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EVIDENCE THAT THE CRYSTALLINE ARRAYS IN THE OUTER MEMBRANE OF *NEUROSPORA* MITOCHONDRIA ARE COMPOSED OF THE VOLTAGE-DEPENDENT CHANNEL PROTEIN

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Antibodies were raised in rabbits against the outer membrane of *Neurospora* mitochondria. Antibodies were obtained that were specific for this membrane's major polypeptide (M_r 31000) and its slower-migrating derivatives on SDS-polyacrylamide gels. These antibodies inhibited the insertion into phospholipid bilayers of voltage-dependent ion channels from detergent extracts of the mitochondrial outer membranes. The same antibodies bound preferentially to membranes containing crystalline surface arrays in outer mitochondrial membrane fractions. These results indicate that the 31 kDa polypeptide is a component both of the ion channels and of the membrane arrays, suggesting identity between the functional and structural entities.

Introduction

Outer membrane fractions isolated from *Neurospora crassa* mitochondria contain crystalline vesicles which can comprise more than one-half the total membrane population following overnight dialysis against low-salt buffer [1,2]. The most prominent structural features in the crystalline membrane arrays are sites of negative-stain accumulation, each 2.5–3 nm in diameter, which are readily visualized in Fourier-filtered electron microscopic images [1,2]. The predominant protein component in the starting membrane preparations is a 31 kDa polypeptide [1–3] which has recently been isolated [3] and strongly implicated as the source of the in vitro voltage-dependent, anion-selective channel activity of these membranes [3,4]. That the stain-accumulating sites in electron images of the crystalline membranes might represent projections of stain-filled channels is suggested by two factors: (i) the diameter of the stain centers is consistent with the expected bore of the channel [3,4]; (ii) the repeat unit in the membrane arrays consists of a group of three, close-packed stain centers and the fungal ion channels insert into phospholipid bilayers as triplets and multiples of triplets [5].

This report presents immunological evidence that the crystalline arrays seen in *N. crassa* mitochondrial outer membrane preparations are composed of the channel protein. Rabbits immunized with the native (detergent-free) membranes produce antibodies which are specific for the 31 kDa polypeptide (and its derivatives on SDS-polyacrylamide gels) and which preferentially bind to the crystalline arrays. The same antisera are also shown to inhibit the in vitro insertion of ion channels from detergent suspensions of the same membranes.

Methods

Outer mitochondrial membrane isolation

Mitochondria were isolated from liquid cultures of *N. crassa* protoplasts (FGSC 326) by procedures previously described [2]. Outer membrane

fractions were obtained by hypoosmotic swelling of the mitochondria, followed by sucrose step-gradient centrifugation, also outlined in detail in [2]. Because of the absence of strong enzymatic markers from the outer membranes of fungal mitochondria [2,6,7], SDS-polyacrylamide gel electrophoresis was used for routine assessment of the purity of the membrane fractions. (Electrophoresis techniques are described below). Preparations of outer mitochondrial membranes free of inner-membrane contamination (based on the absence of inner-membrane marker activities and of cristae-like membranes in negative-stain electron micrographs) show a prominent band on SDS-polyacrylamide gels at 31 kDa and minor bands at 21, 38 and 62 kDa (see Fig. 3 in Ref. 2). Preparations showing numerous other minor bands on such gels, corresponding to prominent inner membrane + matrix proteins, were not used for the immunization procedures described in the next section.

Preparation of antisera against outer mitochondrial membranes

Recently isolated *N. crassa* mitochondrial outer membranes (a total of approx. 1 mg protein) were resuspended in 55 ml high-salt buffer (0.5 M NaCl/10 mM Tris-HCl (pH 7.0)), repelleted by centrifuging at 40 000 × *g* for 90 min, resuspended in 2 ml low-salt buffer (1 mM Tris-HCl (pH 7.0)) and sonicated (three 15-s bursts at 20 W plus one 15-s burst at 70 W, on ice, using a Lab Line Sonifier with microtip).

The 2-ml membrane suspension was mixed with an equal volume of Freund's complete adjuvant (Difco) and 1 ml of this mixture was inoculated into each of two rabbits, type Nys: (FG). Injections were done subcutaneously in the back of the necks of the animals; 3–5 ml of blood had been collected from marginal ear veins of each animal prior to inoculation. After 1 month, the animals were reinoculated with 1 ml of the membrane-adjuvant mixture and, after 7 days, 30–40 ml blood were collected from each rabbit, again from ear veins. (This cycle of immunization and bleeding was repeated three times over the next 6 months. Sera from the four post-immunization bleedings are referred to in the text as first- to fourth-course antisera.) After allowing to clot at 4°C in centrifuge tubes, the blood was spun at 2000 × *g* for 10 min; the serum was carefully decanted and recentrifuged (2000 × *g*, 10 min) prior to storage. A part of each serum specimen was stored at −20° in 1-ml aliquots; the bulk of each specimen was stored in 5-ml aliquots at −60°C.

For some experimental procedures, the antisera were used directly; for others, fractions enriched in immunoglobulins were prepared following the procedure described in Ref. 8. To 0.5 ml antiserum was added 0.125 g ammonium sulfate. After allowing the precipitate to form overnight at room temperature, it was pelleted (7000 × *g*, 30 min). The pellet was dissolved in 0.5 ml H_2O and dialyzed overnight against 3 l H_2O at 4°C, followed by dialysis for several days against 50 mM sodium acetate buffer (pH 5.0). After 3 h of dialysis against cold H_2O, the insoluble lipoprotein was pelleted (7000 × *g*, 10 min) and the supernatant was dialysed overnight against 0.1 N NaCl containing 2 mM NaN_3. Finally, the antibody fraction (final vol., 1.3 ml) was filtered (Gelman Acrodisc, 0.2 μm pore size) and stored at 4°C.

Characterization of the antisera

Antimitochondrial activity of first-course antisera from both animals was assessed by direct immunoprecipitation of mitochondria from suspension. To 0.2 ml suspensions containing either 50 or 500 μg (approx.) mitochondrial protein (in 0.3 M sucrose, 1 mM EDTA, 0.3% bovine serum albumin, pH adjusted to 6.8 with NaOH) were added equal volumes of antiserum serially diluted with 0.15 M NaCl. After incubation for several hours at room temperature, mitochondrial precipitates were easily detected visually in tubes corresponding to final antiserum dilutions as low as 1 : 8 for the 50 μg mitochondrial suspensions and 1 : 40 for the 500 μg suspensions (the latter being the lowest dilution tested). No such precipitates were visualized in control tubes containing either saline alone or undiluted preimmune serum.

Ouchterlony-type double-diffusion reactions [9] were also used to detect antimitochondrial activity of the antisera. The reactions were run in 1% agarose gels containing 100 mM NaCl, 50 mM Tris-HCl (pH 8.6), 0.1% Triton X-100 and 0.01% merthiolate. Precipitin lines could be detected after a few days between wells containing 0.2 ml undiluted first-course antisera and 0.1 ml mitochondrial

suspensions containing 200–500 μg protein (in 2% Triton X-100/500 mM NaCl/25 mM Tris-HCl (pH 7.0)). For the undiluted first-source antisera, precipitin lines were difficult to detect if less than 100 μg mitochondrial protein was added to the antigen wells. No precipitin lines were seen for undiluted preimmune sera with up to 500 μg mitochondrial protein in the antigen wells. To determine the relative titre of the different antisera, comparisons were made of the intensities of the precipitin lines formed by equal aliquots of undiluted antisera with a standard 500 μg mitochondrial suspension. Briefly, the first- and fourth-course antisera from both animals displayed about the same antimitochondrial activity, while the second- and third-course antisera gave noticeably less intense reactions.

The polypeptide specificity of the antisera was determined by the in-gel radioimmunoassay of Adair et al. [10] as modified by Granger and Lazarides [11]. Specimens of whole mitochondria (approx. 50 μg) or isolated outer mitochondrial membranes (approx. 5 μg) were electrophoresed on 12% polyacrylamide slab gels (with an initial 6% polyacrylamide stacking zone) containing 0.1% SDS, using the Laemmli buffer system [12]. Conditions of specimen preparation and electrophoresis were those described in Ref. 2, except that the SDS-membrane mixtures were heated only to 65 °C for 20 s to minimize heat denaturation of the protein. After electrophoresis, the gels were fixed with ethanol/acetic acid, then neutralized and washed with the buffers described in Ref. 11. The gels were incubated with serum (0.4 ml in 200 ml buffer), washed extensively, then incubated in 200 ml buffer containing 2.5 μCi ^{125}I-labeled Staphylococcal Protein A (New England Nuclear, spec. act. 87 μCi/mg protein). After extensive washing, the gels were Coomassie-stained, dried down on filter paper, autoradiographed (several-day exposures on Kodak X-Omat XRP-5 film with a DuPont Cronex Lightening-Plus Intensifier screen) and photographed.

Isolation of the 31 kDa outer-membrane polypeptide

The predominant 31 kDa polypeptide of *Neurospora* mitochondrial outer membranes was isolated by elution from SDS-polyacrylamide gels by the procedure of Hager and Burgess [13]. Specimens of isolated outer mitochondrial membranes were prepared and electrophoresed on a 1.5-mm thick, 12% polyacrylamide slab gel (approx. 12 μg protein in each of nine lanes) as described in Ref. 2. Immediately after electrophoresis, SDS-protein bands were visualized by immersing the gel first in ice-cold 0.25 M KCl for 5 min, then in cold water containing 0.1 mM dithiothreitol for another 20 min. The single, strong, opaque band at M_r 31 000 was cut from each lane and the gel slices were rinsed, minced and crushed in the buffer described in Ref. 13.

This slurry was rotated slowly overnight at room temperature, then centrifuged at 250 × *g* for 5 min. The supernatant was transferred to a siliconized Corex tube and centrifuged at 10 000 × *g* for 10 min. This supernatant was dialyzed overnight at room temperature against 1 l 10 mM Tris-HCl (pH 7.5)/0.1% SDS/5 mM NaN_3. The dialysate was then filtered (Gelman Acrodisc, 0.2 μm pore size), dialyzed several hours against 3 l 10 mM Tris-HCl (pH 7.5) and concentrated, by packing the dialysis bag in solid poly(ethylene glycol) (M_r 20 000, Fisher). The contents of the dialysis bag were then resuspended in a total volume of 100 μl of the same buffer as that used in the final dialysis.

Bilayer conductance measurements

Planar phospholipid bilayer membranes (0.15 mm diameter) were generated by the method of Montal and Mueller [14] as described in Ref. 15. The phospholipids used to form the bilayers were purified [16] from crude soybean phospholipids (type II-S, Sigma). The membrane suspensions were solubilized with 1% Triton X-100 and a small aliquot (10–25 μl) was added to the aqueous phase (1.0 M KCl/5 mM $CaCl_2$) bathing the bilayer membrane. Ion-channel formation was detected as step-conductance increments of 4.3 nS (or its multiples) [4,5]. The experiments were performed under voltage-clamp conditions [15] and a 10 mV driving force was usually used.

Electron microscopy: indirect immunogold assay for antibody binding to membranes

Freshly isolated outer mitochondrial membranes were deposited from suspension (approx. 0.2 mg membrane protein per ml low-salt buffer) onto freshly glow-discharged carbon/formvar-

coated electron microscope grids. Excess liquid was blotted off the grids with filter paper and replaced with 10 μl of either the immunoglobulin fraction from first-course antiserum (1.7 mg protein/ml low-salt buffer), control rabbit immunoglobulin G (Sigma, 0.9 mg protein/ml low-salt buffer) or low-salt buffer alone. (The membranes were not incubated with antibodies in suspension because mixing equal vols. of the fractionated antiserum and membrane suspension resulted in total aggregation of the membranes.) After 10-min incubation at room temperature, the grids were blotted and rinsed twice with phosphate-buffered saline (0.8% NaCl, 0.02% KCl, 0.115% Na_2HPO_4, adjusted to pH 7.5 with KH_2PO_4) containing 1% bovine serum albumin (Sigma, crystallized, lyophilized, fatty-acid-free). To each grid was next added a 10 μl drop of a suspension of colloidal gold (0.01%) adsorbed with Protein A (a gift of Gordon Shore, McGill University [17]), which had first been diluted 1:1 with phosphate-buffered saline. After incubating at room temperature for 60 min, the grids were blotted, rinsed twice with low-salt buffer and negatively stained with 1% uranyl acetate. The grids were examined in the electron microscope (Philips EM301, 100 kV) and randomly selected fields of membranes were photographed at instrument magnification 25 000 ×. Each negative was printed twice at 3-times enlargement. One print was used to classify the membranes in the field according to morphology and to count the colloidal gold particles over each. (The visual sorting of membranes into crystalline and amorphous classes was later confirmed by optical diffraction from the original electron image films [1,2].) The image of each membrane was cut from the second print and weighed, the mass of the cutouts was presumed to be proportional to the exposed surface area of the membrane on the specimen grid. Thus the number of colloidal gold particles per surface area for each membrane in these fields was calculated.

Results

Polypeptide specificity of first-course antisera to outer mitochondrial membranes

Results of the ^{125}I-labeled Protein A assay for detecting antibody binding to mitochondrial polypeptides resolved on polyacrylamide gels are shown in Fig. 1. In this example, the antiserum used was from one rabbit after initial immunization with outer membranes isolated from *N. crassa* mitochondria. (Results obtained with the first-course antiserum from the second animal were essentially identical to those of Fig. 1.)

In the separating (i.e., lower) region of the gel strips corresponding to either isolated outer membranes (lane 1) or whole mitochondria (lane 2), the most strongly labeled band occurs at M_r 31 000, corresponding to the outer-membrane pore protein. A second band, at M_r 62 000, is labeled with

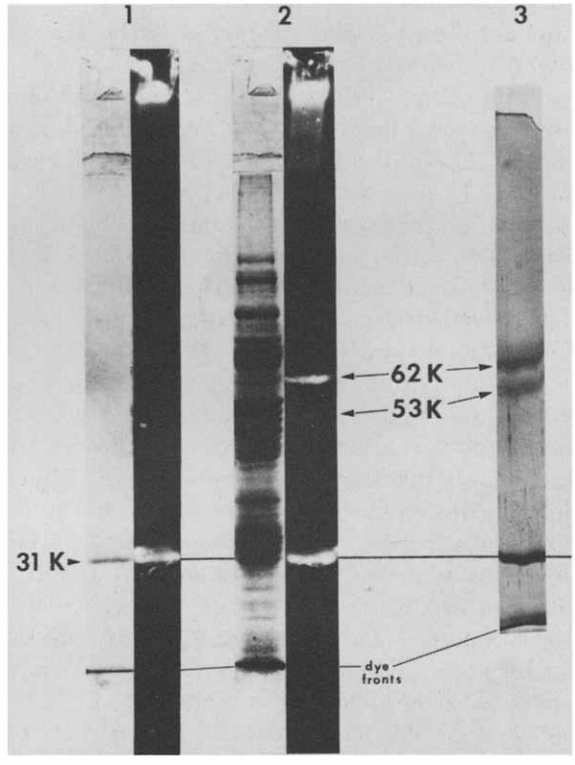


Fig. 1. Polypeptide specificity of first-course antiserum. (1) and (2): Coomassie-stained gel strips (left) and autoradiographs (right) of outer-membrane (1) and whole mitochondrial (2) polypeptides following reaction with first-course antiserum and ^{125}I-labeled Protein A. Arrows point to the three polypeptide components (apparent M_r indicated) which react with antibodies in the serum. (3): Electrophoretic pattern obtained when the 31 kDa polypeptide is eluted from gels, concentrated and reelectrophoresed. This particular gel strip was stained first with Coomassie, then with silver stain (Bio-Rad) after drying and rehydration, to improve detectability of the 53 kDa component.

^{125}I-labeled Protein A in both lanes. (Although very faint in the outer-membrane lane of Fig. 1, this band is clearly seen in longer autoradiographic exposures of the same gel strip.) Finally, a third, weak band is detected in the whole-mitochondria lane at M_r 53000. None of the three labeled bands just described were visible in autoradiographs of electrophoresed whole-mitochondrial or outer-membrane polypeptides for gel strips which were preincubated in parallel experiments with (a) no serum, (b) preimmune serum or (c) commercial control rabbit immunoglobulin G.

In addition to binding at discrete bands in the separating region of SDS-polyacrylamide gels in these experiments, ^{125}I-labeled Protein A also accumulated on the edges of the gel strips, whether or not the strips were preincubated with sera. In our experiments, this artifactual Protein-A binding was always greater in the short stacking-gel region at the top of the strips, resulting in enhanced labeling of the outlines of the specimen wells in Fig. 1. (Similar nonspecific binding of radiolabeled Protein A to the edges of gels is also evident in several autoradiographs in Ref. 11.) Diffuse, sometimes banded labeling also occurred in the specimen lanes in the stacking region of gel strips containing electrophoresed membrane proteins when the strips were preincubated with antisera to outer mitochondrial membranes (e.g., diffuse labeling at the top of lane 2 in Fig. 1). Similar labeling by Protein A in the stacking and upper separating region of gel strips was seen when reactions were run with commercially available antigens and antibodies (e.g., glucose-6-phosphate dehydrogenase and anti-glucose-6-phosphate dehydrogenase, horse-radish peroxidase and anti-horse-radish peroxidase; Sigma Chemical Co.). The labeling in this region of the gels presumably represents binding of ^{125}I-labeled Protein A to antibodies which are complexed to incompletely disaggregated proteins. The aggregates themselves are present in the gels in such small amounts that they escape detection by Coomassie-staining, although faint bands are sometimes seen in these regions when gels containing electrophoresed mitochondrial membranes are silver-stained.

That the immune sera contain antibodies against the major 31 kDa outer-membrane polypeptide is not surprising, since this component represents a large percentage (up to 90% [2]) of the total protein in the outer-membrane fractions used to immunize the animals. The more strongly reacting of the two minor targets of the first-course antisera corresponds in apparent molecular weight (62000) to one of the consistently observed minor protein bands in SDS-polyacrylamide gel electrophoresis pattern of the outer mitochondrial membrane preparations [2]. The fact that its apparent molecular weight is twice that of the major polypeptide of these membranes led us to suspect that this band might represent a dimer of the 31 kDa polypeptide. To test this hypothesis, SDS-protein bands at M_r 31000 were cut out of a preparative gel on which isolated outer mitochondrial membranes had been electrophoresed. The constituent proteins were then eluted, concentrated and reelectrophoresed, as described in Methods. The electrophoretic pattern of the eluted protein (Fig. 1, lane 3) shows the expected strong band at M_r 31000 and, in addition, two weaker bands at M_r 62000 and 53000, in order of intensity. The two slower-migrating species are clearly derived from the 31 kDa component, possibly corresponding to SDS-resistant aggregates of this polypeptide formed at high total-protein concentrations. (They might represent, for example, two conformational states of the dimer, with one migrating anomalously in SDS-polyacrylamide gels, i.e., the band at M_r 53000.) The nature of these slower-migrating derivatives of the 31 kDa outer-membrane polypeptide needs further characterization. Nonetheless, one can conclude that the antibodies in first-course antisera to *N. crassa* mitochondrial outer membranes bind to polypeptides with the same apparent M_r as the pore-forming outer-membrane protein and its derivatives.

Polypeptide specificity of later-course antisera

Although not used in subsequent experiments, antisera obtained after repeated immunization of the animals with isolated outer mitochondrial membranes were also characterized by the gel radioimmunoassay described above. Second-course antisera gave autoradiographs very similar to those obtained with first-course antisera, showing labeling of bands at M_r 31000, 53000 and 62000. Third-course antisera was not tested. (They showed the lowest relative antibody titres by the double-

diffusion immunoassay described in Methods.) The polypeptide-labeling patterns obtained with fourth-course antisera contained two new bands in addition to the three seen with earlier antisera. The M_r of the new bands (38 000 and 50 000) correlate with those of minor bands consistently observed in electrophoretic patterns of isolated mitochondrial outer membranes.

Effect of first-course antiserum on ion-channel insertion

The ion channels characteristic of mitochondrial outer membranes (called VDAC [4,15,18]) can be seen to insert into artificial bilayer membranes when Triton X-100 extracts of the *N. crassa* membranes are added to the aqueous compartment bathing the bilayers (Fig. 2). When aliquots of the immunoglobulin fraction from the first-course antiserum used in the experiment of Fig. 1 were added to the aqueous phase around bilayers already containing channels, the ionic conductance and voltage dependence of the channels were not noticeably altered. However, addition of the purified antiserum in the course of channel insertion was found to inhibit the appearance of additional channels in the bilayers. The reduced rate of ion-channel insertion into bilayers after addition of purified immune serum is demonstrated in Fig. 2, and the mean effect is summarized in Table I. (In both the figure and the table, the absence of similar effects for control protein solutions is also demonstrated.)

The inhibition of channel insertion by first-course antiserum supports the conclusion [3] that the 31 kDa polypeptide, which is the main target of the antisera, is responsible for the voltage-dependent ion-channel activity of these membranes. Complexing of the antibodies with Triton-solubilized channel protein would very likely interfere with subsequent formation of functional ion channels in the bilayers.

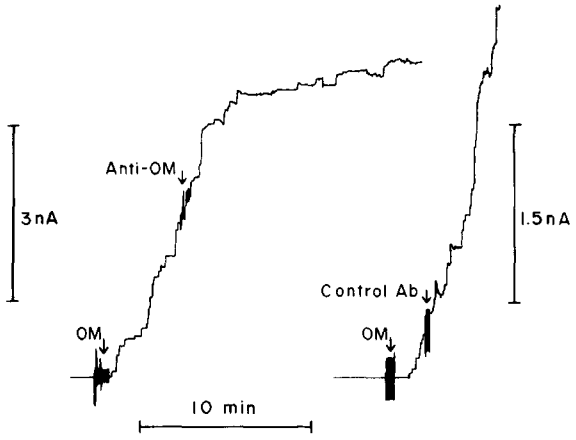


Fig. 2. Inhibition of ion channel insertion by antiserum. Recordings of current across phospholipid bilayers clamped at 10 mV; time increasing from left to right. Left trace: 10 μl 1% Triton X-100 suspension of outer membranes (approx. 0.5 mg protein/ml) were added at arrow labeled 'OM', followed by addition of 20 μl fractionated first-course antiserum ('anti-OM', 6.1 mg/ml). Right trace: consecutive additions of 5 μl of the same membrane suspension and 20 μl control rabbit immunoglobulin G ('Control Ab', 5.0 mg/ml). All additions were made to a 4.5-ml compartment on one side of the bilayer.

Antibody binding to crystalline vs. amorphous outer mitochondrial membranes

As mentioned in Introduction, outer membrane fractions isolated from *N. crassa* mitochondria contain two general classes of membranes, those showing crystalline arrays of stain-accumulating sites in negative-stain electron micrographs and

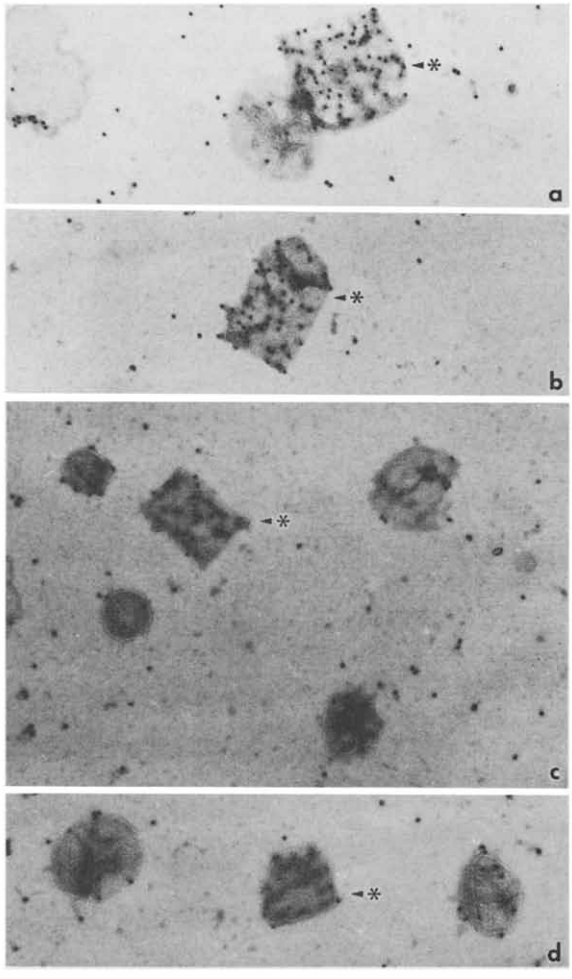
TABLE I

EFFECT OF ANTISERUM ON ION-CHANNEL INSERTION

Addition	Concentration (mg/mg membrane protein)	Number of determinations	(Insertion rate after addition)/(Insertion rate before addition) (±S.D.)
Immunoglobulin fraction from first-course antiserum	6– 18	5	0.08 (±0.05)
Control rabbit immunoglobulin G	14–280	6	1.8 (±1.6)
Bovine serum albumin	50–150	4	1.5 (±1.1)

those with amorphous surfaces. (A fuller discussion of the morphological classes present in these membrane fractions can be found in Ref. 2.) The Protein A-colloidal gold electron microscopic assay described in Methods was used to determine whether antibodies in the first-course antisera bound to either or both classes of outer membranes.

Briefly, the membranes were deposited on a carbon film across an electron microscopic specimen grid and washed first with an antibody (or control) solution and then with a suspension of colloidal gold to which Protein A had been adsorbed. Representative fields from specimens which were treated with Protein A-gold following their preincubation with the immunoglobulin fraction from first-course antiserum (the same serum used in the experiments of Figs. 1 and 2) are presented in Fig. 3.



Figs. 3. Binding of Protein A-gold to outer mitochondrial membranes preincubated with antibodies from first-course antiserum. (a) to (d) are four fields included in the calculations summarized in Table II. Stars point to crystalline membranes in the fields. Magnification: 33000×.

Binding of Protein A-gold to the carbon substrate of the specimens was found to vary considerably from field to field and its occurrence did not require prior washing of the grid surface with antibody solution. Binding of Protein A-gold to amorphous membranes (i.e., those not bearing ordered arrays of stain-accumulating subunits) was generally not significantly greater than background binding in the same fields, whether or not the membranes were first washed with antibody solution. However, binding of Protein A-gold to crystalline vesicles and sheets was noticeably greater than its binding to substrate or to amorphous membranes in specimens preincubated with antibodies from first-course antiserum (see Fig. 3). Table II summarizes mean surface densities of colloidal gold particles over amorphous and crystalline membranes for two specimens, only one of which was prewashed with antibodies from first-course antiserum. (The two data sets were chosen for statistical analysis on the basis of their respective specimens displaying similar background and mean total-membrane binding. The tabulated values represent averages of the surface densities of gold particles over individual membranes, with no correction for background binding in the fields.) The field-to-field variability in binding of colloidal gold to the membranes in these specimens is indicated by the large standard deviations associated with the measurements. In the absence of immunoglobulins from first-course antiserum, binding of Protein A-gold to amorphous and to crystalline membranes is not significantly different. On the other hand, the preferential binding of Protein A-gold to crystalline membranes following incubation of the specimens with antibodies to the 31 kDa polypeptide is statistically very significant (confidence level > 99.9%).

Discussion

The primary polypeptide target of antisera raised against *N. crassa* mitochondrial outer mem-

TABLE II

BINDING OF PROTEIN A-COLLOIDAL GOLD TO OUTER MITOCHONDRIAL MEMBRANES

Condition	Binding statistics *			
	$\bar{\rho}_a (\pm \sigma_a)$ (μm^{-2})	n_a	$\bar{\rho}_c (\pm \sigma_c)$ (μm^{-2})	n_c
− Antiserum	159 (±145)	82	130 (±146)	44
+ Antiserum	91 (±116)	91	275 (±99)	24

* Symbols: $\bar{\rho}$, the mean surface density of gold particles; σ, the standard deviation of surface density measurements; n, number of membranes included in the sample; a, subscript for measurements on amorphous membranes; c, subscript for measurements on crystalline membranes.

branes is the 31 kDa polypeptide, which is the main protein component of these membranes [2]. Secondary targets of the first-course antisera appear to be derivatives (perhaps dimers) of the 31 kDa polypeptide, although antibodies to other minor outer-membrane polypeptides begin to appear in antisera after repeated immunization of the animals with mitochondrial outer membranes. Isolates of the 31 kDa polypeptide have been shown by others to have channel-forming activity [3]. Our demonstration, that antibodies directed against the 31 kDa polypeptide inhibit ion-channel insertion, strengthens the correlation between this polypeptide and the channel activity. In particular, it is now very unlikely that the channel activity of fractions of the 31 kDa polypeptide might have arisen from minor contaminants in these isolates.

Of the membranes present in the mitochondrial outer membrane fractions, only those bearing crystalline arrays of stain-accumulating subunits bind statistically significant amounts of antibodies from first-course antiserum, using indirect immunogold electron microscopy. Thus, the 31 kDa polypeptide is a component of the crystalline arrays and it is present in these arrays either (a) at greater concentration than in the amorphous membranes, or (b) in a state more readily recognized by the antibodies. (The latter might be the case if the proteins adopted different conformations in the two types of membrane or if they were less accessible to the aqueous phase in the amorphous membranes.) Regardless of the situation in the amorphous membranes, the results presented in this report strongly indicate that the crystalline arrays of stain-accumulating subunits present in outer mitochondrial membrane fractions from *N. crassa* are composed of the 31 kDa polypeptide responsible for the voltage-dependent ion channel activity of the membranes.

The antisera raised against intact outer mitochondrial membranes in this study may prove to be useful probes of the functions of the polypeptide components of this membrane. In principal, inhibition of the transfer of molecules (metabolites or polypeptides) across the mitochondrial outer membrane by first-course antiserum would be evidence for the participation of the 31 kDa polypeptide in the transfer process. If fourth-course but not first-course antisera inhibited the activities, the minor (M_r 38000 and 50000) outer-membrane polypeptides would be implicated as functional components. Of course, failure of these antisera to effect mitochondrial activities (without actually precipitating the mitochondria) would be inconclusive. The antibodies might, for example, bind to the polypeptides in mitochondrial outer membranes without interfering with their function. This possibility is raised by the above observation that the antisera do not alter the function of ion channels already inserted into bilayers, although they do interfere with the insertion of these channels.

The sensitive gel-radioimmunoassay of Fig. 1 was used to detect possible reaction of first-course antiserum to *N. crassa* mitochondrial outer membranes with outer-membrane channel-formers from rat-liver mitochondria. The two specimens tested for cross-reactivity were (a) rat-liver VDAC (isolated by M. Colombini) and (b) the hexokinase-binding protein ([19], gift of J.E. Wilson, Michigan State University), fractions of which have been shown to form ion channels similar to VDAC in electrical characteristics ([20,21] also Colombini, M., unpublished observations). *

^{125}I-labeled Protein A did not bind detectably

* When these two protein isolates, VDAC and the hexokinase-binding protein, were electrophoresed on SDS-polyacrylamide gels, the principal polypeptide components displayed different mobilities (apparent M_r 32000 and 33500, respectively). Primary structure data are needed to determine whether these proteins are chemically distinct or different conformers of the same polypeptide.

to the polypeptides of either liver protein fraction following incubation of the respective gel strips with first-course antiserum to *N. crassa* mitochondrial outer membrane. Likewise, there was no detectable reaction of the antiserum with any of the electrophoretically separated polypeptides of whole rat-liver mitochondria (also provided by J.E. Wilson). There was, however, weak labeling of a polypeptide band at M_r 30500 in a specimen of higher-plant mitochondria (gift of W.D. Bonner, Jr., University of Pennsylvania). Whether the latter result indicates cross-reactivity of antisera to fungal outer mitochondrial membranes with the channel-former of higher-plant mitochondria (which can be isolated from outer membranes as a polypeptide of M_r 30000 [22]) is under further investigation.

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