

AD-A199 621

DOCUMENTATION PAGE

Form Approved
OMB No 0704-0188

1a REPORT SECURITY CLASSIFICATION (U)		1b RESTRICTIVE MARKINGS NA	
2a SECURITY CLASSIFICATION AUTHORITY NA		3 DISTRIBUTION / AVAILABILITY OF REPORT Distribution Unlimited	
2b DECLASSIFICATION / DOWNGRADING SCHEDULE NA		5. MONITORING ORGANIZATION REPORT NUMBER(S) NA	
4. PERFORMING ORGANIZATION REPORT NUMBER(S) NA		7a. NAME OF MONITORING ORGANIZATION Office of Naval Research	
6a. NAME OF PERFORMING ORGANIZATION University of Maryland	6b. OFFICE SYMBOL (if applicable) NA	7b. ADDRESS (City, State, and ZIP Code) 800 N. Quincy St. Arlington, VA 22217-5000	
6c. ADDRESS (City, State, and ZIP Code) Dept. of Zoology, College Park, MD 20742		9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER N00014-85-K-0651	
8a. NAME OF FUNDING / SPONSORING ORGANIZATION Office of Naval Research	8b. OFFICE SYMBOL (if applicable) ONR	10. SOURCE OF FUNDING NUMBERS	
8c. ADDRESS (City, State, and ZIP Code) 800 N. Quincy St. Arlington, VA 22217-5000		PROGRAM ELEMENT NO. 61153N	TASK NO. RR04108
11. TITLE (Include Security Classification) (U) Probing the location and dynamics of the sensor of a voltage-gated channel			
12. PERSONAL AUTHOR(S) Colombini, Marco			
13a. TYPE OF REPORT Annual	13b. TIME COVERED FROM 10/87 TO 10/88	14. DATE OF REPORT (Year, Month, Day) 9/21/88	15. PAGE COUNT 13
16. SUPPLEMENTARY NOTATION			
17. COSATI CODES		18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)	
FIELD 08	GROUP	membrane channel, voltage dependence, aluminum, polyanion protein modification, mitochondrion, outer membrane, VDAC selectivity, mutations.	
19. ABSTRACT (Continue on reverse if necessary and identify by block number)			
<p>The research is aimed at eventually determining the molecular mechanism by which the channel-forming membrane protein, VDAC, is voltage dependent. VDAC is located in the outer membrane of mitochondria from all species and its properties are highly conserved. The mitochondria of yeast and <i>N. crassa</i> were the major sources of VDAC for the work reported here. The research effort has moved toward the long-term goal stated above by examining agents that probe VDAC's gating process. Metal trihydroxides strongly inhibit the gating process by effectively neutralizing the voltage sensor that allows VDAC to respond to the membrane potential. Other metal species in solution and metals of lower than valence 3 are inactive. Active metals include aluminum, gallium, indium, scandium and chromium. The binding site of the metal (abstract continued on attached sheet)</p>			
20. DISTRIBUTION / AVAILABILITY OF ABSTRACT <input checked="" type="checkbox"/> UNCLASSIFIED/UNLIMITED <input type="checkbox"/> SAME AS RPT <input type="checkbox"/> DTIC USERS		21. ABSTRACT SECURITY CLASSIFICATION (U)	
22a. NAME OF RESPONSIBLE INDIVIDUAL Dr. Igor Vodyanoy		22b. TELEPHONE (Include Area Code) 202-696-4055	22c. OFFICE SYMBOL ONR

DD Form 1473 JUN 86
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19. Abstract, continued

trihydroxide seems to traverse the membrane as a result of the opening or closing process. A soluble mitochondrial protein (named the VDAC modulator) was isolated which increases VDAC's ability to respond to an electric field. Freeze-fracture studies of N. crassa mitochondrial outer membranes show that VDAC forms ordered arrays composed of regular hexagons and indicate that VDAC's pore may be off-center from the mass of the protein. Site-directed mutations of yeast VDAC have yielded information on the location of groups that contribute to VDAC's anion selectivity. The use of Ficoll to increase the medium viscosity provided further evidence for the process by which polyanions induce an ultra-steep voltage dependence in VDAC.



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Introduction

The regulation of protein function is fundamental to virtually all cellular processes. Certain channel-forming proteins in membranes can be regulated by the transmembrane potential. Relatively small changes in the membrane potential result in large changes in the ability of the channel to conduct ions across the membrane. The molecular mechanism underlying the voltage-gating of channel-forming proteins is poorly understood for any endocytic channel (i.e. channels produced by cells for insertion into their own membranes). This report summarizes the progress made this past year toward understanding the molecular mechanism by which the mitochondrial channel, VDAC, is voltage-gated.

Background

Previous research had shown or provided evidence to support the following view of the nature of the VDAC channel:

- 1) The protein forms a cylinder which spans the membrane but does not protrude significantly above the membrane surface.
- 2) The channel is probably formed by two identical polypeptides, each about 30 kDa.
- 3) The channel is steeply voltage-dependent. It is mainly open at low potentials and the probability of being closed increases sharply with increased membrane potential (both positive and negative).
- 4) There are two separate voltage-gating regions, one at positive and the other at negative potentials.
- 5) The aqueous pore is about 3 nm in diameter and probably shrinks in size to 1.8 nm upon channel closure.
- 6) The voltage sensor is probably composed of a group of lysine residues that allow the channel to respond to the electric field.
- 7) The channel's weak anion selectivity results from a net positive charge within the pore. This charge is partly the result of the presence of lysine residues.
- 8) VDAC's voltage dependence can be greatly augmented by adding small amounts of a polyanion and can be virtually eliminated by addition of aluminum chloride to the medium.

Progress during last year

We had observed that micromolar amounts of aluminum could almost completely inhibit VDAC's voltage dependence. The characteristics of the inhibition were consistent with a neutralization of VDAC's voltage sensor (i.e. that portion that allows

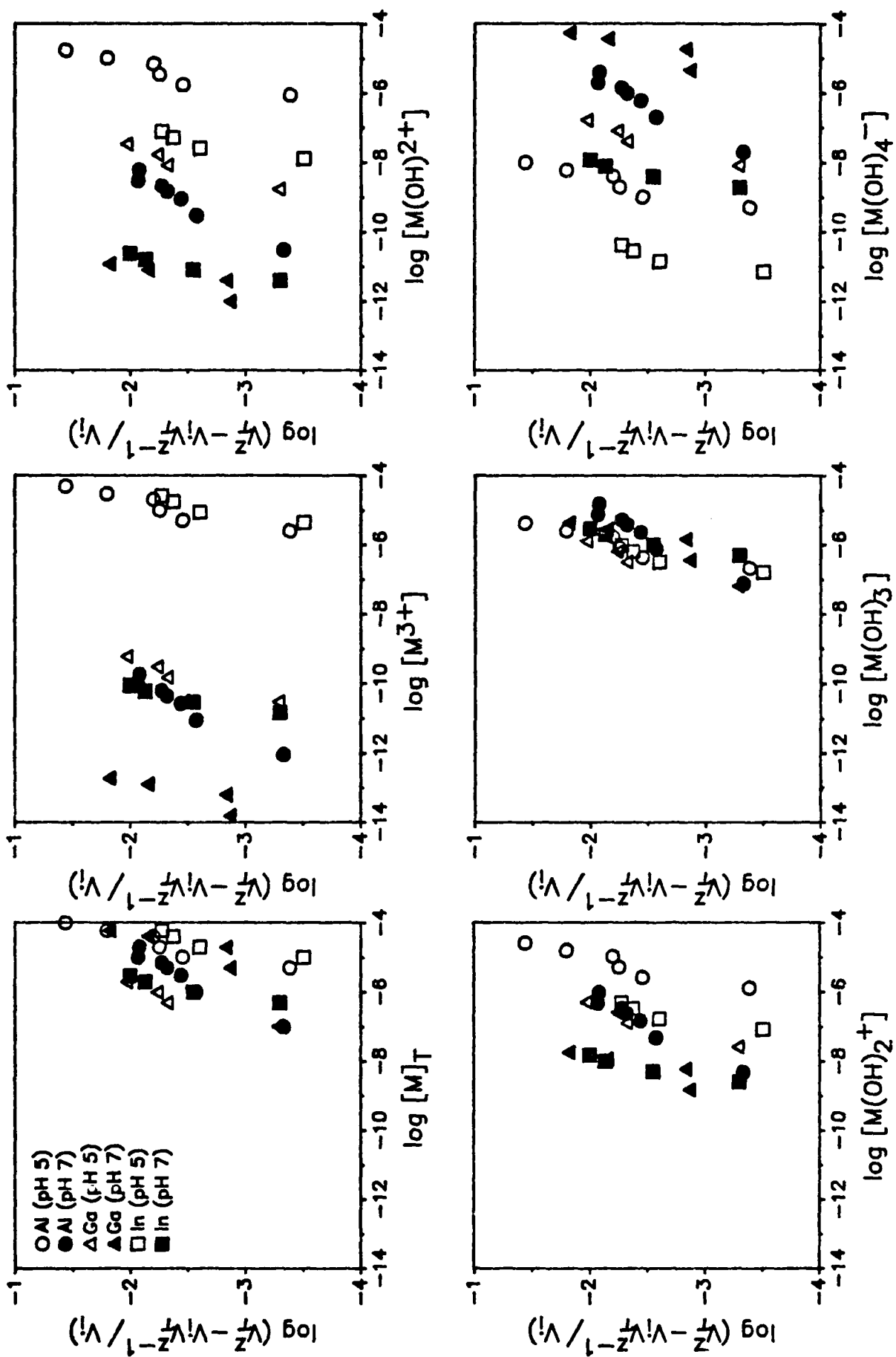
the channel to detect and respond to the membrane potential). Thus one goal was to use this observation to probe the nature and dynamics of the sensor. We first tried to determine what was it about aluminum in aqueous solution that resulted in such a profound effect on VDAC. Due to the fact that aluminum acted at neutral pH and was ineffective at acid pH we suspected that the neutral or negative hydroxylated complexes of aluminum might be the active agents. After testing a number of anions and metals, it became clear that only the active substances were trivalent metals that formed significant amounts of the neutral metal-trihydroxide at the test pH (Table 1). Moreover, the effectiveness of the agent correlated exceptionally well with the amount of metal-trihydroxide it formed in the solution, as calculated from known constants (Table 1, Fig.1). Effective metals included: aluminum, gallium, indium, scandium, and chromium. A manuscript describing this work has been submitted for publication.

Table 1. Comparison of the characteristics of effective and ineffective metals.

Ion (M ⁿ⁺)	Surface Charge Density (e/A ²)	Ionic Radius (Å)	Lowest Effective or Maximum Tested Conc.	
			M(OH) ₃ (M)	Total (M)
<i>Effective</i>				
¹³ Al ³⁺	0.92	0.51	8x10 ⁻⁷	1x10 ⁻⁶
²¹ Sc ³⁺	0.45	0.73	7x10 ⁻⁷	1x10 ⁻⁵
³¹ Ga ³⁺	0.62	0.62	4x10 ⁻⁷	5x10 ⁻⁶
⁴⁹ In ³⁺	0.36	0.81	5x10 ⁻⁷	5x10 ⁻⁷
²⁴ Cr ³⁺	0.60	0.63	1x10 ⁻⁷	5x10 ⁻⁴
²⁶ Fe ³⁺	0.58	0.64	<4x10 ⁻⁴	5x10 ⁻⁴
<i>Ineffective</i>				
¹² Mg ²⁺	0.37	0.66	---	5x10 ⁻³
²⁰ Ca ²⁺	0.16	0.99	---	5x10 ⁻³
²⁷ Co ²⁺	0.31	0.72	3x10 ⁻⁹	5x10 ⁻⁴
²⁹ Cu ²⁺	0.31	0.72	<6x10 ⁻⁶	5x10 ⁻⁴
³⁰ Zn ²⁺	0.29	0.74	3x10 ⁻⁶	5x10 ⁻³
⁵⁷ La ³⁺	0.23	1.02	0.	5x10 ⁻⁴

Fig. 1. Quantitation of the inhibitory effects of aluminum, gallium, and indium measured at pH 5 and 7. The time constants of VDAC closure were fitted to theoretical equations and plotted as a function of the log of either the total metal, or other forms of the metal calculated to be present in solution. In the panel displaying the metal trihydroxide there is remarkable convergence of the results irrespective of the chosen metal or pH.

Fig. 1



In order to gain more insight into the gating process we considered the simplest way in which the charged sensor might move through the electric field (this is a requirement in order to have a voltage-dependent energy change between the open and closed states of VDAC). If the sensor were to move all the way through the membrane, its ability to interact with the metal hydroxide should depend, not only on the side of the membrane to which it is added, but also the conformational state of the protein. Experiments in which the metal is added to one side of the membrane were performed in a variety of ways, using the membrane potential to control VDAC's conformation. Although these experiments are still under way, it seems clear now that when the metal is added to one side, it inhibits almost exclusively the gating process activated when negative potentials are applied to that side. Applying positive potentials to that side results in normal closure followed by an increased tendency of the channels to remain closed. The results indicate that there are two metal hydroxide binding sites, one accessible from each site when VDAC is in the open conformation. In addition, upon channel closure, the site translocates to the other side resulting in binding to metal located on the other side and thus remaining in a closed conformation. However, the sign is such that the metal binding site seems to be moving in the opposite direction to the motion of a positively-charged sensor. Thus, it seems that during the closing process, some domains of the protein migrate through the membrane in one direction while others move in the opposite direction.

These results can be reconciled with the strong evidence that the metal complex results in the neutralization of the sensor (from a detailed study on the metal's effect on VDAC's voltage dependence) by proposing that, upon metal hydroxide binding, the movement of charge through the membrane associated with the opening-closing process is greatly reduced. Although the simplest proposal is that of a direct metal-induced sensor neutralization, the binding of the metal to another site (whose translocation, during the gating process, is coupled to that of the sensor) and changing the charge at that site, is equally effective.

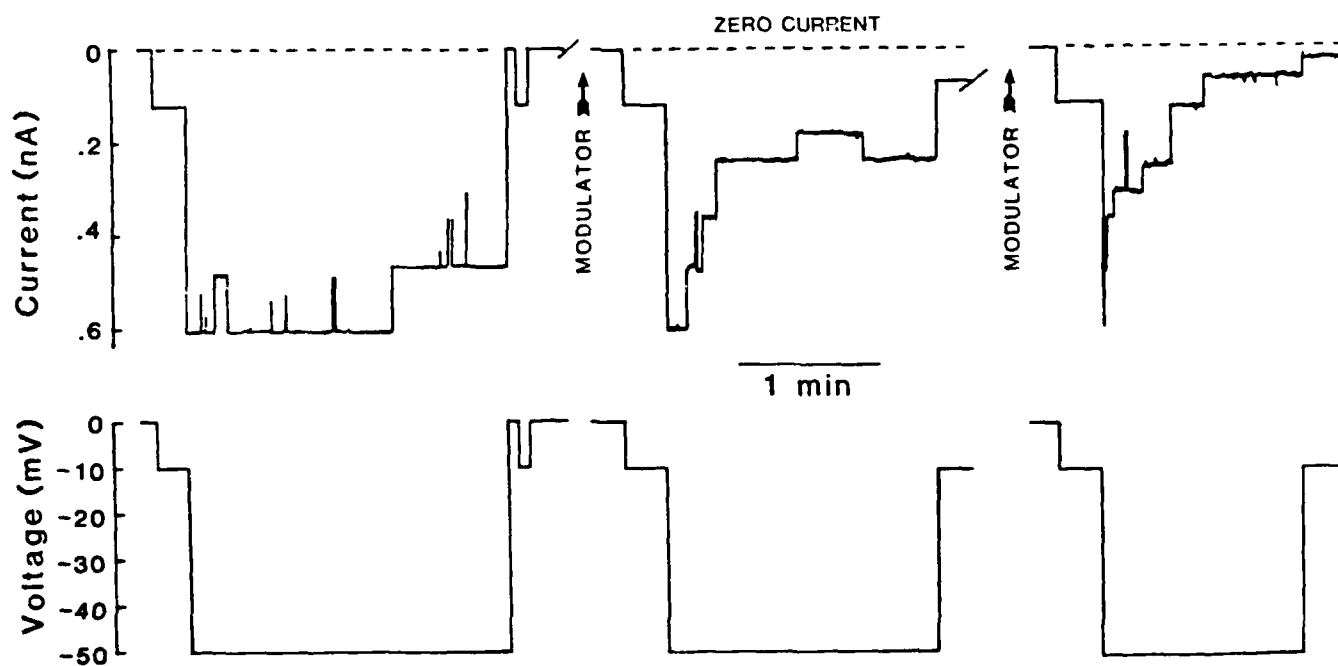


Fig. 2. A membrane containing three VDAC channels was clamped at different potentials before and after the addition of modulator. Following modulator addition, the rate and extent of VDAC closure increased dramatically.

In another line of experiments, a soluble protein capable of greatly influencing the properties of VDAC, has been isolated from mitochondria. Very small amounts (perhaps less than 1 nM) of this protein will greatly increase VDAC's rate and extent of closure at a given potential (Fig. 2). The protein is of high molecular weight (perhaps 85kDa) and highly sensitive to proteases. This protein is of interest both because it may regulate VDAC and the permeability of the outer mitochondrial membrane *in vivo*, and because it may be a useful tool to understand VDAC's gating mechanism. Although the actions of this "VDAC modulator" resemble those of polyanions that increase VDAC's voltage dependence, the modulator's actions are sufficiently different that it probably acts through some other mechanism. A first publication describing the action of this modulator on VDAC is *in press*.

A collaborative project has been initiated with Dr. Michael Forte of the Vollum Inst. in Portland Oregon, in which site-directed mutations are being used to probe VDAC. We are changing amino acid side chains (primarily amino groups) in order to find those responsible for selectivity or voltage dependence. Some progress has been made in identifying amino acids responsible for VDAC's selectivity (Table 2). These should tell us which amino acids line the walls of VDAC's pore. Quite a bit of difficulty is being encountered with altered channels that are unstable. Never-the-less, this approach should give us great insight into the structure of the channel and should prove a good complement to the functional studies.

Table 2 Sample of the results of site-directed mutations in VDAC

Mutation	K65->D	K61->D K65->D	K61->D	K61->A K65->T	Wild Type
Single-channel(nS) conductance (1M KCl 5mM CaCl ₂)	4.1±0.2	4.5	4.5±0.3	4.1	4.1
Reversal Pot.(mV) (measure of selectivity) 1M vs 0.1M KCl	+4,+7 (n=4)	-2.4±1.0	+2.5,1.5 (n=3)	3.0±1	11

A morphological approach to the search for the structural changes associated with VDAC's channel-closing process, was initiated. Freeze-fracture of mitochondrial outer membranes from *N. crassa* has revealed beautiful ordered arrays of VDAC channels. These differ from those reported by the use of negative staining (a technique that visualizes the hole rather than the protein) in ways which indicate that the hole in the channel may be off-center. We are using methods, developed by us in bilayer reconstitution systems, to close the channels and look for the resulting structural changes. The early results indicate that significant changes can be observed.

Finally, experiments have been performed to further test the theory which we developed to explain the dramatic effects of polyanions on VDAC. Addition of polyanions such as dextran sulfate increases VDAC's voltage dependence to levels which are ten times greater than any reported for thus far for any channel. The theory involved the proposal that a significant access resistance exists at the mouth of the channel. By increasing the viscosity of the medium (by adding Ficoll) without increasing the viscosity within the pore, we expected to enhance dextran sulfate's effect by increasing the access resistance relative to the pore's resistance. Ficoll did indeed enhance the action of dextran sulfate to the point that the channels closed in the absence of an applied electric field (if enough was added). This is a greater effect than we expected and we are working to try to understand the result. Irrespective of the reasons for this result, we are using it as a way to close the channels without applying an electric field (such as in the freeze-fracture experiments).

The multi-pronged approach that is being taken is moving toward a molecular understanding of the voltage-gating process in VDAC. I expect that the mechanism will be solved in the near future.

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