Structure of the Outer Mitochondrial Membrane: 
Ordered Arrays of Porelike Subunits in Outer-membrane Fractions from Neurospora crassa Mitochondria

CARMEN A. MANNELLA
Center for Laboratories and Research, New York State Department of Health, Albany, New York 12201

ABSTRACT Light-membrane fractions obtained by hypoosmotic lysis of Neurospora crassa mitochondria exhibit buoyant densities and marker-enzyme activities characteristic of outer mitochondrial membranes. SDS PAGE of these membrane fractions indicates that a polypeptide of Mr 31,000 is the main protein component. Under negative-stain electron microscope examination many of the membranes in these fractions appear as large (0.5-1-μm diameter), collapsed vesicles. Many of the surfaces of flattened, open (i.e., ripped) vesicles exhibit two-dimensional arrays of subunits with central, 2-3-nm diameter, stain-accumulating sites. These porelike subunits are arranged into hexagons within each parallelogram unit cell, 12.6 x 11.1 nm (lattice angle = 109°).

The mitochondrion is a double-membrane system: most respiratory chain components are located on, or in the space bounded by, the inner membrane, which is in turn enclosed by an outer limiting membrane. The intact outer mitochondrial membrane is generally considered to be freely permeable to metabolites, excluding only macromolecules such as holocytochrome c (1-4; however, cf. reference 5).

Electron microscope observations by Parsons and co-workers (6, 7) suggested a physical basis for the high small-molecule permeability of this membrane. The surfaces of outer membranes of plant mitochondria contain dense (but apparently random) arrays of negative-stain-accumulating sites, each ~3 nm in diameter, which could represent hydrophilic openings of channels through these membranes. These outer membrane subunits could not be correlated at the time with particular components of the plant membrane, nor were they found in the outer membranes from mitochondria of rat or guinea pig liver (7).

Mannella and Bonner (8) and Mannella (9) subsequently found that outer membranes isolated from plant mitochondria display x-ray diffraction patterns consistent with random arrays of subunits having in-plane dimensions like those of the subunits seen in electron micrographs. As in the electron microscope studies, previous x-ray diffraction studies of rat liver mitochondrial outer membranes had given no indication of a prominent in-plane subunit structure (10). Mannella and Bonner (4) noted that mitochondrial outer membranes of various organisms generally contain a more or less prominent class of proteins of Mr ~30,000. In the plant membrane these proteins make up over half the total protein mass (4) and are the only proteins insensitive to trypsin, as is the x-ray diffraction from these membranes (8).

These observations led to a hypothesis (8) that the 30-kdalton polypeptides are responsible for the characteristic porelike subunits detected in the plant mitochondrial outer membrane and that the failure to detect similar pore arrays in animal outer membranes is due to their relatively low content of 30-kdalton polypeptides (11). This hypothesis is consistent with recent reports (12-14) that pore-forming activity of protein-lipid or protein-detergent complexes from mitochondrial outer membranes is associated with polypeptides of Mr 30,000-32,000.

We are interested in the structure-function relations of the intact mitochondrial outer membrane, which probably regulates the entry of large (and perhaps small) molecules into the intermembrane space. For this investigation we set out to isolate such membranes with the highest relative content of 30-kdalton polypeptides. A likely source was the fungus Neurospora crassa, from which mitochondrial membranes were first isolated by Cassady (15) and by Neupert and Ludwig (16), whose procedures involved swelling, shrinking, and sonication of the mitochondria. In the latter study outer membrane fractions were found to display a single protein band when electrophoresed on polyacrylamide gels with phenol-formic acid buffer. That the same membranes might also contain prominent porelike subunits was suggested by Stoeckenius's (17)
mention of unpublished electron microscope observations of negative-stain-filled pits in the outer membranes of *Neurospora* mitochondria, like those reported for the plant membrane.

In this report we describe the isolation and electron microscopic appearance of outer membranes isolated from *N. crassa* mitochondria by a procedure which avoids sonication (and detergents) so as to minimize vesiculation and other possible disruption of the membrane structure. Preliminary reports of the composition and ultrastructure of these membranes have been presented earlier (18, 19).

**MATERIALS AND METHODS**

**Neurospora Cultures**

For these studies we used a *N. crassa* mutant which lacks a cell wall, commonly referred to as slime (FGSC 326, /3 [no number], sg [no number], arg-1 / BS369, cr-1 / BS123, aar / BS350], os-1 / BS135], a culture of which was kindly provided by R. L. Metzenberg (University of Wisconsin at Madison). Liquid cultures of slime were grown by shaking in Nelson's Medium B at 30°C (20). Long-term stock cultures were grown for several days on 1.5% agar containing Nelson's Medium B, as first suggested by Selitrennikoff (21), and maintained in storage at −70°C.

**Isolation of Mitochondria**

For mitochondrial membrane isolation we used late-exponential-phase slime protoplasts from 4 l of *N. crassa* protoplasts inclusions with 7 × 107 cells/ml and shaken for 18 h at 150 strokes/min with a reciprocating shaker or at 210 rev/min with a rotatory shaker. Mitochondria were isolated from the protoplasts by a modification of the procedure developed by Lambowitz et al. (22) for *N. crassa* protoplasts.

Slime cells were harvested by centrifugation at 1,000 g for 3–5 min and were resuspended by gentle swirling in 500 ml of a solution containing 0.6 M sucrose, 50 mM NaHPO4, and 5 mM EDTA, pH 6.8 ± 0.2°C. (This and all subsequent solutions were made with deionized, glass-distilled water.) After 5–10 min, the cells were centrifuged at 1,000 g for 10 min and resuspended in 300 ml of a solution containing 0.25 M sucrose, 10 mM Tris- HCl, 1 mM EDTA, and 0.3% bovine serum albumin (BSA) (fraction V, Sigma Chemical Co., St. Louis, MO) at pH 7.2. (This and all subsequent steps were done at 4°C.) Cell lysis in this medium could be followed by light microscopy. Lysis was generally 30–50% complete after 10–20 min and could be increased to 80% or better by three to six manual strokes with a Potter Elvehjem homogenizer (Wheaton Scientific, Millville, NJ). Cell lysates were centrifuged at 1,000 g for 10 min and the supernatant at 25,000 g for 40 min.

The resulting pellets were composed of three layers: a small, clear polysome pellet, above which were the dark-brown mitochondrial layer and a light, somewhat viscous, tan overlay. As much of the overlay as possible was washed off, and the mitochondrial layer was gently resuspended in a few milliliters of isolation medium (0.3 M sucrose, 1 mM EDTA, 0.3% BSA, adjusted to pH 6.8 with NaOH); care was taken to avoid the clear pellet underneath. After all the pellets were resuspended, the total volume was raised to 300 ml with isolation medium to remove any residual light overlay material.

**Fractionation of Mitochondrial Membranes**

The mitochondrial pellets (generally 0.3–0.8 ml, packed vol) were resuspended by gentle pipetting in a final volume of 1 to 3 ml of isolation medium. The suspension was added rapidly to at least 40 vol of cold lysis medium (0.25 mM EDTA, 0.25 mM NaHPO4, adjusted to pH 7.0 with NaOH) and centrifuged at 1,000 g for 5 min. The supernatant was homogenized gently with three strokes of a Potter Elvehjem homogenizer and stirred on ice for another 25 min. At this point solid mannitol was added to the mitochondrial lysate (final concentration, 5%) to encourage contraction of the inner membrane space.

After 5 min the mitochondrial suspension was layered topi over either a 0.3 M sucrose cushion or a 0.9 M sucrose cushion and centrifuged at 10,000 g for 10 min. The supernatant was removed, and the pellet was resuspended in 0.9 M sucrose, pH 7.2, and centrifuged at 25,000 g for 30 min to form the final mitochondrial pellets. Their surfaces were washed gently with a few milliliters of isolation medium to remove any residual light overlay material.

**Enzyme Assays and Protein Determination**

Respiratory activity of the isolated mitochondria was monitored at 25°C by using a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH) and the reaction conditions described by Lambowitz et al. (22).

Antimycin A-sensitive and -insensitive NADPH-cytochrome c oxidoreductase and succinate:cytochrome c oxidoreductase activities of the mitochondrial membrane fractions were monitored as described by Dounce et al. (23). L-Kynurenine, NADPH:oxygen oxidoreductase (3-hydroxylating) was measured by the procedure of Nisimoto et al. (24). The reactions were run at room temperature and monitored with a Gilford Instrument Laboratories Inc. (Oberlin, OH) 2400 spectrophotometer. Protein determinations were made by a modified Lowry procedure (25) with crystallized BSA as the standard. Enzyme and protein determination reagents were purchased from Sigma Chemical Co. (St. Louis, MO), except for antimycin A (Boehringer Mannheim, Indianapolis, IN).

**Electron Microscopy**

Mitochondrial membrane specimens were electrophoresed on 1.5-mm-thick polyacrylamide slab gels consisting of a 6% acrylamide stacking gel (2-cm long) and a 12% acrylamide separation gel (20-cm long), prepared and stained (with Coomassie Brilliant Blue G) as described by Chua and Bennoun (26). The buffer systems of Neville (27) or Laemmli (28) were used with 0.1% SDS in the upper reservoir buffer and in the gels themselves. Mitochondrial membrane specimens containing 10–50 µg of protein were prepared in 60-µl mixtures containing 2% SDS, 5% glycerol, 1.5% β-mercaptoethanol, and 0.04% bromphenol blue. These were heated to 100°C for 1 min before they were loaded onto the gels. For molecular weight calibration protein standards of *M. 14,000-94,000* (Bio-Rad Laboratories, Richmond, CA) were electrophoresed on the same gels. The gels were run at 30 mA for 7.5 h (Neville buffers) or at 50 mA for 6 h (Laemmli buffers). Reagents used in preparing the gels, buffers, and specimens were from Sigma Chemical Co., with the exception of acrylamide and N,N′-methylene-bis-acrylamide, which were from Eastman Chemicals (Rochester, NY).

Electron micrographs were taken on a Philips EM 301 electron microscope at an accelerating voltage of 200 kV. The samples were prepared by negative staining with uranyl acetate or phosphotungstic acid. The sections were examined in the electron microscope within a few hours of preparation.

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RESULTS AND DISCUSSION

Structure and Respiratory Activity of the Isolated Mitochondria

In situ the mitochondria of N. crassa slime grown in liquid culture display varied cross-sectional shapes. Most are circular or elliptical (and sometimes elongated), but many others are donut- or C-shaped (Fig. 1A). The latter types have been previously described for N. crassa, both in wild-type mycelia (30) and in the slime mutant grown as hyphlets on agar (31), and may represent sections through cup-shaped mitochondria.

Mitochondria isolated from slime cells in liquid culture (Fig. 1B) show predominantly circular cross-sections with diameters ranging from 0.3 to 1.5 μm, the upper limit matching that of the largest mitochondrial cross-sections seen in situ. In a few instances adjacent mitochondria appear in thin section to be enclosed by a single outer membrane (arrow, Fig. 1B); these might be derived from the dumbbell-shaped mitochondria occasionally seen in situ (arrow, Fig. 1A). Isolated mitochondria with annular cross-sections are also occasionally detected in these preparations (insert, Fig. 1B).

When, as in Fig. 1B, isolated mitochondria are fixed in media containing 0.25 or 0.3 M sucrose, they show mixed orthodox and condensed inner-membrane configurations with predominantly intact outer membranes. Such mitochondria display state-3 oxidation rates, with NADH as substrate, of 0.9 to 1.2 atoms oxygen·min⁻¹·(mg protein)⁻¹ and respiratory control ratios (i.e. state-3/state-4 respiration rates) of 2.4 to 4.0. Both sets of values are comparable to those of mitochondria isolated from wild-type Neurospora mycelia (32).

Mitochondrial Lysis

The procedure used to isolate outer membranes from Neurospora mitochondria is based on the osmotic shock/density-gradient centrifugation procedure of Douce et al. (23), devised for higher plant mitochondria, which was in turn derived from the original procedure of Parsons et al. (7) for liver mitochondria. This approach avoids the use of sonication to separate the outer membrane, as used by previous workers with Neurospora mitochondria (15, 16) to increase outer membrane yields.

The first step in isolating outer membranes is to dilute the mitochondria in a hypoosmotic medium in order to expand the inner membrane and rupture the outer membrane. In Neurospora slime mitochondria suspended for 0.5 h in hypoosmotic lysis medium (Fig. 1C), the inner membranes display large-scale swelling like that evidenced with animal mitochondria under similar conditions (33).

The extent of outer membrane lysis can be estimated from the relative activities of reactions involving transfer of electrons between inner-membrane redox sites and exogenous cytochrome c, which cannot penetrate the intact outer membrane (3, 4). The unmasking of one such enzyme activity (succinate:cytochrome c oxidoreductase) as a function of decreasing osmoticant (sucrose) concentration is illustrated for a typical mitochondrial preparation in Fig. 2. The freshly isolated mitochondria did not display maximum reaction rates after 0.5 h incubation at low osmolarity (presumably corresponding to maximum outer membrane lysis). Instead, maximum cytochrome c reduction rates were achieved only after the mitochondrial suspension had stood on ice for 2 h. Most prepara-

![Figure 1](https://example.com/figure1.png)

**Figure 1** (A–C) Thin-section electron micrographs of (A) cytoplasm of a N. crassa slime protoplast, (B) isolated N. crassa mitochondria in isolation medium, and (C) isolated N. crassa mitochondria in lysis medium. (D) Spread and negatively stained light (outer) mitochondrial membranes. Each micrograph was taken at 80 kV on a Philips EM301 at X 8,700, and all were enlarged to the same final magnification. Bar, 0.5 μm.

![Figure 2](https://example.com/figure2.png)

**Figure 2** Relative activities of succinate:cytochrome c oxidoreductase of N. crassa mitochondria as a function of osmoticant concentration. The mitochondria (final conc., ~1 mg/ml) were preincubated for 0.5 h at 4°C in solutions containing the specified concentrations of sucrose and either 0.25 mM EDTA plus 0.25 mM EGTA, adjusted to pH 7.0 with NaOH (○), or 0.05 mM CaCl₂ (●). Incubations were initiated 0.5 h (–) or 2 h (——) after isolation of the mitochondria.
tions of N. crassa mitochondria displayed this time-dependent susceptibility to hypotonic lysis. In some preparations the mitochondria lysed fully soon after isolation. In others the outer-membrane lysis was only 30–50% complete after 0.5 h hypotonic incubation, despite several hours preincubation on ice. Electron microscope and light transmission experiments (Mannella, C. A., and S. Rubinstein, work in progress) indicate that the increased susceptibility to osmotic lysis of mitochondria with time after isolation may correlate with slow expansion of the inner membrane space. Preliminary data indicates that treatments which convert freshly isolated Neurospora mitochondria to the orthodox conformation (e.g., preincubation with respiratory substrate) can improve outer membrane yields after hypotonic lysis. In one such experiment, preincubation of mitochondria with 10 mM NADH for 30 s before swelling approximately doubled the yield of outer membranes relative to that obtained with a control (no NADH) mitochondrial suspension.

Biochemical Characterization of Membrane Fractions

The outer mitochondrial membrane fractions characterized biochemically were obtained by hypotonic lysis of mitochondria within 1 h of isolation and were not pretreated in any way to enhance matrix swelling. Metal ion chelators were included in the swelling medium because low concentrations of divalent metal ions can inhibit hypotonic lysis of the outer membranes (Fig. 2), as is also true for higher-plant mitochondria (4).

After osmotic swelling, mitochondrial membranes from animals and higher and lower plants can be fractionated on sucrose-density gradients into light and heavy membranes, which are found to correspond (by biochemical and ultrastructural criteria) to outer and inner membranes respectively (7, 23, 34). After centrifugation for 2 h at 60,000 g, the light membranes of lysed Neurospora mitochondria are stopped by 0.7 M but not by 0.6 M sucrose, whereas the heavier membranes in the lysates pellet through 1.0 M sucrose. (The presence or absence of material at particular gradient interfaces in these experiments was determined both by direct visualization and by electrophoresis of interface fractions on SDS polyacrylamide gels; see below). The buoyant density of the light membranes inferred from these experiments, 1.08–1.09, falls within the range reported for mitochondrial outer membranes of higher plants and yeast, 1.07–1.10 (34, 35).

Specific activities and percentage recoveries of outer- and inner-membrane marker enzymes in the light- and heavy-membrane fractions of lysed Neurospora mitochondria in a typical experiment are summarized in Table I. That the light-membrane fraction contains predominantly outer mitochondrial membranes is indicated by (i) the specific activity of the outer-membrane marker enzyme, kynurenine hydroxylase (15, 16, 35), in this fraction which is sixfold higher than in the starting lysate and (ii) its very low specific activity of the inner-membrane marker, antimycin A-sensitive NADH:cytochrome c oxidoreductase, essentially all of which was recovered in the heavy-membrane fraction. The outer-membrane fractions also display antimycin A-insensitive NADH:cytochrome c oxidoreductase activity but at too low a specific activity to serve as an outer-membrane marker. The activities of this enzyme system for Neurospora mitochondrial outer membranes, 10–17 nmol cytochrome c reduced min⁻¹ mg⁻¹, is comparable to the 18 nmol min⁻¹ mg⁻¹ reported for the corresponding yeast membrane (34) and much smaller than those observed with the higher-plam membrane, 400–700 nmol min⁻¹ mg⁻¹ (23). In fact, the fungal mitochondrial outer-membrane activity is comparable to that which could arise from incomplete (96–98%) inhibition by antimycin A of the inner-membrane NADH:cytochrome c oxidoreductase activity of lysed mitochondria. Thus, the apparent antimycin A-insensitive specific activities of fractions containing either outer or inner membranes of lysed Neurospora mitochondria are similar (Table I). However, the ratios of antimycin A-insensitive to antimycin A-sensitive NADH:cytochrome c oxidoreductase activities in the light (5.5) and heavy (0.04) membrane fractions indicate that the inhibitor-insensitive activity observed in the light-membrane fractions does not arise simply from contamination by inner membranes.

Protein recovery in the light-membrane fraction is routinely low, between 0.6% and 2.0% (vs. 2–4% by the procedure of Neupert and Ludwig [16]), as is the percent recovery of the outer-membrane marker enzyme, generally <10%. The bulk of the outer-membrane marker activity is recovered in the heavy-membrane fraction, presumably reflecting incomplete outer-membrane lysis (Fig. 2) or poor separation of the outer and inner membranes. By comparison, recovery estimates for outer-membrane fractions obtained by mitochondrial swelling and step-gradient centrifugation approach 30% for higher-plant mitochondria (23), but only 4.5% for liver mitochondria (7).

SDS PAGE of three different outer mitochondrial membrane preparations is illustrated in Fig. 3. Similar to the finding of Neupert and Ludwig (16) for outer membranes isolated by swelling and sonication of wild-type Neurospora mitochondria, these light-membrane specimens (Fig. 3, lanes b–d) display a single prominent polypeptide band, in contrast to the numerous bands in the total mitochondrial pattern (Fig. 3, lane a). The mobility of the predominant light-membrane protein on 12% polyacrylamide gels is consistent with a molecular weight of 30,500 to 31,000, close to that of the major polypeptides in the outer membranes of higher-plant mitochondria (28,000 and 29,000, measured on 7.5% to 15% polyacrylamide gels [4, 35]).

In addition to the prominent band at Mₓ 30,000–31,000, minor bands are usually visible in the gel electrophoretic patterns of light-membrane fractions. The strongest and most consistently observed (see Fig. 3) occurs at Mₓ 38,000. Since the mobility of this band does not coincide with that of a major band in whole mitochondrial patterns, it is probably a minor polypeptide component of the outer mitochondrial membrane. Rarely light-membrane fractions are obtained which display numerous light bands coinciding with major heavy-membrane

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**Table I**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>AA-sensitive</th>
<th>AA-insensitive</th>
<th>Kynurenine hydroxylase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysate</td>
<td>130</td>
<td>5.3</td>
<td>2.3</td>
</tr>
<tr>
<td>Light</td>
<td>2 (0.02%)</td>
<td>11</td>
<td>14 (5.3%)</td>
</tr>
<tr>
<td>Heavy</td>
<td>470 (104%)</td>
<td>12</td>
<td>3.3 (40%)</td>
</tr>
</tbody>
</table>

* Light- and heavy-membrane fractions correspond to material at the 0.55/0.9 M sucrose interface and that which pellets through the 0.9 M sucrose layer, respectively.

AA, antimycin A.

§ Percentage recovery of activity in fraction.
bands (indicated by hash marks to the right of Fig. 3). One such fraction was run in lane d of the gel in Fig. 3 and was found to contain (by negative-stain electron microscopy, see below) unusually high numbers of tubular, cristae-like membranes.

Electron Microscopy of Light-membrane Fractions

A wide-angle view of a typical negatively stained field of light membranes obtained by step-gradient centrifugation of hypoosmotically swollen Neurospora mitochondria is shown in Fig. 1 D. The main components of this fraction are membrane vesicles or sheets which vary in width from ~0.1 to >1 μm. When stained with phosphotungstate (as in Fig. 1 D), the membranes are usually irregularly shaped with a distinct white (i.e., stain-excluding) border, similar in appearance to unfixed outer membranes isolated by comparable techniques from animal and higher-plant mitochondria (7, 35). The upper limit of the size of outer membranes isolated by this procedure appears to be about twice that of the same membranes isolated by procedures involving sonication (16).

When large, flat, well-stained membranes in these outer-membrane fractions are viewed at a higher instrument magnification (40,000–60,000), the surfaces of some appear to be covered, entirely or in part, with small close-packed scattering centers, clearly distinguishable (on the phosphor screen and in subsequent micrographs) from the finer granularity in adjacent background fields. These phosphotungstate-accumulating sites are similar in diameter (2.5–3 nm) and center-to-center spacing (4.5–5 nm) to those previously observed by Parsons et al. (7) in images of mitochondrial outer membranes from higher plants. However, unlike the stain centers of the plant membrane, those of the Neurospora membrane are organized into two-dimensional crystalline arrays, whenever they are visible in these images.

Three examples of outer membranes bearing ordered arrays of these subunits are presented in Fig. 4. The insets in A–C of Fig. 4 are the optical diffraction from areas (~150-nm square in each case) at the center of the ordered region of each membrane. The diffraction patterns correspond to two superimposed oblique planar arrays with unit cell parameters (averaged over 23 such lattices) of θ = 109 ± 1°, a = 12.6 ± 0.3 nm, and b = 11.1 ± 0.2 nm. The two reciprocal lattices in such patterns do not show simple preferred rotational orientations (see insets in Fig. 4). Diffraction patterns from obvious single-sheet regions of open membranes consist of one reciprocal lattice, indicating that each of the two reciprocal lattices in the patterns of Fig. 4 originate from the membrane layer on one side of the collapsed vesicles in these micrographs.

The three membranes of Fig. 4 were chosen to illustrate the morphologic classes observed for the crystalline membranes in these outer-membrane fractions. The membrane in Fig. 4 A is a large, flattened vesicle, roughly circular in outline, with ordered arrays of subunits visible only in a central area ~200 nm across. In Fig. 4 B the large, open membrane vesicle has an
FIGURE 5 (A) Electron micrograph of a uranyl acetate-stained outer mitochondrial membrane, obtained at X 57,000 on a Philips EM301 at 100 kV. Bar, 0.1 μm. (B) Computer-filtered image of a 23 x 23 nm² area of one membrane layer at the center of the collapsed vesicle, reconstructed using reflections out to 1/2.05 nm⁻¹ (see text). The unit cell (outlined on this image) contains six stain centers which are arranged in a hexagon with vertices related by a twofold rotation axis, indicated at the center of the hexagon. (C) Optical diffraction from a 170-nm square region at the center of the membrane in A. The h,0 and 0,k axes of one of the two reciprocal lattices in this pattern are indicated, as are several high-order maxima detected in this quadrant of the pattern.
ordered subunit structure across its entire surface. This bag-shaped membrane partially encloses smaller membranes morphologically similar to swollen cristae (7, 35). The apparently single membrane of Fig. 4 C has two regions with distinctly different shapes and substructures. The left region has a rounded outline with amorphous, somewhat patchy surface staining, very similar to that of the noncrystalline region of the membrane of Fig. 4A. In apparent continuity is an elongated region with straight sides and an ordered substructure.

Four or five orders of maxima are usually detected in optical diffraction patterns from images of phosphotungstate-stained membranes, extending on average out to 1/2.5 nm⁻¹ in reciprocal space. Up to six orders of diffraction maxima are obtained with images of uranyl acetate-stained membranes (Fig. 5), corresponding to a resolution of 1/2.0 nm⁻¹. Images of individual membrane layers have been reconstructed, using the SPI-DER image-processing system (37), by inverse transformation of the Fourier spectra corresponding to single reciprocal lattices (38). (For details of this procedure see references 18, 19; also C. A. Mannella and J. Frank, manuscript in preparation.)

The filtered image of a unit cell near the center of the uranyl-stained outer membrane of Fig. 5A shows six approximately circular, negative-stain centers arranged in a hexagon with twofold rotational symmetry (Fig. 5B). The diameters of the negative-stain-accumulating sites in filtered images like Fig. 5B appear somewhat smaller with uranyl (2 to 2.5 nm) than with phosphotungstate (2.5 to 3 nm) as negative stain. Assuming that these negative-stain centers represent the projections of transmembrane channels, an inner pore diameter of 2 to 3 nm would be consistent with the sieving behavior of outer membranes (Fig. 4 C, arrows). These bridgelike structures might represent transfer or segregation of material between, for example, protein-rich (ordered) and lipid-rich (amorphous) membrane regions. Note that the same peripheral, granular, nonstaining material is also present at each end of the ordered region of the membrane in Fig. 4C (double arrows). Experiments are in progress to determine whether the crystalline and amorphous membranes in these outer mitochondrial membrane fractions are compositionally distinct.

The proportion of outer mitochondrial membrane fractions containing crystalline regions before isolation cannot be inferred from these negative-stain electron microscope observations. The apparent inverse correlation between membrane intactness and detection of subunit arrays can have at least two explanations: (i) Visualization of subunits may require that the negative stain have access to both the inner and outer surfaces of these membranes. Thus, all the membranes in these fractions might contain crystalline regions, but this substructure might not be visible in intact membranes due to poor stain penetration. (ii) Crystalline membranes might be initially rare and derive with time from the amorphous membranes in the outer-membrane fractions. The observed association between crystallinity and nonintactness in outer-membrane fractions might then be due to a physical difference between crystalline and amorphous membranes: membranes consisting of ordered subunit arrays may be more prone to ripping or less able to fuse again after rupture.

The morphology of the membrane in Fig. 4 C is suggestive of the latter possibility, i.e., that ordered membranes evolve in vitro from nonordered membrane regions. However, it is also possible that this membrane enclosed in situ one of the complex mitochondria common in Neurospora, e.g., an annular or dumbbell-shaped mitochondrion with one or more large, rounded regions connected to relatively narrow, tubular sections.

Nature of Crystalline Membrane Arrays

Assuming that the porelike subunits which form the crystalline arrays in these membranes are lipid-protein complexes, it is likely that the major protein component of the subunits is the predominant 31-kdalton polypeptide, since (i) no correlation was found between the occurrence of minor bands in gel electrophoretic patterns and the frequency of crystalline membranes in various outer-membrane preparations and (ii) the minor outer-membrane protein components together never comprised >10% of the total Coomassie-Blue-stained intensity in these gels, while the frequency of crystalline membranes can be as high as 25%.

That lipid-protein phase separations may occur in the isolated outer membranes (and that they may be involved in formation of crystalline membranes) is suggested by the granular, white (nonstaining) material sometimes seen to connect the borders of crystalline membranes to smaller, amorphous membranes (Fig. 4C, arrows). These bridgelike structures might represent transfer or segregation of material between, for example, protein-rich (ordered) and lipid-rich (amorphous) membrane regions. Note that the same peripheral, granular, nonstaining material is also present at each end of the ordered region of the membrane in Fig. 4C (double arrows). Experiments are in progress to determine whether the crystalline and amorphous membranes in these outer mitochondrial membrane fractions are compositionally distinct.

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REFERENCES
