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Research Paper

Determination of the Number of Polypeptide Subunits in a Functional VDAC Channel from *Saccharomyces cerevisiae*

Songzhi Peng,[1] Elizabeth Blachly-Dyson,[2] Marco Colombini,[1] and Michael Forte[2]

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Genes encoding VDAC proteins containing specific site-directed amino acid alterations were introduced into wild-type *Saccharomyces cerevisiae*. The mutant VDAC proteins form channels with ion selectivities very different from that of the wild-type channel. Therefore, the resulting yeast strains express two different genes capable of coding for functional, yet distinct, VDAC channels. If VDAC were an oligomeric channel, analysis of VDAC from these strains should have revealed not only the presence of channels with wild-type or mutant selectivity but also channels with intermediate selectivities. While channels with wild-type and mutant selectivities were observed with approximately equal frequency, no channels with intermediate selectivity were observed. Sufficient observations were performed with two different mutant genes (K61E.K65E and K19E.K61E) that the likelihood of having missed hybrid channels was less than 1 in 10^7. These findings favor the hypothesis that each functional VDAC channel is composed of a single 30-kDa polypeptide chain.

KEY WORDS: Mitochondrion; outer membrane; voltage-gated channel; site-directed mutations; yeast; hybrid.

INTRODUCTION

VDAC is a voltage-gated channel found in the mitochondrial outer membrane in species representing all eukaryotic kingdoms (Colombini, 1989). It is likely to function in the control of metabolite flux between the cytoplasm and mitochondrial spaces. While its physiological functions and the molecular basis for its selectivity and voltage dependence are under intense investigation, one fundamental question remains unresolved: whether a single VDAC polypeptide is sufficient to form a functional channel.

The issue of whether the functional VDAC channel is an oligomeric protein has been addressed by a number of investigators. The earliest work by Linden and Gellerfors (1983) clearly showed that VDAC isolated from rat liver forms dimers when solubilized in Triton X-100. This, coupled with observations that the amount of VDAC conductance in planar phospholipid membranes was directly proportional to the amount of detergent-solubilized VDAC added to the aqueous phase (Colombini, 1980; Roos *et al.*, 1982), suggested that the functional channel was a homodimer of two 30-kDa polypeptides. These observations were consistent with the large pore size of the channel and its symmetrical behavior: closure at both positive and negative potentials (Schein *et al.*, 1976; Colombini, 1989), and response to aluminum hydroxide, polyanions, and the VDAC modulator from both sides of the membrane (Zhang and Colombini, 1990; Mangan and Colombini, 1987; Holden and Colombini, 1988). However, structural studies on two-dimensional crystals of VDAC from *N. crassa* raised doubts about the dimer model (Mannella, 1986, 1987). In addition, mass-per-unit-area measurements on these crystals by scanning-transmission electron microscopy showed a one-to-

[1]Laboratories of Cell Biology, Department of Zoology, University of Maryland, College Park, Maryland 20742.
[2]Vollum Institute for Advanced Biomedical Research, Oregon Health Sciences University, Portland, Oregon 97201.

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one relationship between the number of polypeptides and the number of pores (Thomas *et al.*, 1991).

Although the biochemical and structural studies were done on different species, the possibility that a species difference accounts for these findings is unlikely. The properties of the channels are highly conserved (Colombini, 1989) and so are their predicted secondary structures (Colombini *et al.*, 1991), although there is considerable difference in the amino acid sequences among different species (only 24% sequence identity between yeast and humans; see Thinnes *et al.*, 1989). Thus, it is likely that VDAC channels from all sources have the same basic structure.

It is possible, however, that the VDAC molecules in two-dimensional crystals have a different oligomeric structure than that of functional channels in planar bilayers. Therefore, a functional approach was undertaken to identify VDAC channels composed of dimers of VDAC protein. Blachly-Dyson *et al.*, (1990) reported that VDAC's selectivity could be changed in a predictable and graded manner by substituting charged amino acids in its transmembrane strands with residues of opposite charge. In most cases this substitution resulted in little change in the channel's conductance and appeared to cause only a local change in charge. Multiple substitutions produced channels whose selectivity changes appeared to be the result of simple addition of the individual changes. Thus, if the channel were a dimer, the selectivity of a hybrid channel made from one wild-type subunit and one mutant subunit should be intermediate between the selectivities of the mutant and wild-type channels, since the charge change in a hybrid dimer would be half the change in an all-mutant dimer. Our previously reported experimental observations show that VDAC's reversal potential changes linearly with the change in net charge within the pore (Blachly-Dyson *et al.*, 1990).

Starting with the assumption that the channel was a dimer, we tried to detect a channel with a reversal potential intermediate between that of the wild-type and the mutant channel in VDAC isolated from cells producing both mutant and wild-type proteins. The identification of such a channel would provide support for the dimer hypothesis. In order to clearly distinguish among mutant, wild-type, and hybrid channels, we used double point mutations in which two positive charges were replaced by two negative charges, dramatically shifting the selectivity of the channels. Measurements of the reversal potentials of the membranes containing single or multiple channels were made to see if channels with intermediate selectivity could be detected. In no case were hybrid channels detected, suggesting that a functional VDAC channel is composed of a single VDAC protein.

MATERIALS AND METHODS

Preparation of Mutant Channels

Single-copy plasmids containing the mutant gene were introduced into wild-type yeast cells. Mitochondria were isolated from these cells essentially as previously described (Blachly-Dyson *et al.*, 1990). The mitochondria were hypotonically shocked (1 mM KCl, 1 mM Tris-HCl, pH 7.5) and the membranes collected by centrifugation at 27,000 *g* for 20 min. The membranes were solubilized in 2.5% Triton X-100, and VDAC was purified on a mixed hydroxyapatite–celite column. Samples were supplemented with DMSO to 15% and stored frozen at −80°C.

Channel Reconstitution

Planar phospholipid membranes were made across a 0.1 mm hole in a Saran partition by the monolayer method (Montal and Muller, 1972) as revised by Colombini (1987). The monolayers were produced by layering 1% soybean phospholipids (w/v) and 0.2% cholesterol (w/v) in hexane (Sigma Chemical Co.) on salt solutions. The membranes were generated in the presence of a salt gradient: 1 M KCl on the *cis* side and 0.1 M KCl on the *trans* (both sides also contained 5 mM $CaCl_2$ and 1 mM MES buffer, 2-[*N*-morpholino]-ethanesulfonic acid, at pH 5.8). The voltage was clamped (Colombini, 1987) by maintaining the *trans* side at virtual ground and the *cis* side at the desired voltage. Matched calomel electrodes interfaced with the solutions. Their asymmetry was measured before and after the experiment and used to correct the values of the applied voltage. The drift in electrode asymmetry during an experiment was less than 0.2 mV (typically less than 0.1 mV).

VDAC channels were inserted by adding an aliquot (2 to 5 μl) of the purified channels in detergent solution to the *cis* compartment. Conditions were chosen which resulted in the insertion of one or a few channels.

RESULTS

Yeast cells, containing both a chromosomal gene

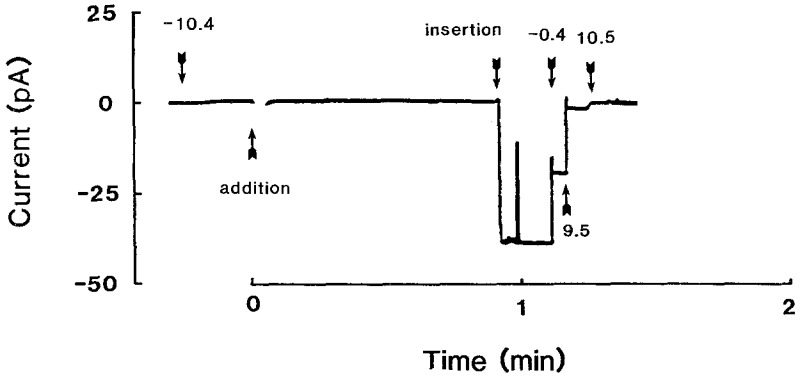

Fig. 1. An example of the measurement of the reversal potential of a VDAC channel isolated from yeast cells containing two copies of the VDAC gene, one coding for wild-type VDAC, and the other for a mutant K61E.K65E (lysines at positions 61 and 65 substituted by glutamates). The channel was inserted into a planar phospholipid membrane (conditions as in Materials and Methods) almost 1 min after sample addition. The membrane potentials (as shown in mV) were applied at points indicated by the arrows and maintained until the next potential is shown. The final value, 10.5 mV, brought the current to zero and therefore was the reversal potential. All potentials were corrected for a 0.4 mV electrode asymmetry.

coding for wild-type VDAC and a plasmid-borne gene coding for a VDAC polypeptide in which lysines at positions 61 and 65 were replaced with glutamates (K61E.K65E), or the lysines at positions 19 and 61 were replaced by glutamates (K19E.K61E), were grown in suspension culture, mitochondria isolated, and VDAC channels purified. The channels were reconstituted into planar membranes so as to try to insert a single channel in the membrane. The reversal potential of these channels in their open state was estimated in the presence of a KCl gradient.

The K61E.K65E mutant channels have a reversal potential of -2.4 mV, quite different from that of the wild-type channel of 10.3 mV, and K19E.K61E mutant channels have a reversal potential of -12.5 mV. Previous analysis of mutant VDAC channels has indicated that individual substitutions affect channel selectivity independently, i.e., multiply substituted VDAC proteins produce channels whose selectivity changes appear to be the result of simple addition of the individual charge changes (Blachly-Dyson *et al.*, 1990). Thus, a hybrid channel composed of a wild type protein with the mutant K61E.K65E protein should have a reversal potential of one half of the summation of 10.3 and -2.4, i.e., 4.0 mV. By the same reasoning, a hybrid of wild-type and mutant K19E.K61E protein should have a reversal potential of -1.1 mV.

Twenty-six successful reconstitution experiments were performed using the mutant K61E.K65E. An example is shown in Fig. 1. In this example, the initial insertion event resulted in a conductance of 1.8 nS indicating the insertion of a single channel (under these conditions, the single-channel conductance is 1.80 ± 0.06 nS; see Blachly-Dyson *et al.*, 1990). The reversal potential of this conductance was 10.5 mV, very close to the average reversal potential for wild type channels of 10.2 mV (Blachly-Dyson *et al.*, 1990). A hybrid channel would be expected to have the intermediate reversal potential of 4.0 mV. Thus, in the example, the channel must have been a wild type. Classifications of the observations into wild type, mutant, or hybrid were made either on observations on single-channel insertions or when two simultaneous insertions yielded clear wild type or mutant selectivity.

In the 26 experiments, 11 channels could be clearly categorized as wild-type and 15 as mutant. No single channels with selectivity characteristic of a hybrid were observed. Five insertions were pairs of channels whose combined selectivity was intermediate between that of the wild type and that of the mutant. Based on these observations, the fraction of wild type polypeptides observed was $16/36 \approx 0.44$ and that of mutant polypeptides was $20/36 \approx 0.56$. From these data and the binomial distribution, it is possible to estimate the probability of not having observed hybrid channels in these experiments as a result of random chance:

Probability of observing a wild type channel

$$= P_w = (16/36)^2 = 0.20$$

Probability of observing a mutant channel

$$= P_m = (20/36)^2 = 0.31$$

Probability of either a mutant or a wild type

$$= P_{mw} = 0.51$$

Probability of observing a hybrid channel

$$= P_h = 2 \times (16/36) \times (20/36) = 0.49$$

Since, of the 26 events that could be unambiguously assigned, all were either mutant or wild, the likelihood of missing a hybrid channel, should it exist (association occurring in a random manner), is $(P_{mw})^{26} = 0.51^{26} = 2 \times 10^{-8}$, i.e., 2 times in 100 million. A second set of experiments was performed in the same way using a mutant in which lysines at positions 19 and 61 were replaced by glutamates (K19E.K61E). In reconstitution experiments, 13 channels were wild type and 10 were mutant. Another 29 channels were observed in 2- and 3-channel events with intermediate selectivities. In this set of experiments, both of the fraction of wild type and mutant polypeptides were 0.5. Thus $P_{mw} = 0.5$. However, of the unambiguously assignable events, 23 were either wild type or mutant and none were hybrid. The likelihood of missing hybrids in this case is $0.5^{23} = 1 \times 10^{-7}$, i.e., 1 in 10 million.

The reversal potentials of the observed insertion events are plotted (circles) in Figs. 2 and 3 for the K61E.K65E and the K19E.K61E experiments, respectively. The A panels are for the single-channel insertions and B panels are 2-channel insertions. On the same figures are plotted the expected probabilities (bars) of observing a particular reversal potential assuming each channel is a dimer. The observed values are inconsistent with the expectations of a dimer model but quite consistent with a monomer model.

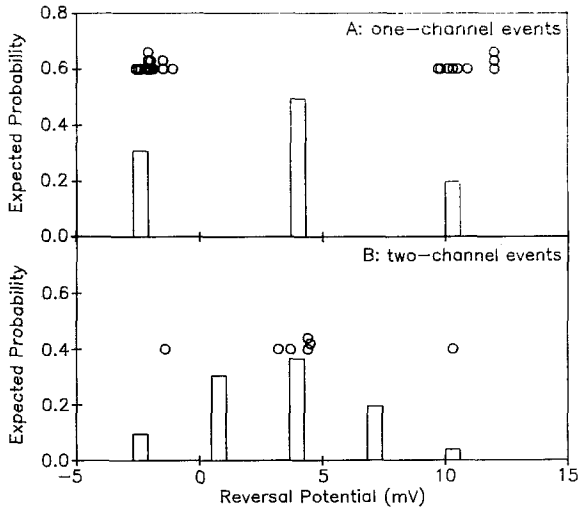

Fig. 2. The reversal potentials of observed one- and two-channel insertions compared to values expected from the dimer hypothesis. Channels were from cells possessing both wild and mutant (K61E.K65E) VDAC genes. The data were collected as in Fig. 1. The probabilities of observing intermediate reversal potentials were calculated from those for observing wild and mutant (i.e., homodimer) channels (see text) by using the binomial distribution and assuming the hybrid-channel reversal potential to be intermediate between that of the wild-type and mutant. In panel A the data collected from single-channel recordings were plotted (circles). The vertical locations of the data points are arbitrary, just above the histogram. The bars represent the expected reversal potentials (values on X-axis) of wild, mutant, and hybrid channels with their corresponding probabilities (values on Y-axis). Observations and expectations for two-channel insertions are illustrated in panel B.

DISCUSSION

We have failed to observe VDAC channels with selectivities expected of dimers containing one wild type protein and either of the two mutant proteins. It is possible that the synthesis and assembly of VDAC channels, or outer mitochondrial membrane proteins in general, favor the formation of homodimers. If so, this putative mechanism must be extremely efficient since no hybrids were observed despite roughly equal expression of the two VDAC genes in the same cell. The likelihood of having missing hybrids by random chance is less than 1 in 10 million.

A more reasonable conclusion from these observations is, however, that VDAC channels are formed from a single VDAC protein. This conclusion is consistent with results obtained by examining two-dimensional crystals of VDAC (Thomas, *et al.*, 1991) in which insufficient mass per channel was found to account for a dimer. The observations of Linden and Gellerfors (1983) that detergent-solubilized VDAC exists as a dimer is not formally inconsistent with our observations since VDAC protein in Triton micelles may associate in ways not observed in membranes.

The conclusion that VDAC channels are formed from individual VDAC proteins is surprising because, in many ways, VDAC functions in planar bilayers in a symmetrical manner; each channel closes at both positive and negative potentials and each channel responds to dextran sulfate, aluminum hydroxide, and the VDAC modulator from both sides of the membrane. The simplest explanation of such symmetrical behavior is to assume that each channel is

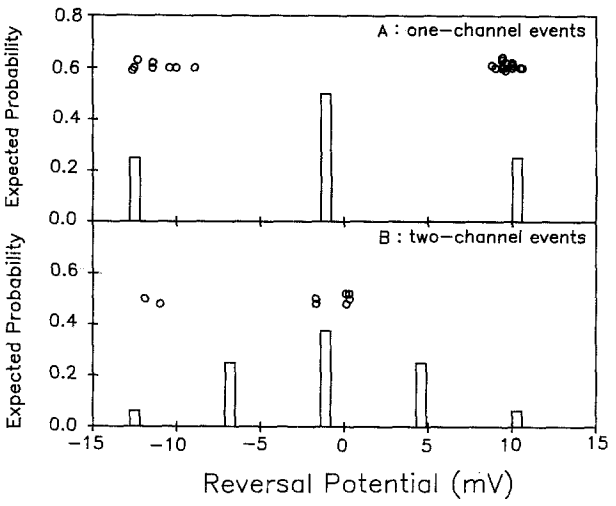

Fig. 3. The reversal potentials of observed one- and two-channel insertions compared to values expected from the dimer hypothesis for a different mutant. Channels were from cells possessing both wild-type and mutant (K19E.K61E) VDAC genes. The illustration is as described in Fig. 2.

composed of a symmetrical dimer of VDAC proteins. However, if, as our observations indicate, a channel is composed of one 30-kDa polypeptide, the task of forming a 3-nm-diameter pore with symmetrical biophysical properties is more formidable. Blachly-Dyson *et al.*, (1990) identified 1 α helix and 12 β strands that form the transmembrane portion of the channel. Such a structure could form a cylinder with an appropriately sized aqueous pore if the strands were severely tilted (approximately 55°). Tilting of β strands in a β barrel pore is expected on theoretical grounds (Forte *et al.*, 1987) and is actually observed in the structure of bacterial porin (Weiss *et al.*, 1990). Such tilting would result in a pore shorter than the thickness of the lipid bilayer. This is consistent with reconstructed images of the surface topography of VDAC in the two-dimensional crystals (Thomas, *et al.*, 1991) which reveal that the walls of the channel are depressed relative to the rest of the crystal. The weight of evidence now favors the conclusion that one 30-kDa polypeptide forms one VDAC channel.

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