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Characterization of Translocation Contact Sites Involved in the Import of Mitochondrial Proteins

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Abstract. Import of proteins into the mitochondrial matrix requires translocation across two membranes. Translocational intermediates of mitochondrial proteins, which span the outer and inner membrane simultaneously and thus suggest that translocation occurs in one step, have recently been described (Schleyer, M., and W. Neupert, 1985, *Cell*, 43:339–350). In this study we present evidence that distinct membrane areas are involved in the translocation process. Mitochondria that had lost most of their outer membrane by digitonin treatment (mitoplasts) still had the ability to import proteins. Import depended on proteinaceous structures of the residual outer membrane and on a factor that is located between the outer and inner membranes and that could be extracted with detergent plus salt. Translocational intermediates, which had been preformed before fractionation, remained with the mitoplasts under conditions where most of the outer membrane was subsequently removed. Submitochondrial vesicles were isolated in which translocational intermediates were enriched. Immunocytochemical studies also suggested that the translocational intermediates are located in areas where outer and inner membranes are in close proximity. We conclude that the membrane-potential–dependent import of precursor proteins involves translocation contact sites where the two membranes are closely apposed and are linked in a stable manner.

In recent years a number of steps that are involved in the transport of proteins into mitochondria have been described (for reviews see 16, 26). Precursor proteins are synthesized in the cytosol and appear to contain targeting signals which direct them to mitochondria (15, 18, 19, 20, 42). In many cases these signals are NH_2-terminal sequences which are cleaved off upon entry into the mitochondrial matrix by a processing peptidase (3, 8, 24, 25, 38), though non-cleavable signals also exist (1, 45). Proteinaceous structures are involved in the initial binding step of the precursors to the outer mitochondrial membrane (17, 30, 46, 47). Transfer of proteins across the inner membrane depends on the membrane potential (10, 28, 36). Recently, nucleoside triphosphates have also been shown to be required for import (29).

Since mitochondria are organelles with two membranes, many proteins must traverse the outer membrane to insert into the inner membrane, or must cross two membranes to be imported into the matrix. In a recent study, import at low temperature was found to lead to correct processing of precursors while they remained sensitive to externally added proteases. This indicated that translocational intermediates that were spanning the two membranes had been trapped. Similar translocational intermediates were also generated by reacting precursor proteins with a specific antibody before allowing import to occur (35). It was concluded that import into the mitochondrial matrix proceeds via translocation contact sites where the two membranes come so close together that they can be spanned simultaneously by the precursor proteins.

To further characterize the translocation contact sites, we studied whether these sites persist in mitoplasts and if mitoplasts retain the ability to generate translocational intermediates and to import proteins. Furthermore, we enriched a membrane fraction containing the translocational intermediates. Immunochemical studies were performed to define the translocation sites morphologically.

From the data obtained we conclude that proteins which are imported in a membrane-potential–dependent fashion use translocation contact sites where the two membranes are closely apposed. The translocation contact sites persist even upon removal of most of the outer membrane by digitonin treatment or by shearing forces. The membrane contacts are present in the absence of precursor proteins and they are not stabilized by the membrane potential, but most likely also exist in the absence of the potential. A factor that is removed from mitoplasts by the combination of digitonin and salt has been found to be essential for import. Fractionation of mitochondria yielded vesicles which were enriched in translocation contact sites and had an intermediate density in relation to those of inner and outer membranes. We conclude that in the areas where mitochondrial proteins are imported, the two mitochondrial membranes are in such close contact that components of the two membranes can interact in a specific and stable manner.

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Materials and Methods

Materials

[^{35}S]Methionine (1,000 Ci/mmol) and [^{35}S]sulfate (800–1,300 Ci/mmol) were purchased from Amersham Buchler (Braunschweig, Federal Republic of Germany). [^{14}C]Formaldehyde was from New England Nuclear (Dreieich, FRG). Protein A-Sepharose and Percoll were from Pharmacia (Freiburg, FRG). Triton X-100, Genapol X-100, digitonin, ascorbic acid, sodium succinate, and phenylmethylsulfonyl fluoride (PMSF) were from Merck (Darmstadt, FRG). Proteinase K was from Boehringer Mannheim GmbH (Mannheim, FRG). Hydroxylapatite and protein assay reagent were from Bio-Rad Laboratories (München, FRG). *N,N,N′,N′*-tetramethylphenylenediamine (TMPD),[1] nucleotides, protein A, antimycin A, oligomycin, and horse heart cytochrome *c* were purchased from Sigma (Deisenhofen, FRG). Sodium malate was from Serva (Heidelberg, FRG). Glutaraldehyde was from Ladd Research Industries, Inc. (Burlington, VT). *Neurospora crassa* cytochrome *c* was a gift from Dr. D. W. Nicholson of this institute. All chemicals were of the highest grade available.

Isolation of Mitochondria

N. crassa wild-type 74A was grown and harvested as described (40). When cells were labeled with [^{35}S]sulfate, the sulfate concentration in the growth medium was reduced to 0.08 mM, and 3 mCi of [^{35}S]sulfate were added per liter of culture. Mitochondria were isolated by differential centrifugation (28) in SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM morpholino propane sulfonic acid (MOPS)/KOH, pH 7.2). In some experiments, mitochondria were further purified on a Percoll gradient, as described previously (35), except that MOPS buffer was used instead of Tris buffer. Unless otherwise indicated all steps were carried out at 0–4°C.

Fractionation of Mitochondria

Subfractionation with Digitonin. Treatment of mitochondria with digitonin was performed as described (38) with the following modifications. Samples of mitochondrial suspensions (30 μl at 10 mg protein/ml in SEM buffer containing 1 mM PMSF) were incubated with the equivalent volume of digitonin solutions (0–1.6% wt/vol in SEM buffer containing 1 mM PMSF) for 5 min at 0°C. After sevenfold dilution with SEM buffer, mitochondria were reisolated by centrifugation at 14,000 *g* for 7 min. Supernatants were saved for measuring enzyme activities and for immunoblotting. The loosely packed pellets were gently resuspended in SEM buffer at 5 mg initial protein per milliliter, tested for marker enzymes, and used for import studies. In experiments where the salt-extractable factor was analyzed, digitonin fractionation was performed in the same way except that 50 mM KCl was added to the SEM buffer (SEM-K buffer).

Preparation of Desalted Extract. To prepare desalted extract, 5 mg mitochondrial protein in SEM-K buffer were extracted with 0.25% (wt/vol, final concentration) digitonin as described above. After 5 min at 0°C the sample was spun for 10 min at 48,000 *g*. The supernatant was removed and desalted by passage through a Sephadex G-25 column (38) that had been equilibrated with SEM buffer. The pass-through fraction was collected and stored in aliquots at −80°C.

Fractionation of Mitochondria into Outer and Inner Membranes. In experiments where mitochondria were fractionated into outer membrane and mitoplasts by shearing forces, 300 μg mitochondrial protein were incubated in 1.5 ml of reticulocyte lysate to generate translocational intermediate as described below. The mitochondria were then reisolated by centrifugation at 14,000 *g* for 7 min and resuspended together with 10 mg of unlabeled mitochondria in 4 ml swelling buffer (5 mM KPi, pH 7.2, 1 mM PMSF, 2 mg cytosolic protease inhibitor fraction from *N. crassa* (38)). After 15 min at 0°C, the mitochondria were ruptured by five strokes in a homogenizer with a tight-fitting teflon pestle. The homogenate was layered on a discontinuous sucrose gradient (1 ml 70% sucrose, 5 ml 32% sucrose, 2 ml 15% sucrose (wt/vol), each containing 1 mM EDTA and buffered with 10 mM MOPS/KOH, pH 7.2) and spun at 134,000 *g* for 60 min in a rotor (SW 41; Beckman Instruments, Inc., Fullerton, CA). The outer membrane fraction was recovered from the 32/15% interface, and mitoplasts from the 70/32% interface.

Preparation of Submitochondrial Vesicles. For analysis of membrane vesicles that contained translocational intermediates, 1 mg protein of metabolically ^{35}S-labeled mitochondria was incubated in 1.5 ml reticulocyte lysate to generate translocational intermediates as described below). The mitochondria were then treated (together with 10 mg of labeled mitochondria which did not contain translocational intermediates) in 2 ml swelling buffer as described above. Submitochondrial vesicles were prepared by sonication with a sonifier (B15; Branson, Danbury, CT) using a microtip for 3 min at 20% duty cycle and intensity setting 3. The homogenate was adjusted to 55% sucrose wt/vol by addition of solid sucrose and put at the bottom of a linear gradient from 20–50% wt/vol sucrose in 10 mM MOPS/KOH, pH 7.2, 1 mM EDTA. The gradient was spun for 18 h at 274,000 *g* in a Beckman SW 41 rotor and then fractionated into 500-μl aliquots for analysis. The protein concentration of the fractions was determined by measuring radioactivity. Marker proteins were quantified from peroxidase-stained immunoblots and translocational intermediate was assayed as described below. In a control where no translocational intermediate had been generated, ADP/ATP carrier was directly immunoprecipitated, resolved by SDS PAGE, fluorographed, and quantitated by densitometry. The result of this quantitation agreed well with the quantitation from the peroxidase blot (data not shown).

Protease Treatment of Mitochondria

Freshly isolated mitochondria were suspended at 2 mg protein/ml in ice-cold SEM buffer and then proteinase K (at the concentrations indicated) was added. After 30 min at 0°C, the protease was inactivated by the addition of 1 mM PMSF (from a 200-mM stock solution in ethanol). Mitochondria were then reisolated by centrifugation at 14,000 *g* for 7 min. The pellets were gently resuspended in SEM buffer at 10 mg protein/ml and used for import experiments.

Synthesis of Precursor Proteins and Import into Mitochondria

N. crassa precursor proteins were synthesized from poly-A RNA in reticulocyte lysates (27) in the presence of [^{35}S]methionine. After translation postribosomal supernatants were prepared as described previously (36). Lysates were stored in aliquots at −80°C and were thawed immediately before use. For the generation of translocational intermediates, lysate (1 ml) was first reacted with 200 μg IgG (in 10 μl) against F_1-ATPase subunit β ($F_1\beta$) for 15 min at 25°C. The lysate was then centrifuged at 27,000 *g* for 15 min and aliquots of 50 μl of the supernatant were taken and used for import reactions. Routinely, 5 or 10 μg of mitochondrial protein were incubated in a final volume of 60 μl (50 μl of lysate plus 10 μl additions, or SEM buffer) for 30 min at 25°C. The mitochondria were reisolated by centrifugation at 27,000 *g* for 10 min and the supernatant was removed. The mitochondria were washed once in 100 μl of SEM buffer and then solubilized in Triton buffer as described below. The translocational intermediate was defined as the mature size $F_1\beta$ that could then be harvested with protein A–Sepharose. For import reactions, the same protocol was applied except that no antibody was added to the lysate. Both formation of the translocational intermediate and import of $F_1\beta$ were linear up to 250 μg of mitochondrial protein per milliliter. In cases where complete translocation was tested by resistance to protease digestion, samples were put on ice for 5 min directly after the import reaction, and then proteinase K was added from a 50-fold concentrated stock solution to a final concentration of 20 μg/ml. After 30 min at 0°C, the protease was inactivated by addition of 1 mM PMSF and the mitochondria were reisolated as described above.

Harvesting of the Translocational Intermediate and Immunoprecipitation

Mitochondrial pellets containing translocational intermediates were dissociated in 500 μl of Triton-buffer (1% Triton X-100, 300 mM NaCl, 10 mM Tris/HCl, pH 7.4) (44) containing 1 mM PMSF. After a clarifying spin (20,000 *g* for 15 min) the supernatants were transferred into new cups containing 2 mg of swollen and prewashed protein A–Sepharose. The samples were shaken for 90 min at 4°C and then the protein A–Sepharose beads were washed (36) and the immunocomplexes were dissociated in sample buffer (23) without mercaptoethanol. When import of $F_1\beta$ was tested, 2 mg of protein A–Sepharose (prewashed) were preincubated with an appropriate amount of anti-$F_1\beta$ immune serum for 45 min in 100 μl Triton buffer. The antibody/protein A–Sepharose complex was washed and 500 μl Triton buffer containing the dissociated sample was added and immunoprecipitated as described above.

1. *Abbreviations used in this paper*: $F_1\beta$, subunit 2 of the F_1-ATPase; α-IDH, α-subunit of isocitrate dehydrogenase; SEM buffer, 250 mM sucrose, 1 mM EDTA, 10 mM MOPS, pH 7.2; SEM-K buffer, SEM buffer plus 50 mM KCl; TMPD, *N,N,N′,N′*-tetramethylphenylenediamine.

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Electron Microscopic Analysis of Mitochondria Labeled with Protein A-Gold

Mitochondria (30 μg) were incubated in 300 μl reticulocyte lysate containing pre-$F_1\beta$ IgG complex to generate translocational intermediates. After 30 min of incubation the mitochondria were reisolated by centrifugation at 14,000 *g* for 7 min. The resulting pellet was resuspended in 100 μl SEM buffer, transferred into new cups, sedimented as above and resuspended in 150 μl SEM buffer containing protein A coupled to 3 nm colloidal gold particles (2×10^9 particles/ml). The mitochondria were allowed to react with the protein A-gold at 4°C for 45 min with occasional stirring. The labeled mitochondria were then recovered by centrifugation at 14,000 *g* for 2 min and washed once in SEM buffer. In control experiments, mitochondria were treated in the same way except that anti-malate-synthase antibody was used instead of the $F_1\beta$ antibody. In cases where the mitochondria were treated with hypotonic buffer, 10 mM MOPS/KOH (pH 7.2) was used in the washing step instead of the SEM buffer and outer membranes were partially removed by forcing the mitochondrial suspension through a narrow plastic pipette tip. Mitochondria were fixed in 0.5% wt/vol glutaraldehyde for 2 h at room temperature, sedimented by centrifugation at 10,000 *g* for 5 min, treated with 1% wt/vol OsO_4, and embedded in Epon. Thin sections were lightly stained with lead citrate and examined in a Siemens Elmiskop 102 (Siemens AG, Munich, Federal Republic of Germany).

Morphometric Analysis of Gold Particle Distribution

For morphometric analysis, electron micrographs from two independent experiments were viewed at a magnification of 60,000. The outer mitochondrial membranes and the contact sites were measured using a semiautomatic image-analyzing system MOP/AMO3 (Kontron, München, FRG). Regions where the distance between centers of the inner and outer mitochondrial membranes was <20 nm were considered as contact sites. The lengths of these sites of close proximity were then measured. Only gold particles within a distance of <33 nm from the outer membrane were considered to be membrane-attached and were counted. For statistical analysis see legend to Table I.

Miscellaneous

Protein was determined with the assay according to Bradford (Bio-Rad Laboratories, Richmond, CA) (4). The Laemmli procedure (23) for SDS-PAGE was used. Gels were fluorographed after impregnation with sodium salicylate (6). X-ray films (Kodak X-omat) were quantitated using a Hirschmann gel scanner. Immunoreplica analysis was performed according to Towbin et al. (41) except that 0.04% wt/vol SDS was added to the blotting buffer. Proteins were visualized by either lactoperoxidase staining or by labeling with [^{14}C]protein A. Enzyme assays were performed according to established procedures (35, 38) except that, where necessary, Genapol X-100 was added to the samples to a final concentration of 1% wt/vol, which was diluted in the test so that it never exceeded 0.03%. [^{14}C]Protein A was prepared according to Dottavio-Martin and Ravel (9) and protein A-gold was prepared as described by Roth (32).

Results

A Limited Number of Sites Exist in Mitochondria for Translocational Intermediates Spanning Outer and Inner Membranes

Complexes between the precursor to ATPase subunit $F_1\beta$ and $F_1\beta$-specific antibody were generated by reacting precursor proteins, synthesized in the reticulocyte lysate, with antibody as previously described (35). Precursors in these complexes were processed by mitochondria in an energy-dependent fashion (Fig. 1 *A*, lanes *7* and *10*). They did not, however, become resistant to protease digestion, in contrast to the processed $F_1\beta$ under normal import conditions (cf. lanes *2* and *4* with *7* and *9*). $F_1\beta$, without prebound antibody did not bind to the protein A-Sepharose (lane *1*). The amount of translocational intermediate was therefore determined as the amount of mature-sized $F_1\beta$ that could be bound to protein A-Sepharose. The antibody/precursor complexes were stable throughout the experiment. The amount of radioactive translocational intermediate could not be reduced by adding an excess of unlabeled mitochondria to the mitochondria containing the translocational intermediate before harvesting with protein A-Sepharose (cf. lanes *7* and *8*). Imported radioactive $F_1\beta$, however, was no longer immunoprecipitated if unlabeled mitochondria were added before lysis with Triton and immunoprecipitation (cf. lanes *2* and *3*).

Increasing amounts of mitochondria were allowed to generate translocational intermediates of $F_1\beta$ from reticulocyte lysate containing the precursor/antibody complex. The import reaction was stopped after 30 min by pelleting the mitochondria and the translocational intermediate was quantitated (Fig. 1 *B*). The amount of translocational intermediate formed depended linearily on the concentration of mitochondria in the reticulocyte lysate up to 250 μg mitochondrial protein per milliliter lysate. The same relationship was seen if mitoplasts were used in the import reaction instead of mitochondria (see below, cf. Fig. 6 *B*).

The Translocation Intermediate Cofractionates with the Mitoplasts

Mitochondria were incubated in reticulocyte lysate containing the pre-$F_1\beta$ IgG complex to generate the translocational intermediate. They were then reisolated and treated with increasing concentrations of digitonin to remove increasing amounts of the outer membrane. The translocational intermediate was completely resistent to extraction up to a digitonin concentration of 0.4%, whereas 80% of porin, a marker for the outer membrane, was removed from the mitochondria at this concentration (Fig. 2 *A* and *B*). At higher digitonin concentrations the translocational intermediate was partly extracted, as was the ADP/ATP carrier, an integral inner membrane protein. Imported $F_1\beta$ cofractionated with the α-subunit of isocitrate dehydrogenase (α-IDH), a marker enzyme for the mitochondrial matrix (Fig. 2 *A* and *B*) and is therefore regarded as a soluble protein.

In a second experiment, mitochondria were swollen by hypotonic treatment, then exposed to shearing forces and subsequently resolved by density gradient centrifugation. Fig. 3 *A* shows the protein pattern of the starting mitochondrial preparation (lane *1*), the resulting mitoplast fraction (lane *2*), and the outer membrane fraction (lane *3*). The distribution of porin, ADP/ATP carrier, and α-IDH is presented in Fig. 3 *B*. The outer membrane fraction is essentially free of the inner membrane marker (lane *3*). We always observed, however, that 10–30% of the outer membrane marker (porin) remained with the mitoplast fraction (lane *2*). The translocational intermediate exclusively cofractionated with the inner membrane and matrix markers (Fig. 3 *C*).

In summary, we conclude that the translocational intermediates spanning the two membranes are concentrated in areas where the two membranes are in close proximity. The regions where the membranes make contacts that mediate import of the precursors were not destroyed by digitonin or shearing forces under conditions that removed the majority of the outer membrane from the mitochondria.

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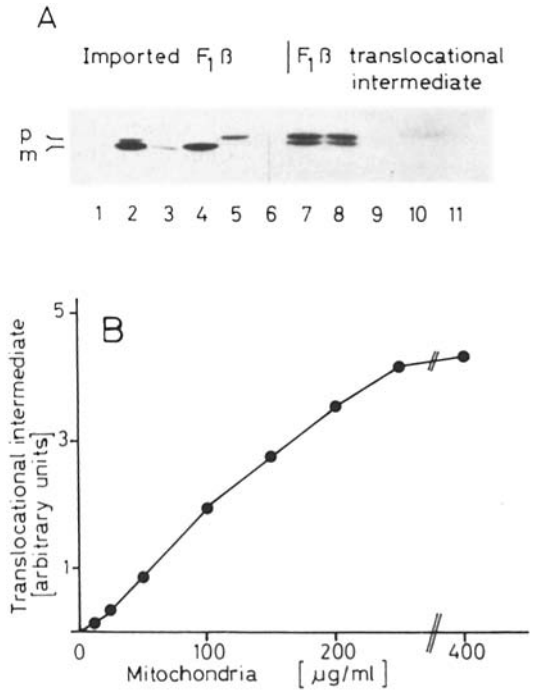

Figure 1. Characterization of the translocational intermediate of $F_1\beta$. (*A*) Precursor proteins, synthesized in reticulocyte lysate, were incubated either with a control antibody (anti-malate-synthase IgG, lanes *1–6*) or with anti-$F_1\beta$ IgG (lanes *7–11*). After 15 min at 25°C, the lysates were centrifuged at 27,000 *g* for 15 min, to remove any precipitated protein, and mitochondria (10 μg) were incubated with the supernatants. Membrane-potential dependence of import was tested by adding 4 μM antimycin A and 10 μM oligomycin to reactions *5*, *6*, *10*, and *11*. After 30 min at 25°C, to allow import, samples *4*, *6*, *9*, and *11* were treated with 10 μg of proteinase K/ml at 0°C to test for sequestration. Protease treatment was stopped after 30 min by the addition of 1 mM PMSF. The mitochondria were then reisolated as described in Materials and Methods. After addition of 200 μg fresh mitochondria to reactions *3* and *8*, all samples were subjected to immunoprecipitation. Samples *2–6* were incubated with 10 μl anti-$F_1\beta$ antibodies prebound to protein A–Sepharose. Samples *1* and *7–11* received protein A–Sepharose alone. Immunoprecipitates were analyzed by SDS-PAGE and fluorography. *p*, precursor to $F_1\beta$; *m*, mature $F_1\beta$. (*B*) Increasing amounts of mitochondria were incubated in reticulocyte lysate containing the $F_1\beta$–IgG complex. After 30 min the samples were cooled to 0°C, the mitochondria were reisolated, and the amount of translocational intermediate was determined. The translocational intermediate was quantified by scanning the fluorographs.

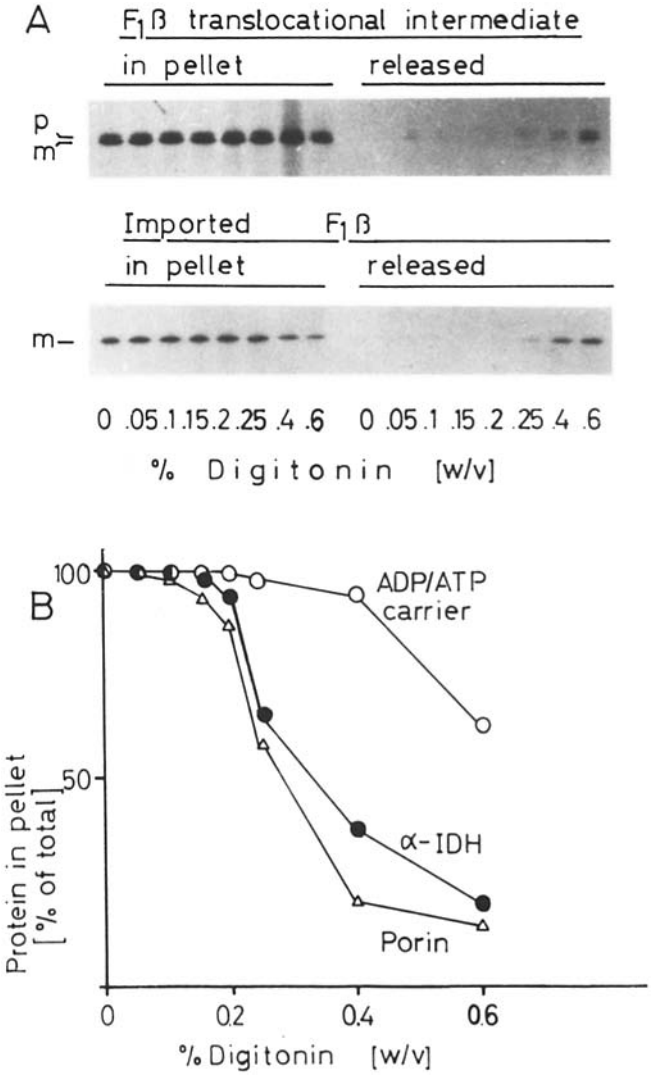

Figure 2. The translocational intermediate of $F_1\beta$ cofractionates with mitoplasts. Complex of ^{35}S-labeled pre-$F_1\beta$ and anti-$F_1\beta$ antibody was formed with 800 μl of reticulocyte lysate as described in Materials and Methods. Mitochondria were added to the lysate to allow import and the formation of the $F_1\beta$ translocational intermediate. The mitochondria were reisolated, suspended in SEM buffer at 10 mg protein/ml, and divided into eight aliquots. The aliquots were incubated with increasing concentrations of digitonin as indicated. (*A*) Supernatants and pellets were assayed for translocational intermediate and for imported $F_1\beta$ as described in Materials and Methods. The immunoprecipitates were dissociated, analyzed by SDS-PAGE, and fluorographed. (*B*) Pellets were subjected to SDS-PAGE and proteins were transferred to nitrocellulose membranes. Markers for the outer membrane (porin), inner membrane (ADP/ATP carrier), and matrix (α-IDH) were visualized with antibodies and [^{14}C]protein A. Bands of the resulting autoradiographs were quantitated by densitometry. The sum of the signal in the pellet and supernatant at a given digitonin concentration was set at 100%.

Mitoplasts Can Import Proteins and Can Form the Translocational Intermediate

We tested the ability of mitoplasts to import precursor proteins or form the translocational intermediate in order to determine whether the two membranes are held together by the translocational intermediate, or whether they exist as stable complexes in the absence of precursor proteins. For this purpose, mitochondria were treated with increasing amounts of digitonin and then diluted, reisolated, and resuspended in detergent-free buffer. The mitochondria treated with varying digitonin concentrations were characterized by determining marker enzyme activities and proteins. At low digitonin concentrations (<0.2%) the outer membrane became permeable; adenylate kinase was released and succinate cytochrome *c* reductase became accessible to added cytochrome *c* (Fig. 4, *B* and *C*). At higher concentrations, porin was released from the mitochondria (50% at 0.3% digitonin, Fig. 4 *B*). At even higher concentrations, the inner membrane was labilized and the matrix enzyme fumarase was released. Eventually, the ADP/ATP carrier, a marker for the inner membrane, was also released.

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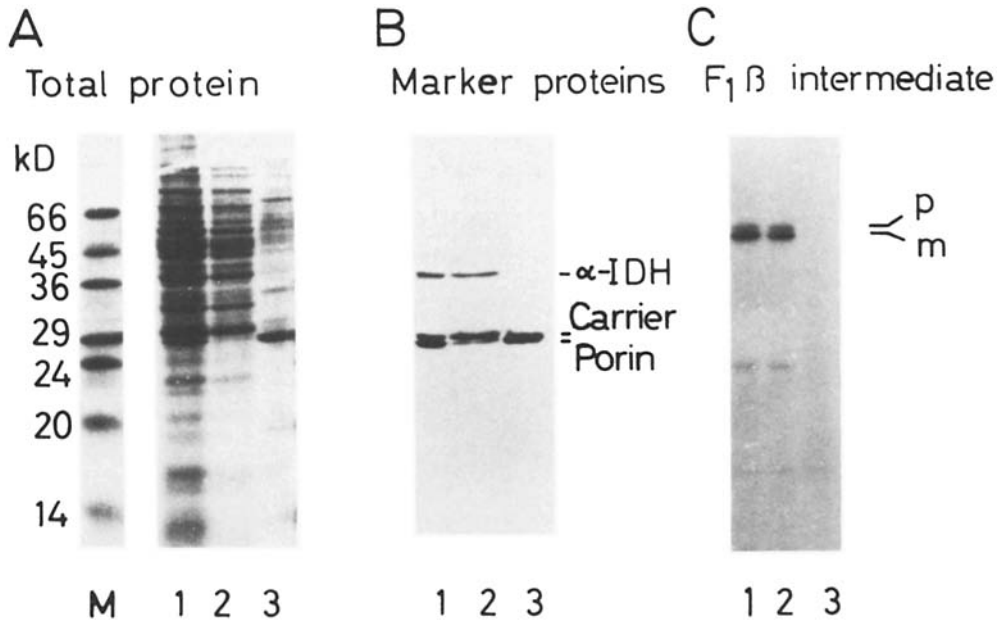

Figure 3. The translocational intermediate remains with the mitoplasts after removal of the outer membrane by shearing forces. Mitochondria (300 μg) were incubated in 1.5 ml reticulocyte lysate containing precursor proteins that had been preincubated with anti-$F_1\beta$ IgG to generate the $F_1\beta$ translocational intermediate. The mitochondria were reisolated by centrifugation and subjected to swelling and rupture, and outer membranes and mitoplasts were separated as described in Materials and Methods. Fractions were subjected to SDS-PAGE and stained for protein (*A*), blotted for marker proteins (*B*), and analyzed for the presence of translocational intermediates (*C*). The markers were porin (outer membrane), ADP/ATP carrier (inner membrane), and α-IDH (matrix). Lanes *1*, starting mitochondria; lanes *2*, 70/32% sucrose interface plus 32% sucrose fraction; lanes *3*, 32/15% sucrose interface plus supernatant. In lanes *3*, five times the equivalent volumes were applied. Carrier, ADP/ATP carrier.

Mitochondria treated at varying digitonin concentrations were incubated in reticulocyte lysate to generate the translocational intermediate of $F_1\beta$ (from the antibody-bound precursor) or to import the protein into the matrix. Both the translocational intermediate and imported $F_1\beta$ were obtained with the various preparations in a membrane-potential–dependent fashion (Fig. 4 *A*). The translocation activity cofractionated with the ADP/ATP carrier (Fig. 4 *B*). The import of $F_1\beta$ was somewhat more efficient than the formation of the translocational intermediate. This may be due to repeated use of translocation contact sites by free precursors, whereas the intermediate may saturate and block contact sites. Since the outer membrane marker (porin) was released from the mitochondria at digitonin concentrations that only slightly affected import, we conclude that an intact inner membrane, but not an intact outer membrane, is required for import of $F_1\beta$. If the amount of preexisting ADP/ATP carrier in these digitonin-treated mitochondria is considered to be a measure of "intact mitoplasts", then even at the highest digitonin concentration used the formation of the translocation intermediate was not inhibited. The situation was different, however, for the import of the ADP/ATP carrier. With the precursor to this protein, binding (data not shown) as well as import declined with increasing digitonin concentrations (Fig. 4 *A*) in a manner which paralleled the release of porin (Fig. 4 *B*). This may suggest that the receptors for the ADP/ATP carrier can be removed or inactivated by digitonin under conditions where the components of the import machinery for $F_1\beta$ are not affected.

Import of $F_1\beta$ into Mitoplasts Depends on an Extractable Factor That Can Be Replenished

When the digitonin fractionation described above was performed in the presence of 50 mM KCl, import as well as the formation of the translocational intermediate was completely blocked at digitonin concentrations >0.2% (Fig. 5 *A*). Distribution of the markers was not altered by including 50 mM KCl into the medium. This indicates that a component of the intermembrane space, which is membrane associated and can be removed from the membrane by detergent in the presence of salt, is involved in the translocation reaction.

We tried to elucidate the nature of the salt-extractable factor. Mitoplasts that had been depleted of the factor by KCl treatment were incubated with desalted mitochondrial extracts prepared as described in Materials and Methods. After this treatment the mitoplasts regained the ability to import $F_1\beta$ (Fig. 5 *B*, lanes *3* vs. lanes *8*). The factor seems to be a protein since it fractionated with the void volume of a Sephadex G-25 column and it was destroyed by heating the extract to 95°C (lanes *9*). Treatment of the extract with protease also abolished its ability to reestablish the import into detergent plus salt-treated mitoplasts; however, protease-treated extract inhibited import into intact mitochondria as well (data not shown). This may be due to uncoupling of the mitochondria by the peptides generated by proteolysis (31).

Import into intact mitochondria was unaffected by pretreatment with 300 mM KCl (data not shown). The target of the detergent plus salt extraction is therefore likely to be a membrane component that cannot be removed while the outer membrane is intact (e.g., exposed to the intermembrane space). Components known to be indirectly involved in the import of precursor proteins that are inside the outer membrane include the proteins of the membrane-potential generating system. Cytochrome *c* added to the reticulocyte lysate during the import reaction did not substitute for the factor (lanes *5*) nor did ATP (lanes *7*), which creates a membrane potential via the F_1-ATPase. Similarly, TMPD plus ascorbate (which donates electrons at the level of the cytochrome oxidase) (33) did not support import (lanes *4*), nor did the combination of cytochrome *c* plus ascorbate and

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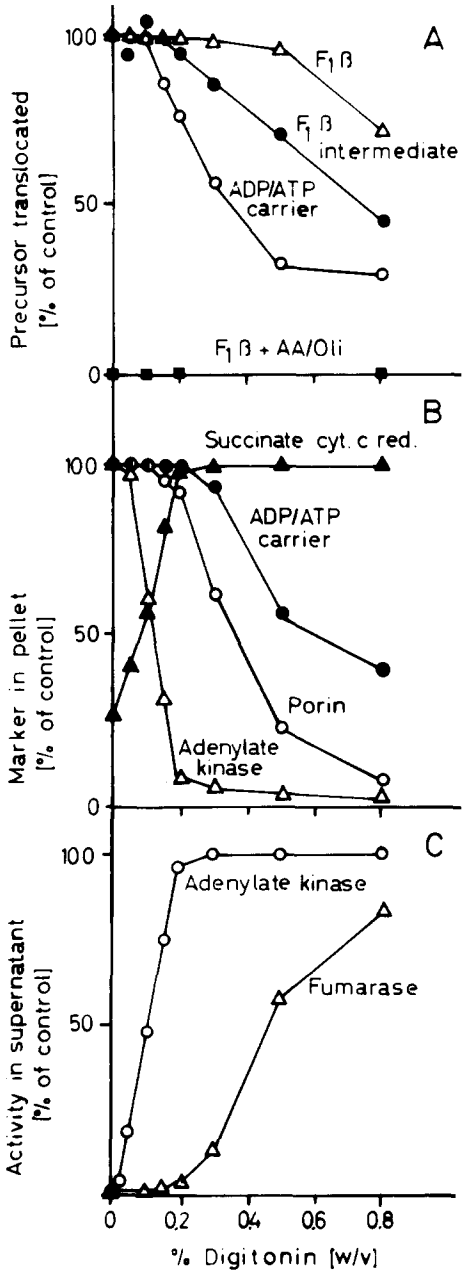

Figure 4. Mitoplasts can form translocational intermediates when the inner membrane is energized. Mitochondria were treated with increasing concentrations of digitonin as described in Materials and Methods. They were then tested for the ability to import proteins and to generate the $F_1\beta$ translocational intermediate (*A*). Mitoplast preparations were also tested for $F_1\beta$ import after destroying the membrane potential with 4 μM antimycin A plus 10 μM oligomycin (AA/Oli). The distribution of marker proteins was determined in parallel (*B* and *C*). Adenylate kinase (intermembrane space) and fumarase (matrix) were quantified by measuring enzyme activities; porin (outer membrane) and preexisting ADP/ATP carrier (inner membrane) were immunoblotted and decorated with [^{14}C]protein A. Imported ADP/ATP carrier was determined by adding 12 μM carboxyatractyloside at the end of the reaction period and then passing the Triton X-100–solubilized mitoplasts over a hydroxyapatite column as previously described (34). Exposure of the inner membrane after digitonin treatment was analyzed by testing the accessibility of cytochrome *c* to succinate cytochrome *c* reductase (*C*). (100% succinate cytochrome *c* reductase activity is defined as the maximal activity obtained after exposing mitochondria to shearing forces in 5 mM potassium phosphate buffer (pH 7.2)).

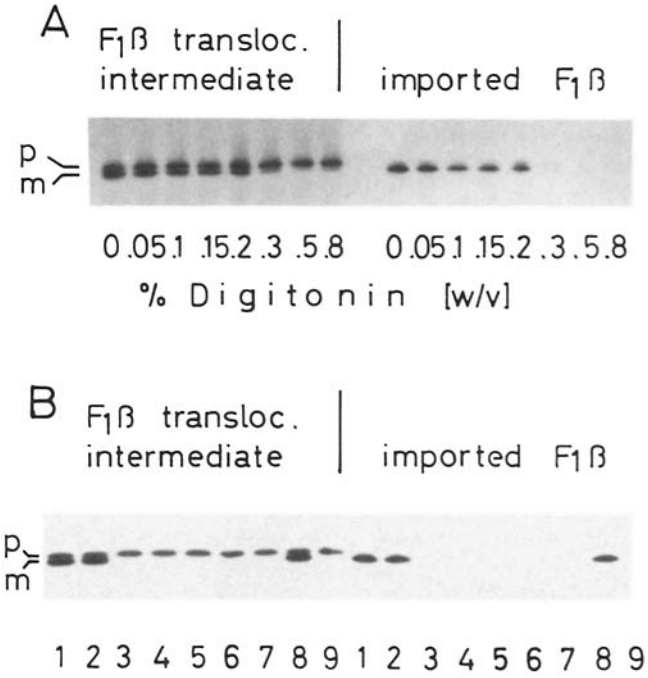

Figure 5. Import into mitoplasts depends on a factor that is extracted by digitonin plus 50 mM KCl. (*A*) Mitochondria were treated with digitonin as described in Fig. 4, with the exception that SEM-K buffer was used instead of SEM. The mitoplasts generated by this treatment were tested for the ability to form the $F_1\beta$ translocational intermediate and to import p-$F_1\beta$. (*B*) An extract was prepared from mitochondria by treatment with digitonin in a buffer containing 50 mM KCl. This extract was desalted as described in Materials and Methods. Mitochondria were treated with 0.25% digitonin in SEM buffer (reaction *2*) or in SEM-K buffer (reactions *3–9*). Mitoplasts were then reisolated either in SEM buffer (reactions *2–7*), or in desalted extract (reaction *8*), or in boiled desalted extract (reaction *9*), and used in reactions to determine formation of the translocational intermediate or import of $F_1\beta$. Reaction *1* is a control with untreated mitochondria. Import reactions *1–3* received no further additions. Reaction *4* received 8 mM ascorbate plus 0.2 mM TMPD. Reaction *5* received 7.5 μM *N. crassa* cytochrome *c*. Reaction *6* received both ascorbate plus TMPD, and cytochrome *c*. ATP (2 mM, pH 7.2) was added to reaction *7*.

TMPD (lanes *6*). Therefore the role of the factor must be other than for the generation of a membrane potential.

The Inner Mitochondrial Membrane Does Not Contain a Second Set of Import Receptors

Since mitoplasts did import proteins and generated the translocation intermediate, we tested whether the inner membrane alone could support import. First, it was determined how much protease was needed to destroy the import receptors on the outer membrane and thereby abolish import (47) while leaving the intermembrane space unaffected. For this purpose, intact mitochondria were treated with increasing concentrations of proteinase K. At 0°C, 10 μg proteinase K per milliliter reduced import of $F_1\beta$ by 94%. Nearly 100% of the adenylate kinase activity was recovered with the mitochondria (Fig. 6 *A*). In the presence of detergent, adenylate kinase activity was destroyed by the concentrations of protease used (data not shown). Under these conditions components on the outer surface of the outer membrane that are

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necessary for import could apparently be destroyed without affecting components exposed to the intermembrane space.

In a second experiment, mitochondria were pretreated with 10 μg of proteinase per milliliter. After inactivation of the protease by addition of PMSF, the mitochondria were incubated with 0.3% of digitonin to remove (part of) the outer membrane and to expose the inner membrane. The mitoplasts were then assayed for import activity by incubation in reticulocyte lysate. The protease-treated mitochondria had a strongly reduced import activity (Fig. 6 *B*, lane *5*). The mitoplasts from protease-pretreated mitochondria showed the same residual activity (lane *6*), whereas the untreated mitoplasts (lanes *3* and *4*) imported $F_1\beta$ in a manner comparable to that of the control mitochondria (lanes *1* and *2*). This suggests that a separate import machinery on the inner membrane does not exist. The translocational intermediates formed by mitoplasts apparently span remnants of the outer membrane and the inner membrane and are functionally the same as those observed in intact mitochondria.

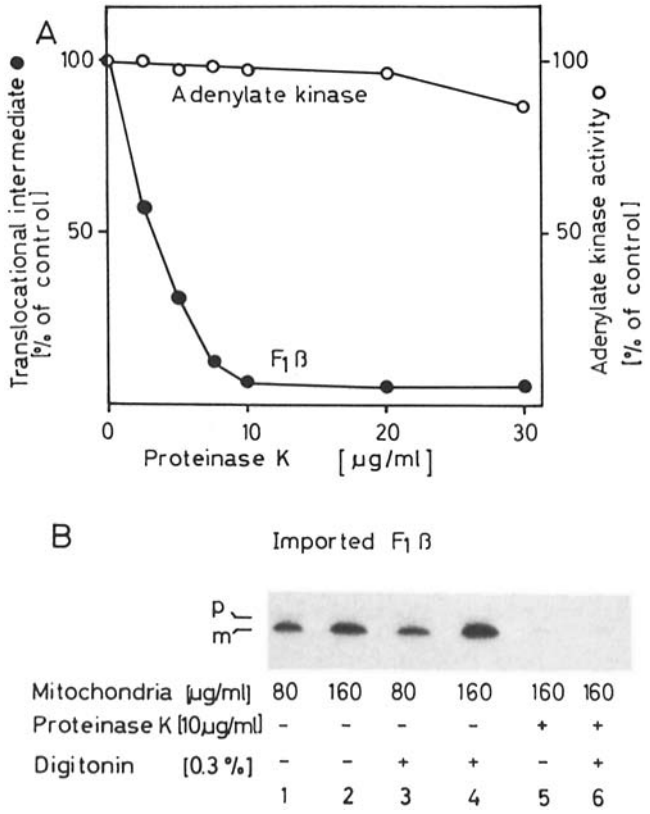

Figure 6. The inner mitochondrial membrane lacks translocation activity. (*A*) Mitochondria were suspended in SEM buffer at 2 mg protein/ml and treated for 30 min at 0°C with the indicated concentrations of proteinase K. The protease was inactivated by the addition of 1 mM PMSF and the mitochondria were reisolated. Adenylate kinase activity and formation of the $F_1\beta$ translocation intermediate were measured in aliquots of the mitochondria as described in Materials and Methods. (*B*) A mitochondrial suspension (2 mg/ml) was divided into two equal portions. One portion was pretreated with 10 μg/ml of proteinase K as described above. The other portion received proteinase K that had been inactivated with PMSF. After inactivation of the protease each portion was halved again, and one-half was treated with 0.3% digitonin. After reisolation in detergent-free buffer, all four samples were then assayed for the ability to import the $F_1\beta$ precursor. To be sure the import activity was proportional to the amount of mitochondria, two concentrations of mitochondria and mitoplasts (80 and 160 μg/ml) were used in the import reaction.

Dissipation of the Membrane Potential Does Not Lead to Destruction of Translocation Contact Sites

An early step in the translocation process depends on the membrane potential; once a translocational intermediate has been formed, further import is independent of the potential (35). We wanted to know if the presence of translocation contact sites, as defined by the ability to form translocational intermediates, is dependent on the membrane potential.

Mitochondria were isolated under the following conditions (Fig. 7, lanes *3–5*): in the presence of antimycin A plus oligomycin, which block the generation of the membrane potential (28); (lanes *6* and *7*) in the presence of succinate and ADP, which let mitochondria phosphorylate (16); (lanes *8* and *9*) in the presence of ascorbate plus TMPD; (lanes *10* and *11*) in a buffer containing 30% vol/vol glycerol, which was reported to reduce the number of membrane contacts in liver mitochondria (22) instead of sucrose. Then mitoplasts were prepared by treating mitochondria with 0.3% digitonin to limit the potential reformation of dissipated contact site regions in the subsequent reenergization step. (Control mitochondria received SEM buffer plus the appropriate additions instead of digitonin.) The mitoplasts and mitochondria were

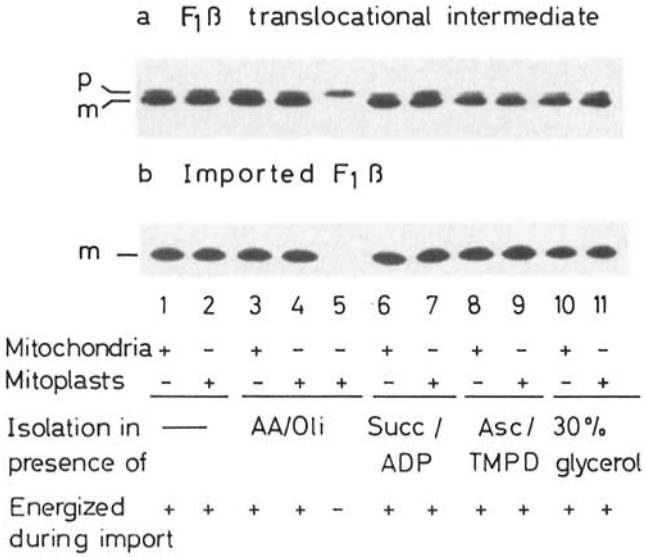

Figure 7. Deenergization of mitochondria during digitonin treatment does not affect translocation contact sites. Mitochondria were suspended in SEM buffer (lanes *1* and *2*); in SEM buffer containing 4 μM antimycin A plus 10 μM oligomycin (lanes *3–5*); in SEM buffer containing 2.5 mM succinate plus 0.5 mM ADP (lanes *6* and *7*); in SEM buffer containing 8 mM ascorbate plus 0.2 mM TMPD (lanes *8*, *9*); or in 10 mM MOPS buffer (pH 7.2), containing 1 mM EDTA and 30% glycerol (lanes *10* and *11*). Half of the preparations were then treated with 0.3% digitonin while controls were mock treated (no digitonin). After reisolation, the mock-treated mitochondria and mitoplasts were assayed for formation of the translocational intermediate. Import assays *1–4* and *6–11* were performed in the presence of 8 mM ascorbate plus 0.2 mM TMPD. Lanes *1*, *3*, *6*, *8*, and *10* are controls with mitochondria. In lanes *2*, *4*, *5*, *7*, *9*, and *11* mitoplasts were used in the import reactions.

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reisolated in ascorbate plus TMPD-containing buffer to regenerate, or sustain, the membrane potential. With all mitoplast preparations, roughly half of the outer membrane was removed by the digitonin treatment (data not shown). No difference, however, was detected in the ability of the different mitoplast preparations to form the translocational intermediate (Fig. 7 *a*) or to import pre-$F_1\beta$ (Fig. 7 *b*). Both reactions depended on reestablishing the membrane potential (lanes *5*).

These results are consistent with the concept of stable contact sites whose presence is independent of membrane potential. If the number of translocation contact sites was modulated by the energy state of the mitoplasts, we would assume that mitoplasts from phosphorylating mitochondria would contain more and mitoplasts from deenergized mitochondria would contain less translocation contact sites and thus more or less translocation intermediate.

Translocational Intermediates Are Enriched in a Vesicle Fraction Derived from Mitochondria by Sonication

Since the translocation contact sites appeared to be resistant to a variety of disruptive treatments, we tested whether a membrane fraction could be purified from mitochondria which was enriched in these sites, as determined by the presence of translocational intermediates.

Cells were grown in the presence of [^{35}S]sulfate and the specific radioactivity of the mitochondria was determined. 1 milligram of these mitochondria was then incubated in reticulocyte lysate containing ^{35}S-labeled precursors of $F_1\beta$, which had been reacted with antibody to generate the translocational intermediate of $F_1\beta$. (As a control an aliquot of the labeled mitochondria was not incubated in reticulocyte lysate, but only treated with protein A-Sepharose. No radioactivity labeled $F_1\beta$ could be detected with the protein A-Sepharose beads (data not shown)). After reisolation from the reticulocyte lysate, the mitochondria were suspended in swelling buffer and sonicated together with 9 mg mitochondria that had not been incubated in reticulocyte lysate. The submitochondrial vesicles were subjected to centrifugation on a linear sucrose gradient. The protein profile of the gradient is shown in Fig. 8 *A*. The distribution of porin and the ADP/ATP carrier (Fig. 8 *B*) indicated that the outer membrane was enriched at 32% sucrose and the inner membrane banded at 43% sucrose. The specific content of the translocational intermediate (radioactivity in translocational intermediate per milligram mitochondrial protein) was highest in fractions that were of lower density than the bulk of inner membrane vesicles and banded at 40% sucrose (Fig. 8 *C*).

We conclude that vesicles were generated upon sonication which were enriched in translocation contact sites. These vesicles containing translocation contact site areas appear to be less dense than most of the inner membrane. Total distribution of the $F_1\beta$ intermediate roughly followed the inner membrane marker. This is not surprising since the contact site areas are small compared with the inner membrane area. Vesicles of a wide size range were generated upon sonication (data not shown). Large vesicles contain large portions of inner membrane and thereby exhibit density properties of the inner membrane.

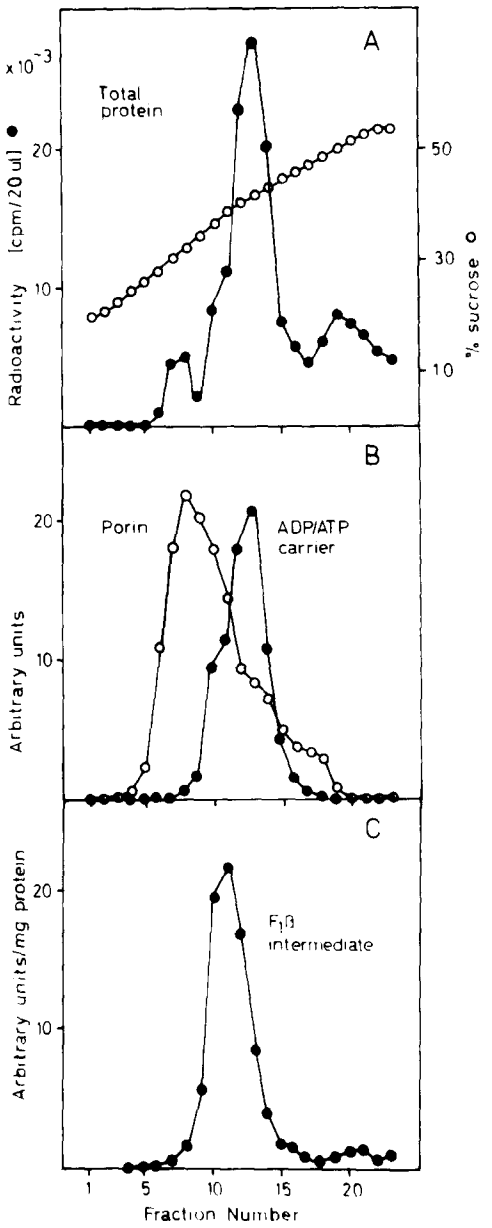

Figure 8. Separation of submitochondrial vesicles by sucrose gradient centrifugation. Metabolically labeled mitochondria containing translocational intermediates were sonicated in swelling buffer as described in Materials and Methods. The homogenate was then made 55% wt/vol sucrose and covered with a linear sucrose gradient from 20–50% wt/vol. After centrifugation for 18 h at 274,000 *g*, 0.5-ml fractions were taken and analyzed. (*A*) Distribution of protein. Radioactivity in an aliquot of each gradient fraction was determined by scintillation counting. (*B*) Distribution of membrane markers. Aliquots of the gradient fractions were subjected to SDS-PAGE, immunoblotted for ADP/ATP carrier and for porin, and quantified by densitometry. (*C*) Specific distribution of the translocational intermediate on the sucrose gradient. A volume corresponding to 70,000 cpm (15 μg protein) was taken from each fraction and assayed for translocational intermediate by harvesting with protein A–Sepharose. After SDS-PAGE and fluorography the signals of the translocational intermediates were quantified by densitometry.

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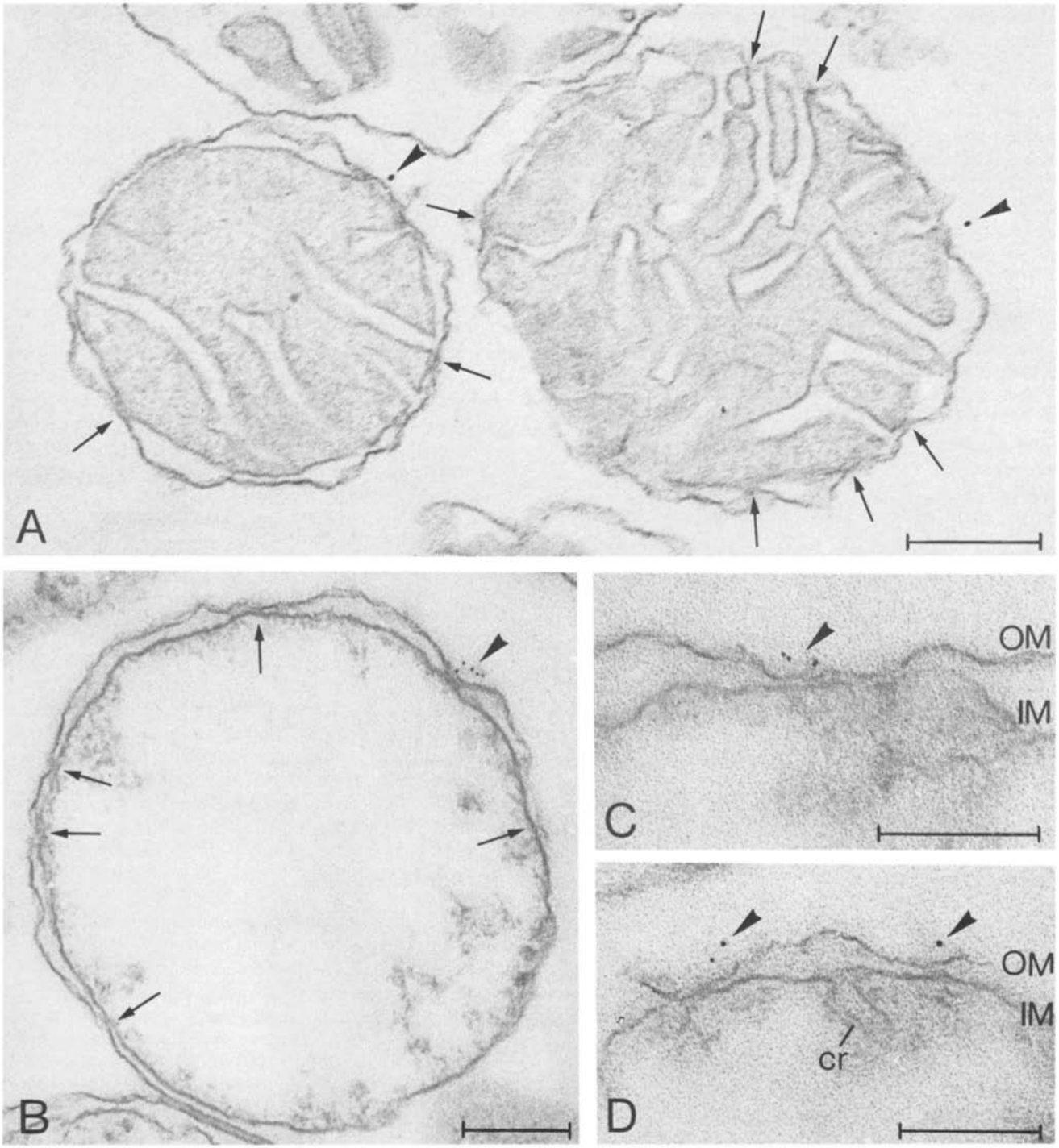

Figure 9. Visualization of the translocational intermediate by labeling with protein A–Gold. Mitochondria containing translocational intermediates were incubated with protein A–gold. They were then washed and fixed as described in Materials and Methods and examined by electron microscopy. (*A*) Mitochondria labeled with gold particles; (*B*) same as in *A*, except that the mitochondria were swollen in 10 mM MOPS buffer (pH 7.2) before fixation; (*C*) detailed view of a swollen mitochondrion (prepared as in *B*) at higher magnification (a translocation contact site area is shown); (*D*) detail as in *C* except that before fixation the mitochondria were subjected to shearing forces. The outer membrane is ruptured on both sides of the contact site. (*Arrowheads*) Contact sites, labeled with protein A–gold. (*Arrows*) Sites of close contact of the outer and inner membranes, not labeled with protein A–gold. *OM*, outer membrane; *IM*, inner membrane; *cr*, cristae. Bars, 0.2 μm.

Visualization of the Sites of Translocation by Labeling with Protein A–Gold

In an initial attempt to answer the question of whether the contact sites seen by electron microscopy in mitochondrial cross sections are related to the biochemically defined translocation contact sites, we tried to identify the location of the

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Table I. Quantitation of Protein A–Gold Bound to the Mitochondrial Surface

Experiment	Total No.	Distribution of gold particles: At translocation contact sites	Distribution of gold particles: Not associated with contact sites	P
Experiment 1				
f observed	134	116	18	≪0.001
(f' expected)	(134)	(27.7)	(106.3)	
Experiment 2				
f observed	174	153	21	≪0.001
(f' expected)	(174)	(36.5)	(137.5)	
Both experiments				
Distribution of gold particles (%)	100%	87.3%	12.7%	

Mitochondria were incubated in reticulocyte lysate containing the pre-$F_1\beta$ IgG complex, reisolated, washed, and reacted with protein A–gold as described in Materials and Methods. Counting of gold particles and morphometry was performed on a total length of 124.2 μm (experiment 1) or 166.7 μm (experiment 2) of outer mitochondrial membranes containing a length of 25.7 μm (experiment 1) or 34.9 μm (experiment 2) of contact sites. The expected distribution was computed from the total number of gold particles counted and the relative length of contact sites and outer mitochondrial membranes. The value for *P*, based on the X^2 statistics ($X^2 = [\frac{f - f'}{f'}]^2$), indicates that the predominant labeling of translocation contact sites is highly significant.

translocational intermediates on the mitochondrial surface. Since the translocational intermediates are the only IgG-containing components of the mitochondrial surface it was possible to visualize them by labeling with protein A–linked gold particles.

Mitochondria containing translocational intermediates were incubated with protein A–gold and ultrathin sections were examined for the distribution of the gold particles. Most gold grains were observed in areas where the two membranes were closely apposed (Fig. 9 *A*). Due to the complex stereological relationships of the sections, an unequivocal assignment of a gold grain to a contact site was difficult. The localization of the gold grains was unambiguous, however, when the mitochondria were incubated in swelling buffer before fixation (Fig. 9, *B–D*) so that inner and outer membranes were well separated. They remained apposed only in small areas (~20% of the outer membrane surface). The morphometric analysis of the gold particle distribution on the outer mitochondrial membrane showed a highly significant labeling of contact sites (see Table I). Gold particles were concentrated in these areas (87% of the gold grains in contact sites). The distribution of gold grains was similar to the distribution reported for cytoplasmic ribosomes on yeast mitochondria (21). These ribosomes, specifically engaged in the synthesis of mitochondrial proteins (39), were found to be preferentially located in places where the two mitochondrial membranes were in close proximity.

In some experiments, mitochondria were exposed to shearing forces before fixation. This, as described above, removed part of the outer membrane. The outer membrane was frequently ruptured next to a site where the two membranes were in contact (Fig. 9 *D*). This can be interpreted as evidence that the two membranes form stable complexes in the areas of the translocation contact sites.

In control experiments, where no antibody-trapped translocational intermediate was present (e.g., mitochondria were incubated in reticulocyte lysate in the presence of IgG against the glyoxysomal enzyme malate synthase) a negligible amount of gold particles was associated with the mitochondria (data not shown).

Discussion

Contacts between outer and inner mitochondrial membranes have been observed in electron micrographs (11, 12) and appeared to be of stable nature since they were preserved even if a large part of the outer membrane had been removed (13). It is not clear, however, whether the sites defined biochemically by the formation of translocational intermediates (35) are identical to the sites defined morphologically in electron micrographs. The experiments presented here support the existence of stable contact sites involved in import of mitochondrial proteins. These contacts did not depend on the existence of translocational intermediates and they were not broken by sonication. A vesicle fraction that was enriched in translocational intermediates could be prepared.

Translocational intermediates remained with mitoplasts after removal of a large part of the outer membrane. Apparently, those portions of the outer membrane that were removed are not involved in the translocation process, although they may contain receptors for some proteins. Such mitoplasts were still capable of importing proteins. Since the inner membrane of these mitoplasts was also exposed to the precursor proteins, it was necessary to discount the inner membrane per se as a translocation-competent membrane. This was done by pretreating the mitochondria with protease to remove the import receptors on the outer membrane (47). No translocation activity could be measured with mitoplasts that were subsequently prepared from protease-treated mitochondria. This indicated that the inner membrane by itself is not competent for translocation, but that import can occur even if most of the outer membrane is absent. In addition, the translocation step of import seems to be restricted to the contact sites. It should be kept in mind, however, that the inner membrane is translocation competent for proteins approaching from the matrix side, e.g., mitochondrially made proteins are translocated to the outer surface of the inner membrane (26), and, as recently shown, the precursor of the FeS protein of complex III is imported into the matrix via translocation contact sites and then routed back across the inner membrane to the outer surface of the inner membrane (14).

Neither import of $F_1\beta$ nor the formation of its translocational intermediate was sensitive to the removal of most of the outer membrane with digitonin. Therefore, in the case of $F_1\beta$ all the components required for import seem to be located in the area of the translocation contact sites. The protease-sensitive surface structures required for import may be part of the translocation contact site. The situation seems different for the import of the ADP/ATP carrier. Its precursor binds to protease-sensitive high affinity sites (37, 47) in the absence of a membrane potential (36) and can, upon reenergization, be imported via translocation contact sites (Pfanner, N., and W. Neupert, manuscript submitted for publication). We observed a decrease of carrier binding and import in response to digitonin extraction of the outer mem-

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brane. A possible explanation for this is that the receptors for the ADP/ATP carrier were distributed over the outer membrane and were not concentrated in the translocation contact sites.

Recently, the structure of rat liver mitochondria, which had been isolated in various energy states or which had been incubated in buffer containing divalent cations, was studied using freeze-fracture techniques (22, 43). A modulation of the number of fracture-plane jumps (which were thought to occur because of fusions of the outer and inner mitochondrial membranes) in response to the energy state or the cations added was observed. The formation of translocational intermediates in our studies, however, was independent of whether mitochondria were in an energized or deenergized state before and during preparation of the mitoplasts. Therefore, translocation contact sites seem to be different from the associations described in these reports. Formation and dissociation of translocation contact sites in response to the membrane potential may not occur or may occur only to a limited degree. At present it is not clear whether there is a relation between the translocation contact sites defined here (both biochemically and morphologically) and the fusion sites observed in freeze-fracture experiments by others (22, 43). Upon digitonin treatment of rat liver mitochondria, outer membrane vesicles were observed that remained attached to the inner membrane (13). It seems unlikely, however, that such mitoplasts would be competent for protein import since the outer face of the outer membrane would not be accessible in the inverted vesicles. With *Neurospora* mitochondria we have never seen formation of inverted outer membrane vesicles upon digitonin treatment. Instead, membrane sheets attached to the inner membrane were observed (our unpublished observation).

Translocation contact sites appear to mediate the membrane-potential-dependent import of precursor proteins (14, 35, Pfanner, N., and W. Neupert, manuscript submitted for publication). In addition to authentic mitochondrial proteins, fusion proteins consisting of mitochondrial targeting signals and a nonmitochondrial "passenger" protein are imported via translocation contact sites (Schwaiger, M., H. Müller, M. Tropschug, and W. Neupert, manuscript in preparation). Passage through translocation contact sites seems to be an obligatory step in the import of most mitochondrial proteins. Exceptions appear to include proteins of the outer membrane and of the intermembrane space that are imported independently of the membrane potential.

Translocation of proteins across two membranes is not restricted to mitochondria. Chloroplasts, which must import most of their proteins, and gram-negative bacteria are also surrounded by two membranes. In both cases, structural equivalents to mitochondrial translocation contact sites have been observed and speculations about their function in import of proteins have been suggested (2, 5, 7), although no direct evidence for the involvement of these contact sites in protein translocation has been presented yet. It will be interesting to learn whether the contact sites in bacteria and eucaryotic cell organelles are related, either functionally, structurally, or throughout evolution.

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