmembrane topology of VDAC from *N. crassa* have employed peptide-specific antibodies. Stanley *et al.* (17) reported that lysis of *N. crassa* mitochondrial outer membranes increased the binding of antibodies raised against synthetic peptides corresponding to regions 1–20, 195–210, and 272–283, suggesting that these epitopes were exposed at the intermembrane side of the mitochondrial outer membrane. The binding of antibodies raised against regions 129–145 and 251–268 was not increased by mitochondrial lysis, suggesting that these epitopes were exposed at the cytosolic surface. In addition, the use of external proteases has identified a number of accessible regions defining sites exposed at the cytosolic surface, including 20–60, 80–100, 110–130, 145–180, and 190–230.

In order to compare results obtain in these studies to those presented here, we need to establish which surface faces the cytosol. Studies using antibodies directed to peptides representing the 20 amino acids at the N terminus seem to allow this assignment with the highest level of confidence. This region was previously established to be a transmembrane region in yeast VDAC based on changes in the channel's ion selectivity resulting from two mutations (D15K and K19E) (15). These mutations also influenced the steepness of the voltage dependence as expected, indicating that they are moving through the electric field during voltage gating (9). In addition, biotin-modification or cysteine introduced at positions Ser-7, Ser-12, and His-23 were all shown to result in type 2 responses, confirming their location on a mobile domain (10). Here, we find that biotin modification of cysteines at Ser-7 and His-23 is accessible to added streptavidin only from the same side of the membrane. The results of Stanley *et al.* (17) require this region (or portions of it) to face the intermembrane space. Thus, we conclude that all sites on the Thr-53 side of the membrane face the intermembrane space.

With this assignment, our folding pattern is consistent with the antibody results in the region 272–283. Regions 129–145 and 251–268 are too long to be useful discriminators. Our results conflict with the placement of region 195–210 facing the intermembrane space by the antibody experiments. However, this placement also conflicts with the protease accessibility results. Outer membrane lysis may result in structural changes in VDAC that expose the antibody binding site. Of the other protease-accessible regions, 20–80, 110–130, and 190–230 are consistent with our folding pattern. The other two, 80–100 and 145–180, are not.

The other proposed transmembrane folding patterns for VDAC (13, 16, 23, 24) are very inconsistent with the results presented in this paper. Two of these patterns are for mammalian VDAC, but species differences is an unlikely explanation due to the highly conserved nature of VDAC and its properties and the conserved patterns in the primary sequence that likely reflect the molecule's secondary structure (22). A closer look at the proposed transmembrane strands for *N. crassa* based on a Gibbs sampler approach (24) shows that proposed transmembrane strand 200–210 is in conflict with our observation that positions 190 and 211 are located on the same side. Previous data showing that charge changes at Lys-205 and Lys-211 had no effect on selectivity also argue against this region as being transmembrane (15).

The alternative patterns are largely based on theoretical arguments. The folding pattern presented here is supported by multiple independent sets of experiments. We invite others to design experiments to test this pattern further or at least propose folding patterns that are consistent with most of the experimental data.

In conclusion, by introducing pairs of cysteine residues into the VDAC sequence and observing their accessibility to streptavidin, we have probed the topology of VDAC and proposed a new modified folding pattern for VDAC. This approach may prove useful for other channel-forming proteins.

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REFERENCES

1. Colombini, M. (1989) *J. Membr. Biol.* **111,** 103–111
2. Rostovtseva, T., and Colombini, M. (1997) *Biophys. J.* **72,** 1954–1962
3. Lee, A. C., Xu, X., Blachly-Dyson, E., Forte, M., and Colombini, M. (1998) *J. Membr. Biol.* **161,** 173–181
4. Thomas, L., Kocsis, E., Colombini, M., Erbe, E., Trus, B. L., and Steven, A. C. (1991) *J. Struct. Biol.* **106,** 161–171
5. Peng, S., Blachly-Dyson, E., Colombini, M., and Forte, M. (1992) *J. Bioenerg. Biomembr.* **24,** 27–31
6. Mannella, C., Guo, X, W., and Cognon, B. (1989) *FEBS Lett.* **253,** 231–234
7. Mannella, C., Forte, M., and Colombini, M. (1992) *J. Bioenerg. Biomembr.* **24,** 7–19
8. Peng, S., Blachly-Dyson, E., Forte, M., and Colombini, M. (1992) *Biophys. J.* **62,** 123–135
9. Thomas, L., Blachly-Dyson, E., Colombini, M., and Forte, M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 5446–5449
10. Song, J., Midson, C., Blachly-Dyson, E., Forte, M., and Colombini, M. (1998) *Biophys. J.* **74,** 2926–2944
11. Hodge, T., and Colombini, M. (1997) *J. Membr. Biol.* **157,** 271–279
12. Colombini, M., Yeung, C. L., Tung, J., and König, T. (1987) *Biochim. Biophys. Acta* **905,** 297–286

[2] C. Byrd, E. Blachly-Dyson, M. Forte, and M. Colombini, unpublished results.

13. Forte, M., Guy, H. R., and Mannella, C. A. (1987) *J. Bioenerg. Biomembr.* **19,** 341–350
14. Colombini, M., Peng, S., Blachly-Dyson, E., and Forte, M. (1992) *Methods Enzymol.* **207,** 432–444
15. Blachly-Dyson, E., Peng, S. Z. Colombini, M., and Forte, M. (1990) *Science* **247,** 1233–1236
16. De Pinto, V., Prezioso, G., Thinnes, F., Link, T. A., and Palmieri, F. (1991) *Biochemistry* **30,** 10191–10200
17. Stanley, S., Dias, J. A., D'Arcangelis, D., and Mannella, C. A. (1995) *J. Biol. Chem.* **270,** 16694–16700
18. Qiu, X. Q., Jakes, K. S., Finkelstein, A., and Slatin, S. L. (1994) *J. Biol. Chem.* **269,** 7483–7488
19. Blachly-Dyson, E., Song, J., Wolfgang, W. J., Colombini, M., and Forte, M. (1997) *Mol. Cell Biol.* **17,** 5727–5738
20. Colombini, M. (1987) *Methods Enzymol.* **148,** 465–475
21. Guo, X. W., Smith, P. R., Cognon, B., D'Arcangelis, D., Dolginova, E., and Mannella, C. A. (1995) *J. Struct. Biol.* **114,** 41–59
22. Song, J., and Colombini, M. (1996) *J. Bioenerg. Biomembr.* **28,** 153–161
23. Rauch, G., and Moran, O. (1994) *Biochem. Biophys. Res. Commun.* **200,** 908–915
24. Mannella, C., Neuwald, A. F., and Lawrence, C. E. (1996) *J. Bioenerg. Biomembr.* **28,** 163–169