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Fusion of the mitochondrial outer membrane: use in forming large, two-dimensional crystals of the voltage-dependent, anion-selective channel protein

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Phospholipase A_2 induces crystallization of the channel protein, VDAC (also called mitochondrial porin), in the outer membrane of *Neurospora crassa* mitochondria. The channel crystals formed in native membranes typically contain a few hundred unit cells. To increase the size of these membrane crystals for low-contrast electron microscopic imaging and diffraction studies, fusion of the isolated mitochondrial outer membranes was attempted before and after phospholipase treatment. Successful fusion of the untreated membranes was achieved by a procedure involving slow dehydration at acid pH. Single crystals of channels obtained by subsequent action of soluble phospholipase A_2 on fused mitochondrial outer membranes may contain several thousand unit cells.

Introduction

The availability of two-dimensional crystals of biological macromolecules offers many advantages for structural studies. A good example is the application of computer image processing techniques to electron micrographs of crystalline membrane proteins, e.g., Refs. 1 and 2. In a few instances, well-ordered membrane crystals occur naturally, the prototypical example being bacteriorhodopsin in the purple membrane of *Halobacterium halobium* [1]. More often, crystalline arrays of integral membrane proteins must be manufactured in vitro, a difficult process that usually entails isolation of the protein of interest and replacement of detergents used with appropriate lipids under conditions that promote crystallization of the protein. Recently, a third approach to the crystallization of membrane proteins has been introduced, namely to induce crystallization by slow depletion of membrane lipids using soluble phospholipase A_2. This technique has been developed during studies of the ordering of the channels in the mitochondrial outer membrane of *Neurospora crassa* [3,4]. It has since been successfully applied to several other systems, in particular the Ca^{2+}-ATPase of sarcoplasmic reticulum [5] and the Na^+/K^+-ATPase of kidney plasma membrane [6].

An apparent prerequisite for the success of the phospholipase/dialysis technique is the availability of membranes already enriched in the protein of interest. In the case of the mitochondrial outer membrane, the channels (named VDAC and later referred to as mitochondrial porin) are formed by a 30 kDa polypeptide that is a prominent protein component of the membrane [7–9]. When dilute suspensions of the membranes are incubated with soluble bee venom phospholipase A_2 during continuous dialysis against low-salt buffer, there is quantitative conversion of the vesicles to crystalline arrays of channels [3]. The arrays typically consist of several hundred (maximum about 1000) unit cells, each of which contains six transmembrane pores and each formed by one or two 30 kDa polypeptides [4]. Thus, the maximum number of 30 kDa proteins per array is about the same as the total number of channel polypeptides per *Neurospora* mitochondrion estimated from quantitative immunoprecipitation, 10 000 [10]. This strongly suggests that each channel array induced by phospholipase A_2 treatment is derived from the channel proteins in a single outer mitochondrial membrane vesicle. This represents a potentially significant limitation of the technique, in particular for low-dose, high-resolution electron microscopic applications. (The more

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coherent unit cells there are in a two-dimensional crystal, the fewer electrons per unit cells are needed to record a usable image or diffraction pattern; see Ref. 1.

Experiments were undertaken to determine whether the size of single membrane crystals of the VDAC protein could be increased by fusion of the membranes. The approaches taken to induce fusion were based, for the most part, on techniques that have been successful in fusing other biomembranes. These techniques (described in the next section) span a wide range of physical and chemical factors. Despite this apparent diversity, certain general principles appear to apply to the fusion process. (See, for example, the review of Prestegard and O'Brien [11] on which the following summary is primarily based.) Fusion of membranes can be conceptualized as occurring in three stages: (1) contact and aggregation of the membranes, (2) bilayer destabilization, and (3) merging of the components of the two bilayers. In general, treatments that encourage or enhance one or more of these processes may be fusagenic. For example, aggregation of membranes containing acidic lipids (such as the outer membrane of *Neurospora* mitochondria [12]) may be enhanced by decreasing the electrostatic repulsion between the bilayers, e.g., by lowering the pH or by adding divalent metal ions. Divalent metal ions also tend to dehydrate membrane surfaces, as does poly(ethylene glycol), which leads to closer interfacial contacts and to destabilization of the bilayer phase. Other agents that promote lipid phase separations or phase transitions, especially to nonbilayer phases, also tend to be fusagenic, such as increased temperature and lipophiphilic or amphiphilic compounds like dimethylsulfoxide and detergents. Physical disruption of the bilayer by ice crystals (during cycles of freezing and thawing) may also lead to membrane fusion.

In the experiments described below, both fusion of already formed crystalline arrays and of native outer membranes (prior to phospholipase/dialysis treatment) were attempted. We report success using the latter approach at producing single crystals of outer mitochondrial membrane channels that are approximately 4-times larger than those obtained with native membranes.

Materials and Methods

Membrane isolation

Mitochondrial outer membranes were isolated from liquid cultures of the slime variant of *Neurospora crassa* (FGSC 326) by procedures described previously in Ref. 9. Crystallization of the channels in the outer mitochondrial membranes [3,4] was induced by incubating the membranes (1–10 μg protein/ml) with soluble bee venom phospholipase A_2 (0.2–2 U/ml; Sigma, St. Louis, MO) during dialysis against low-salt buffer (10 mM Tris-HCl/0.25 mM EDTA (pH 7.0)). For the fusion experiments described below, membranes were concentrated to 0.2–0.5 mg protein/ml by centrifugation (60000 × *g*, 90 min) and resuspension in low-salt buffer unless otherwise indicated. (EDTA was omitted from buffers for experiments involving divalent cations.)

Membrane fusion techniques

Several different procedures were used in attempts to fuse isolated outer mitochondrial membranes before or after induction of channel crystallization. These included the following treatments and combinations thereof.

Heating. Membrane suspensions were heated for 10–30 min to temperatures ranging from 35 to 50 °C.

Freeze-thawing. Membrane suspensions in polyethylene vials were plunged in liquid nitrogen for 10 min, then thawed slowly in a bath of water at room temperature or 37 °C.

Divalent metal ions. Membrane suspensions were incubated for 30–60 min with 0.1–10 mM $MgCl_2$ or $CaCl_2$ at room temperature or 37 °C.

Organic fusagens. Membrane suspensions were incubated with the following chemical fusagens (all purchased from Sigma): (a) 35% (w/v) poly(ethylene glycol) (PEG-4000) for 15 min to several hours at room temperature or 30 °C; (b) 1–30% (v/v) dimethylsulfoxide (DMSO) for several hours at room temperature or 30 °C; (c) glycerol mono-oleate (25–200 μg/ml dispersions prepared as described in Ref. 13) for 30 min at 37 °C; (d) 6 mM octyl glucoside/0.2 mM dodecyltrimethylammonium bromide (purple membrane 'annealing solution' [14]) for one to several hours at room temperature and for several days at 4 °C.

Low pH dehydration. Membranes were pelleted and resuspended to a protein concentration of 0.5–1.0 mg/ml with a buffer containing 5 mM potassium phosphate/5 mM sodium azide (final pH 5.5–6.0). 30–50 μl aliquots of the suspension were transferred to wide-bottom, 60 ml nitrocellulose tubes which were stored overnight (15–18 h) at room temperature in a dessicator which contained fresh dessicant and which was flushed with nitrogen before sealing. After drying, the membranes form a thin, translucent film about 1 cm in diameter on the bottom of the centrifuge tube. The membranes are rehydrated by overlaying with 100 μl of cold low-salt buffer and stored on ice for 30 min prior to thorough resuspension (by gentle pipetting).

Electron microscopy and image processing

Following the various treatments, membrane suspensions were generally cleared of fusagens by dilution with and/or dialysis against low-salt buffer, pelleted and resuspended in low-salt buffer to a protein concentration of approx. 0.2 mg/ml. The membranes were negatively stained with uranyl acetate and electron micro-

scopic images were recorded as described in Refs. 2 and 9. The crystallinity of the phospholipase-treated membranes was initially assessed by optical diffraction from the micrographs [2,9]. Correlation techniques were used to evaluate the degree of order of the channel arrays in images of the negatively stained membranes [2]. Basically, this involved cross-correlation of a digitized membrane crystal field with a reference consisting of a few unit cells (obtained by lattice filtration of the array image). A peak search program was then applied to the cross-correlation function to precisely map unit cell positions within the arrays. All digital image processing was done with the SPIDER system [15].

Results

The aim of these experiments was to determine whether the size of single crystals of mitochondrial outer membrane channels formed by the phospholipase/dialysis technique of Mannella [3,4] could be significantly increased by fusion either of the arrays themselves or of the membranes prior to crystallization of the channel protein.

Fusion of existing membrane arrays

In general, no procedure was found which would fuse mitochondrial outer membranes already bearing crystalline arrays of channels into larger coherent arrays. Heating membrane suspensions above 40°C has been shown to be effective for the fusion of periodic arrays of bacteriorhodopsin [16]. However, this technique not only failed to induce fusion of mitochondrial outer membrane channel arrays, it also had a disordering effect on the membrane crystals (as determined by optical diffraction from micrographs of the negatively stained arrays). In parallel experiments, it was determined that heating suspensions of mitochondrial outer membranes above 40°C significantly inhibited VDAC activity, measured as a reduced rate of channel insertion into phospholipid bilayers from detergent extracts of the membranes (Ref. 7 and Colombini, M. personal communication). Thus, it is likely that the disordering effect of heating on the channel arrays is related to irreversible heat denaturation of the 30 kDa VDAC protein. Because of this finding, temperatures above 40°C were generally avoided in subsequent fusion protocols.

The mixture of octyl glucoside and dodecyltrimethylammonium bromide which, like heating, has been used to fuse purple membranes [14], appeared to dissolve the mitochondrial channel arrays. Membrane fragments, smaller than the original membrane arrays, were found after treatment with this detergent mixture, which contained periodic channel arrays with several different types of previously unobserved geometries.

Dimethylsulfoxide at low concentrations (1–10%) had no obvious effect on the overall size and shape of the membrane arrays. Higher concentrations of this organic solvent caused dissolution of the membranes.

Incubation of the membrane arrays with divalent cations, poly(ethylene glycol) or glycerol monooleate generally caused considerable aggregation of the arrays, which might be considered a first step in the fusion process [11]. Aggregation was especially severe in the presence of Ca^{2+} at even submillimolar concentrations. However, no increase in the average size of individual membrane crystals was observed under any of these conditions.

Freeze-thawing of the phospholipase-treated mitochondrial outer membranes also resulted in their aggregation. Occasionally, aggregates were seen in which crystalline arrays appeared to be fused in an end-to-end manner. However, arrays joined in this fashion were not coherent, i.e., the rows and columns of the lattice were not in register, and so these were not considered useful fusions.

Fusion of native mitochondrial outer membranes

Application of commonly used membrane fusion protocols (freeze-thawing, treatment with divalent metal ions or organic fusagens) to freshly isolated mitochondrial outer membranes yielded results similar to those obtained with phospholipase A_2-treated crystalline membrane arrays. The procedures resulted at best in aggregation of the membranes with no real evidence of increased vesicle size.

Successful fusion of mitochondrial outer membranes was achieved with a procedure not previously described, employing slow dehydration of the membranes from low pH, dilute phosphate buffer (see Materials and Methods). The procedure was derived from one used to fuse inner mitochondrial membranes [17], in which mitoplasts (mitochondria stripped of outer membranes) are close-packed by settling on a glass slide and then washed with buffer containing a fusagen (Ca^{2+}). Because outer membranes are much lighter than mitoplasts, they could not be densely packed by settling at $1 \times g$. In the course of experiments aimed at controlling outer membrane packing (by combining centrifugation and buffer evaporation), it was found that the membranes fused in the course of slow drying, without employing chemical fusagens. While fusion of these membranes could be achieved using phosphate buffers of pH 5–7, the most consistent results were obtained in the pH 5.5–6.0 range.

Fusion of outer mitochondrial membranes by slow dehydration from acid pH buffer is illustrated in the electron micrographs of Figs. 1–3. The native isolated membranes (Fig. 1) have collapsed diameters in negatively stained specimens of 0.5–1.5 μm. After dehydration, considerable aggregation of these membranes is evident, and significant numbers of larger vesicles, approx. 2–4 μm in diameter, can be found among the

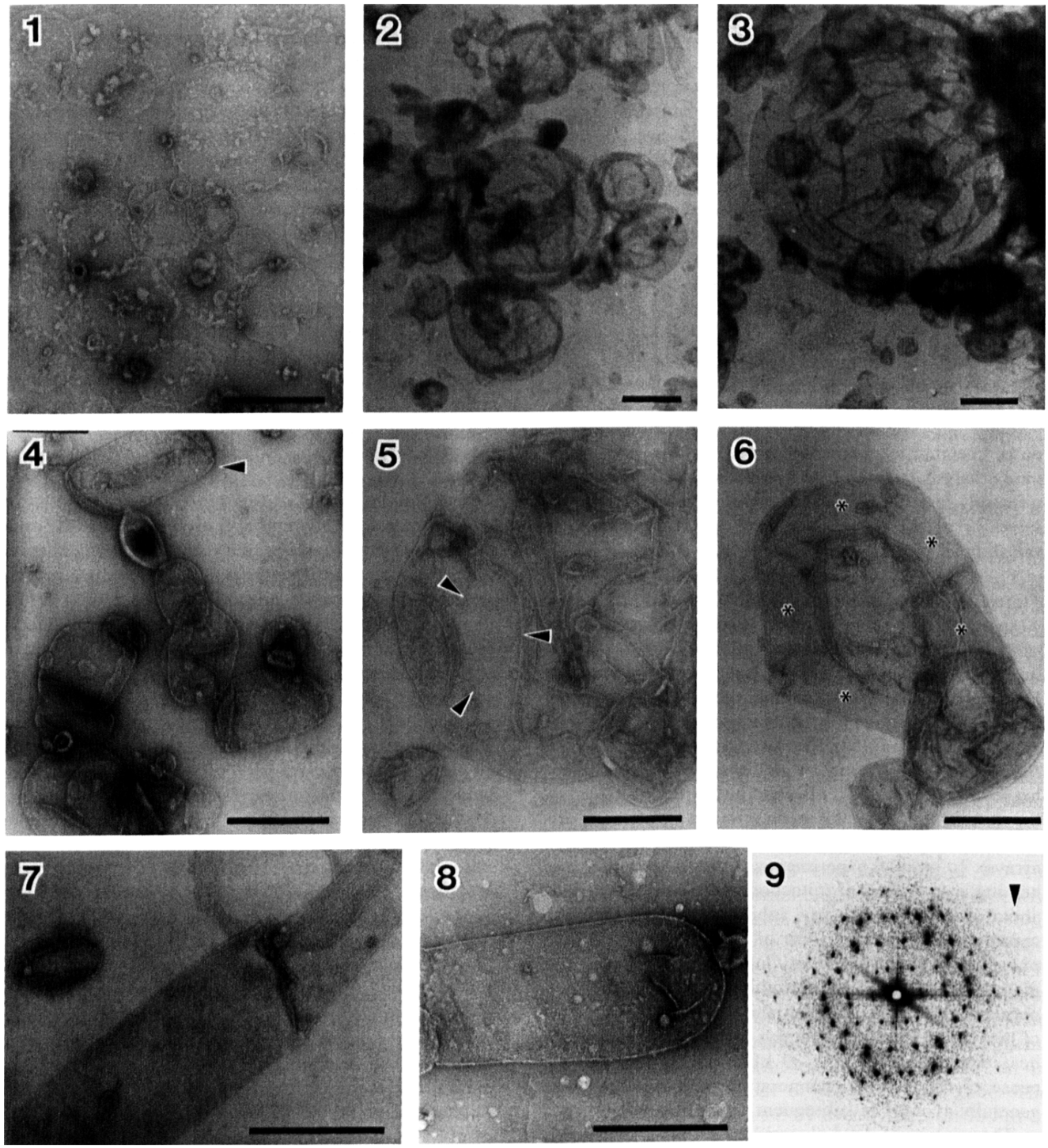

Fig. 1. Electron micrograph of negatively stained outer membranes isolated from *Neurospora crassa* mitochondria. Scale bar = 1 μm.

Fig. 2–3. Outer mitochondrial membranes after low-pH dehydration and rehydration. Scale bars = 1 μm. Note change of scale relative to Fig. 1.

Fig. 4. Crystalline VDAC arrays generated by phospholipase A_2 treatment of isolated mitochondrial outer membranes. Scale bar = 0.5 μm.

Fig. 5–8. Large crystalline VDAC arrays generated by phospholipase A_2 treatment of fused mitochondrial outer membranes. Scale bars = 0.5 μm.

Fig. 9. Computed diffraction pattern from the two periodic layers in the cylindrical membrane array of Fig. 8. Arrow points to a reflection at 1/(2.1 nm).

clumps of membranes (Fig. 2). Usually a few vesicles can be found in these fused preparations that are sufficiently large (diameters 5–10 μm, Fig. 3) for use in patch-clamp studies [18].

Channel-protein crystallization

When outer mitochondrial membranes from *Neurospora* are treated with phospholipase A_2, crystallization of the 30 kDa VDAC polypeptide occurs. A typical field of membrane crystals obtained by treatment of isolated, native mitochondrial outer membranes is presented in Fig. 4. Single crystals are generally collapsed vesicles with one or more straight edges, linear dimensions varying from 0.25 to 0.75 μm (about one-half the range observed with the starting membranes). The largest membrane crystals observed are collapsed cylindrical tubes 0.3–0.4 μm wide and 0.5–0.6 μm long (one of which is indicated by an arrow in Fig. 4). These membrane arrays contain typically 600–800 unit cells, which are divided evenly between the top and bottom membrane layers in the collapsed vesicles. (The two array layers are never in register and so they represent two independent two-dimensional crystals.)

Treatment of fused mitochondrial outer membranes with phospholipase A_2 also results in crystallization of the channel protein (although the fused membranes tend to require more extensive treatment to induce crystallization, see Discussion). Following dialysis in the presence of lower levels of phospholipase A_2 (0.2–0.5 U/ml), numerous large (1–2 μm diameter) round vesicles can be found containing one or more regions of crystallinity, interspersed with non-crystalline regions. As shown in Fig. 5, these membrane crystals often contain numerous folds which can limit the size of ordered crystalline fields (one of which is demarcated by arrows). With increased phospholipase A_2 treatment (0.5–2 U/ml), different types of membrane crystals are obtained. Fig. 6 is an example of a VDAC polycrystal obtained with a preparation of fused membranes that contains at least five distinct (non-coherent) two-dimensional crystalline regions (indicated with *). Since the individual single-crystal regions in membranes like those of Figs. 6 and 5 are usually not much larger than those in crystals derived from native (i.e., not fused) mitochondrial outer membranes, these types of large membrane crystals offer no advantage for electron microscopic experiments. However, in addition to the partially crystalline and polycrystalline membranes, large single-crystal arrays of VDAC are also found following phospholipase A_2 treatment of fused mitochondrial outer membranes. In particular, collapsed tubes composed of single cylindrical arrays can now be found that are considerably wider (0.5–0.6 μm) and longer (1–2 μm) than those derived from non-fused preparations. Two examples are presented in Figs. 7 and 8. The areas of the single crystal regions in these large VDAC arrays are, on average, 2–4-times greater than those obtained without pre-fusion of the membranes, containing upwards of 4000 unit cells.

Computed or optical diffraction patterns from electron micrographs of the large membrane arrays embedded in uranyl acetate (Fig. 9) are essentially identical to those obtained with arrays derived from non-fused membranes. These diffraction patterns generally show reflections out to 1/2 nm^{-1}, a typical resolution cutoff for negatively stained biological specimens. (This appears to be a limit imposed by the stain itself and not by lattice disorder or radiation damage [2]). Maps of unit cell positions within the channel arrays obtained by correlation methods (see Materials and Methods) indicate that the lattice order is not discernably different for arrays derived from native and from fused mitochondrial outer membranes. (The majority of unit cell positions deviate less than 1 nm from the ideal lattice positions.) Of course, not every array in these prefused preparations is a large, perfect single crystal. Folds, rips and other kinds of lattice dislocation occur in crystals derived from both fused and native membranes. Also, there is a variable degree of membrane aggregation in the prefused crystalline membrane preparations, which represents a net loss of usable arrays.

Nonetheless, the availability of larger crystalline fields on average in the prefused preparations represents an important advantage for electron microscopic diffraction and imaging studies. This advantage is not evident with stained specimens, which have high contrast and, as noted above, resolution limits imposed by the stain and not by crystal size. In general, maximum resolution (about 1/2 nm^{-1}) can be achieved for negatively stained VDAC arrays with a few as 150–200 unit cells [2], an array size readily found in unfused preparations. The advantage of larger crystals is realized in experiments on unstained specimens, which have low inherent contrast and greater sensitivity to the damaging effects of the electron beam. Because of the unfavorable signal-to-noise ratio in unstained (versus stained) specimens and the need to image with electron doses that are as low as possible, there should be a direct correlation between array size and attainable resolution in optical or electron diffraction patterns (at least up to the limits imposed by lattice disorder). This has been confirmed by electron imaging experiments with frozen-hydrated VDAC crystals (Mannella, C., unpublished data). Optical diffraction patterns from low-dose images of arrays derived from non-fused membranes generally show only 2–3 orders of diffraction; those from prefused preparations contain 6–7 diffraction orders.

Discussion

The reason for the success of the low-pH dehydration technique, as described above, in fusing the

mitochondrial outer membrane is not entirely understood. It is reported because it is clearly effective in this case and may have wider application, in particular when used in conjunction with the phospholipase A_2/dialysis technique to produce large crystals of membrane proteins from small vesicles or liposomes. There is a previous report of fusion of a biological membrane (sarcoplasmic reticulum) by room-temperature dehydration [19] and freeze-drying has been reported to induce fusion of liposomes [20]. It is worth noting that the mechanism of action of such fusagens as poly(ethylene glycol) and Ca^{2+} is thought to involve local dehydration of membrane surfaces, allowing closer interbilayer contact than is otherwise possible [11]. Our results and those of Ref. [19] suggest that bulk evaporation of water from the membrane specimen may directly produce the same effect with certain membranes. The efficacy of low pH at inducing fusion of mitochondrial outer membranes may be related to their relatively high content of acidic phospholipids, 17% [12]. Low pH favors fusion of liposomes containing acidic phospholipids, an effect that may be attributed to protonation and subsequent dehydration of headgroups [21].

Compared to native mitochondrial outer membranes, the fused membranes generally require somewhat higher levels of soluble phospholipase A_2 to induce channel crystallization. This may be due to the initially aggregated state of the membranes following dehydration. (Outer membranes aggregated by divalent cations are also resistant to phospholipase A_2; Mannella, C., unpublished data.) Resistance to phospholipase action might also indicate that oxidation of membrane phospholipids has occurred [22], despite the N_2 atmosphere in which drying is carried out. However, we found that inclusion of an inhibitor of lipid peroxidation (reduced glutathione) during fusion and dialysis had no effect on subsequent crystallization of the channels.

There may also be concern about the effects of dehydration on the state of the channel protein itself. Slow dehydration of membranes is commonly used for the preparation of partially ordered, stacked multilayer specimens for X-ray scattering studies. There are indications from such experiments that extensive dehydration may disrupt fundamental membrane organization (e.g., Ref. 23). However, in the present case with mitochondrial outer membranes, the fact that the channel protein forms the same crystalline arrays in fused as in native membranes following phospholipase A_2 treatment suggests that any structural changes that might be associated with drying are reversed during rehydration. Also, the observation [18] that the conductance characteristics of the fused fungal membranes are essentially the same as those of outer membranes on mega-mitochondria from liver is evidence that the majority of the channels have not been irreversibly denatured by dehydration. There were indications in the present experiments that the extent and rate of dehydration is critical for successful fusion of these membranes. For example, we found that poorer fusions were obtained if the drying rates were increased or if drying times were significantly longer than those reported above. Therefore, it may be possible to find conditions for improved fusion of these membranes by varying the rates of dehydration (e.g., by controlling the humidity with concentrated salt solutions [24]) and by monitoring the extent of dehydration of the specimens.

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