ELECTRON MICROSCOPIC STAINS AS PROBES OF THE SURFACE CHARGE OF MITOCHONDRIAL OUTER MEMBRANE CHANNELS

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To enhance contrast for electron microscopic imaging, biological structures are often "negatively stained," i.e., dried down in a thin layer of a glass-forming heavy-metal salt. An ideal negative stain is one which fills in the surface relief of specimen molecules. However, electron-microscopic stain distribution may also reflect "positive staining," e.g., binding of charged stain molecules to oppositely charged sites on the specimen. Recent model studies on proteins of known primary structure (1–4) have shown that both kinds of staining may occur simultaneously in crystalline and paracrystalline arrays.

We have found (5) that outer membranes isolated from *Neurospora* mitochondria contain distinctive ordered arrays of roughly circular features, 2–3 nm in diam (Fig. 1), which accumulate a wide variety of electron microscopic stains. The nonspecific accumulation of stain suggests that these features are topological, i.e., depressions in the membrane surface that might represent the openings of hydrophilic pores. Their pore nature is supported by recent immunological evidence (6) that the arrays are composed of the same 31,000-dalton polypeptide which, when extracted from the outer mitochondrial membranes, forms ion channels in phospholipid bilayers.

Unlike the nonspecific accumulation of stain inside the putative channels, there are indications of specificity in the distribution of different stains elsewhere on the membrane arrays (5). The existence of specific distribution patterns for different stains has been confirmed by multivariate statistical analysis of the computed diffraction patterns from many electron images of differently stained outer mitochondrial membrane arrays (7). The results of the present report demonstrate that the distribution patterns of two of these stains, potassium phosphotungstate (KPT) and uranyl acetate (UA), may be explained in terms of the local surface charge at different sites on the channel arrays.

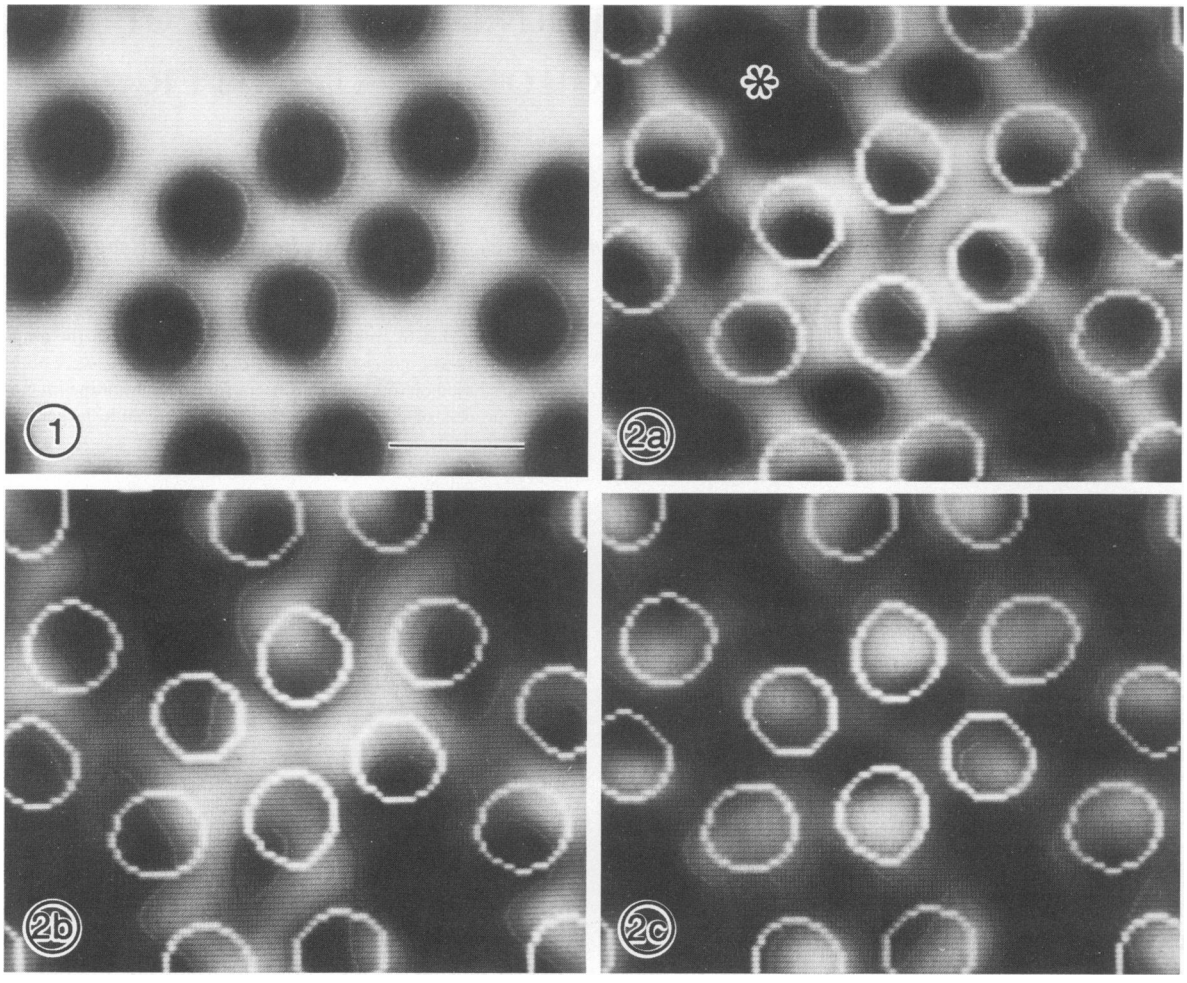
METHODS

We use Fourier-filtration procedures to construct planar projections of individual ordered layers of mitochondrial channels from electron images of crystalline vesicles stained with either 2% KPT or 1% UA (5). Average images of these channel arrays are obtained by summing several such projections for a particular stain, following low-pass filtration (to 1/3.0 nm^{-1}), imposition of twofold symmetry, and rotational and translational alignment (8). (An averaged KPT image is shown in Fig. 1.) We then construct difference images by subtracting averaged images that have first been scaled by minimizing the *R*-factor between the amplitudes of their computed Fourier transforms. The results below were obtained with averages based on four to six different filtered membrane images. We are in the process of extending these studies to include larger numbers of images for wider varieties of staining conditions.

RESULTS AND DISCUSSION

Fig. 2 *a* is a (UA) – (KPT) difference image of a region on the outer mitochondrial membrane array corresponding to that of Fig. 1. The dark maxima on this map are sites of UA accumulation, which occur primarily away from the putative pore openings (the white circular contours). The largest dark areas (one is marked with an asterisk in Fig. 2 *a*) probably correspond to phospholipid since they are eliminated when the channels rearrange after phospholipase treatment of the membranes (9). The outer membranes of *Neurospora* mitochondria contain ~4/5 zwitterionic phospholipids and 1/5 acidic phospholipids (10). Bilayers formed from such a phospholipid mixture would be expected to carry a net negative surface charge at the pH (6.9) used for KPT staining and to be uncharged at the pH (4.3) of the UA stain (11). The staining characteristics of the membrane regions away from the pores are consistent with those expected for such bilayer surfaces, i.e., they repel the phosphotungstate anions but not the UA stain, which consists predominantly of uncharged molecules at the concentration and pH used (3).

The white maxima in the immediate vicinity of the putative pore openings in the (UA) – (KPT) difference image of Fig. 2 *a* represent loci of relative KPT accumulation. These maxima may represent binding of the anionic stain to positively charged sites on the rims of the mitochondrial channels. As a test of this hypothesis, the membrane surfaces were chemically modified with succinic anhydride, a reagent that attaches anionic carboxyl groups to previously positively charged amino groups of accessible basic amino acids (12). Fig. 2 *b* is a difference image for KPT-stained succinylated-minus-normal channel arrays. The white maxima around the circular pores in this image indicate less KPT accumulation at these sites after succinylation, which is consistent with the positive-staining hypothesis. (Loss of fixed cationic sites on the periphery of the pores would be expected to diminish electrostatic binding by the KPT anions.) Interestingly, when the ion channels isolated from liver mitochondrial outer mem-



FIGURES 1 and 2 Fig. 1: Averaged electron microscopic image of KPT-stained outer membrane channel arrays of *Neurospora* mitochondria. Each dark circle is thought to represent one stain-filled pore opening. The six stain centers in the middle of this image can fit within one unit cell in the planar crystalline array. The bar equals 5 nm. Fig. 2: Difference images corresponding to the same array region as in Fig. 1. (*a*) (UA) − (KPT), normal membranes. (*b*) KPT stain, (succinylated) − (normal) membranes. (*c*) (KPT + heavy metal) − (KPT), normal membranes. Membranes were succinylated by incubating for 5 min at room temperature with 0.3% succinic anhydride in 3% DMSO and 50 mM MOPS buffer (pH 7.5) and immediately extensively dialyzed (final dialysis against 1 mM Tris HCl, pH 7) prior to negative staining.

branes are succinylated to about the same extent, their functional characteristics are altered: ion selectivity is reversed and their usual voltage dependence is lost (13). Whether the white maxima in the difference images of Figs. 2 *a* and 2 *b* represent functionally important clusters of basic amino acids is under investigation.

Finally we have noticed that inclusion of 5 to 50 μM Pb^{++} or Cd^{++} in KPT-stain solutions tends to improve the long-range order in the planar crystalline arrays of the mitochondrial outer membrane channels. The heavy-metal-minus-normal difference image for KPT-stained membranes (Fig. 2 *c*) shows relatively greater KPT accumulation (black maxima) in the presumed lipid regions, which could be due to metal-cation screening of the anionic head groups of the acidic phospholipids. There are also strong white maxima (indicating relatively less KPT accumulation in the presence of heavy metals) at the centers of the pores in this difference image. Whether heavy metals induce a transition to a less-open channel conformation is also currently under investigation.

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FLUORESCENCE PROBES FOR THE STUDY OF ACETYLCHOLINE RECEPTOR FUNCTION

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INTRODUCTION

Our group initiated the use of pyrene derivatives for functional and structural studies of the membrane-bound Acetylcholine Receptor (AcChR) system. Our studies have taken two approaches. We have recently used a hydrophobic derivative (pyrene-1-sulfonyl azide) that is able to bind covalently to regions of the AcChR molecule accessible from the membrane lipid (1) to monitor ligand-induced effects at those hydrophobic regions of the AcChR protein exposed to the membrane matrix (2). The structural effects induced by ligand binding are interpreted in relation to the AcChR "desensitization" process. We have also used a hydrophilic pyrene derivative (1,3,6,8-pyrene tetra sulfonate [PTSA]) to develop a fast spectroscopic method for measuring AcChR functionality, i.e., the formation of a short-lived cation channel, within the physiological time scale. The procedure is based on the fluorescence quenching of intravesicularly entrapped PTSA by externally added Tl^+ which substitutes for the physiologically occurring Na^+. Addition of cholinergic agonist to preparations of AcChR membranes containing entrapped PTSA promotes the formation of the AcChR cation channel, which allows the added Tl^+ into the internal volume of the vesicles where collisional quenching between the cation and the entrapped fluorophore occurs. This Tl^+ influx is spectroscopically monitored as a time-dependent decrease of the fluorescence emitted by PTSA in the millisecond time scale.

In this communication, we use the latter, "stopped-flow/fluorescence quenching," procedure to examine several functional aspects of the membrane bound AcChR, including the effect of alkaline extraction of peripheral proteins from the AcChR membranes, and the effect of binding of specific anti-AcChR antibodies, which serves as a molecular model for the autoimmune disease *Myasthenia gravis*(4, 5).

Abbreviations used in this paper: AcChR, acetylcholine receptor; PTSA, 1,3,6,8-pyrene tetrasulfonate; EDTA, ethylenediaminetetracetic acid; PMSF, phenylmethanesulfonyl fluoride; α-Bgt, α-bungarotoxin; DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-[4-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene.

MATERIALS AND METHODS

AcChR-enriched membranes were purified for *Torpedo californica* electric tissue. The electroplax was minced and homogenized 1:2 (wt/vol) in ice-cold buffer (10 mM Tris, pH 7.4, 5 mM EDTA, 5 mM iodoacetamide, 0.5 mM PMSF and 0.02% $NaNO_3$). The homogenate was centrifuged at 3,500 rpm for 10 min in a GS-3 Sorvall rotor (DuPont Instruments-Sorvall, Newton, CT). The supernatant was filtered through cheesecloth and centrifuged at 30,000 rpm for 30 min in a Beckman 35 rotor (Beckman Instruments, Inc., Fullerton, CA). The resulting pellet, referred to as crude AcChR-membranes, was resuspended in HEPES buffer (10 mM HEPES, pH 7.4, 100 mM $NaNO_3$) at ~10 mg of protein/ml. This crude AcChR had specific activity values of 10 μg of α-Bungarotoxin (α-Bgt) bound/mg of protein. Routinely, three-fourths of that membrane preparation was submitted to alkaline extraction of the peripheral membrane proteins (5).