Protein Import into Mitochondria: ATP-dependent Protein Translocation Activity in a Submitochondrial Fraction Enriched in Membrane Contact Sites and Specific Proteins

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Abstract. To identify the membrane regions through which yeast mitochondria import proteins from the cytoplasm, we have tagged these regions with two different partly translocated precursor proteins. One of these was bound to the mitochondrial surface of ATP-depleted mitochondria and could subsequently be chased into mitochondria upon addition of ATP. The other intermediate was irreversibly stuck across both mitochondrial membranes at protein import sites. Upon subfraction of the mitochondria, both intermediates cofractionated with membrane vesicles whose buoyant density was between that of inner and outer membranes. When these vesicles were prepared from mitochondria containing the chaseable intermediate, they internalized it upon addition of ATP. A non-hydrolyzable ATP analogue was inactive. This vesicle fraction contained closed, right-side-out inner membrane vesicles attached to leaky outer membrane vesicles. The vesicles contained the mitochondrial binding sites for cytoplasmic ribosomes and contained several mitochondrial proteins that were enriched relative to markers of inner or outer membranes. By immunoelectron microscopy, two of these proteins were concentrated at sites where mitochondrial inner and outer membranes are closely apposed. We conclude that these vesicles contain contact sites between the two mitochondrial membranes, that these sites are the entry point for proteins into mitochondria, and that the isolated vesicles are still translocation competent.

In mitochondria, chloroplasts, nuclei, and Gram-negative bacteria, some proteins are transported across two biological membranes. Mounting evidence suggests that these transport processes do not represent two successive translocations across two separate, single membranes. In all four membrane systems mentioned above, sites of contact between the two membranes have been observed and suggested to act as transport sites. The first detailed description of such sites and their possible involvement in protein translocation was provided for mitochondria (Hackenbrock, 1968; Hackenbrock and Miller, 1975; Kellems et al., 1975) and Gram-negative bacteria (Bayer, 1979). More recently, import of proteins into the nucleus was found to occur through the nuclear pore that connects the two membranes (reviewed by Dingwall and Lasky, 1986; Newport and Forbes, 1987). Finally, a component involved in the import of proteins into chloroplasts was found (Pain et al., 1988) to be enriched at points on the chloroplast surface where the two membranes are in close apposition (see also Cremers et al., 1988).

A role of the mitochondrial "contact sites" in protein import is suggested by two observations. First, mitochondria isolated from cycloheximide-inhibited yeast cells contain cytoplasmic polysomes, which are selectively bound to those surface regions where the two membranes are closely apposed (Kellems et al., 1975). Because these polysomes are enriched in mRNAs for imported mitochondrial proteins, they are probably bound to mitochondria by nascent, arrested chains of these proteins (Ades and Butow, 1980; Suissa and Schatz, 1982). Second, precursor proteins that have been synthesized in vitro and trapped during translocation into isolated mitochondria by low temperature or antibodies become stuck across both mitochondrial membranes (Schleyer and Neupert, 1985; Schwaiger et al., 1987). However, neither of these probes has provided direct evidence that mitochondrial contact sites contain import activity.

In this study, we have used purified artificial mitochondrial precursor proteins to generate two translocation intermediates which are trapped at different points along the import pathway. These proteins, which contain the first 22 residues of the cytochrome oxidase subunit IV precursor fused to the cytosolic enzyme, mouse dihydrofolate reductase (COXIV-DHFR)[1] (Hurt et al., 1984), can be purified in relatively large amounts, and are particularly useful tools for identifying mitochondrial membrane components involved in protein import. The first precursor protein studied was a chimeric protein composed of bovine pancreatic trypsin inhibitor (BPTI) coupled to the COOH terminus of a COXIV-

1. *Abbreviations used in this paper*: BPTI, bovine pancreatic trypsin inhibitor; COXIV-DHFR, cytochrome oxidase subunit IV fused to mouse dihydrofolate reductase.

DHFR derivative. If this chimeric precursor is presented to energized mitochondria in the presence of ATP, a translocation intermediate is generated that is irreversibly trapped at a late step of the import pathway: its DHFR moiety is located in the matrix, its amino-terminal presequence is cleaved off by the matrix protease, and its carboxy-terminal BPTI moiety is still exposed on the mitochondrial surface (Vestweber and Schatz, 1988*b*). Thus, the intermediate must span both mitochondrial membranes, which implies that it is stuck across membrane contact sites. Moreover, because this translocation intermediate inhibits import of several authentic mitochondrial precursors, it appears to be irreversibly trapped at sites through which proteins are imported into mitochondria.

The second import intermediate used for these studies was also produced using the purified COXIV-DHFR fusion protein. However, unlike the chimeric contact site marker described above, this intermediate is reversibly trapped along the import pathway. Previously, Eilers et al. (1988) reported that binding of the COXIV-DHFR precursor protein to isolated mitochondria is stimulated by a membrane potential, but does not require ATP. Thus, if the precursor is added to respiring mitochondria depleted of ATP, it becomes bound to the mitochondrial surface, and retains its amino-terminal presequence. Upon addition of ATP, this bound precursor can be chased into mitochondria, and cleaved to its "mature" form, even in the absence of a membrane potential. This "ATP-depletion intermediate" proved to be useful for assaying the translocation activity of submitochondrial fractions.

These two import intermediates allowed us to tag and isolate the mitochondrial membrane regions with import activity. In confirmation of previous studies (Schleyer and Neupert, 1985; Schwaiger et al., 1987), we find that partly translocated precursors are enriched at sites of contact between the two mitochondrial membranes. In addition, we find that the submitochondrial membrane fraction that cofractionates with the contact and import site marker (*a*) is composed of attached inner and outer membrane vesicles, (*b*) remains productively associated with intermediates in the import pathway, (*c*) retains the capacity to transport proteins across membranes, (*d*) is associated with cytoplasmic ribosomes, and (*e*) is enriched in specific mitochondrial proteins.

Materials and Methods

Isolation of Mitochondria and Generation of Intermediates in the Import Pathway

Mitochondria were isolated (Daum et al., 1982) from the wild-type *Saccharomyces cerevisiae* strain D273-10B (ATC 25657, American Type Culture Collection, Rockville, MD; MAT α). To produce EDTA-washed mitochondria, spheroplasts were homogenized in breaking buffer (0.6 M sorbitol, 20 mM Hepes-KOH, pH 7.4) containing 10 mM EDTA. EDTA-free buffers were used for all other steps in the mitochondrial preparation. $MgCl_2$-treated mitochondria were prepared using buffers containing 2 mM $MgCl_2$ throughout.

Import intermediates were produced using two different polypeptides, both of which are derived from the COXIV-DHFR fusion protein. The ATP depletion intermediate was generated with ^{35}S-labeled fusion protein purified from an *Escherichia coli* strain harboring the expression plasmid pKK223-pCOXIV-DHFR (Eilers and Schatz, 1986). The chimeric translocation intermediate was generated from the ^{35}S-labeled purified DV12-COXIV-DHFR derivative (Vestweber and Schatz, 1988*a*), which was covalently coupled via its COOH-terminal cysteine residue to BPTI (Vestweber and Schatz, 1988*b*).

Generation or manipulation of these intermediates was carried out in import buffer (0.6 M sorbitol, 20 mM Hepes-KOH pH 7.4, 40 mM KCl, 25 mM KPi, pH 7.4, 10 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 1 mg/ml fatty acid–free BSA). For reactions driven by a membrane potential in the absence of ATP, samples were incubated in import buffer in the presence of succinate and malate (10 mM each), 12.5 U/ml potato apyrase (Pfanner and Neupert, 1986), and 2.5 μg/ml efrapeptin under conditions that allowed for sufficient aeration of the mitochondrial suspension. For ATP-driven reactions, samples were incubated in import buffer containing ATP and an ATP-regenerating system (1 mM ATP, 9 mM creatine phosphate, 28 U/ml creatine kinase).

Submitochondrial Fractionation

Submitochondrial membrane vesicles were produced and fractionated using a modification of the procedure of Riezman et al. (1983). Mitochondria were resuspended to 10 mg/ml in breaking buffer and swollen by incubation for 30 min at 0°C in 9 vol of 20 mM Hepes-KOH (pH 7.4) containing 1 mM PMSF and 0.5 mM EDTA. Mitochondria were condensed by addition of sucrose to 0.45 M and incubation for 10 min at 0°C. Samples were then sonicated in a cell disruptor (Heat Systems-Ultrasonic, Inc.) equipped with a microtip for 90 s at 0°C at 80% duty cycle and maximum power. Residual intact mitochondria and large fragments were removed by centrifugation at 32,000 *g* for 20 min at 4°C, and submitochondrial membrane vesicles in the supernatant were collected by centrifugation at 200,000 *g* for 45 min, at 4°C. 0.2-ml aliquots of the vesicles (15 mg; equivalent to 100 mg of starting mitochondria) were layered onto linear sucrose gradients (4 ml, 0.85-1.6 M sucrose in 10 mM KCl and 5 mM Hepes-KOH, pH 7.4) and centrifuged at 100,000 *g* for 16 h at 4°C.

Electron Microscopy

To stain for the inner membrane marker protein cytochrome oxidase (Seligman et al., 1968), submitochondrial membrane vesicles were fixed in 2% highly purified glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) in 0.1 M sodium dimethylarsenate (pH 7.4) for 1 h at 4°C. Samples were then washed repeatedly by centrifugation and resuspension in 0.1 M sodium dimethylarsenate, pH 7.4, over a period of 24 h and incubated with 1 mg/ml reduced cytochrome *c*, 0.2% (wt/vol) 3,3'-diaminobenzidine, 50 mM KPi, pH 7.4 at 37°C for 1 h with occasional agitation. The mixture was then chilled to 0°C, and washed by centrifugation and resuspension once with 50 mM KPi, pH 7.4, and once with 0.1 M dimethylarsenate, pH 7.4. Samples were then incubated in 1% OsO_4 for 1.5 h at 4°C, washed with 0.1 M dimethylarsenate, pH 7.4, immobilized in agar (2% wt/vol), de-

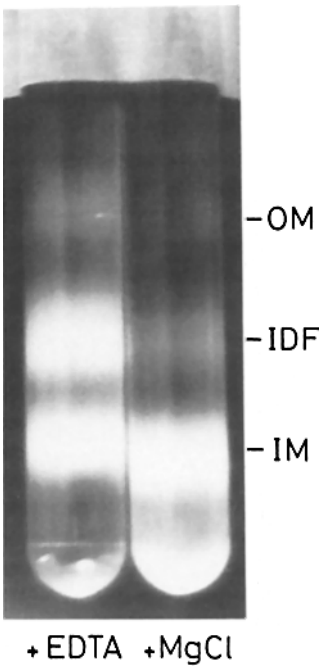

Figure 1. The effect of $MgCl_2$ on preparation of submitochondrial membrane vesicles. Submitochondrial membrane vesicles were prepared from isolated yeast mitochondria by sonication and separated by centrifugation through sucrose density gradients as described in Materials and Methods. (*Left*) Vesicles prepared from EDTA-treated mitochondria in $MgCl_2$-free buffers; (*right*) vesicles prepared from untreated mitochondria in buffers containing 2 mM $MgCl_2$. *OM*, *IDF*, and *IM*, outer membrane, intermediate-density fraction and inner membrane, respectively.

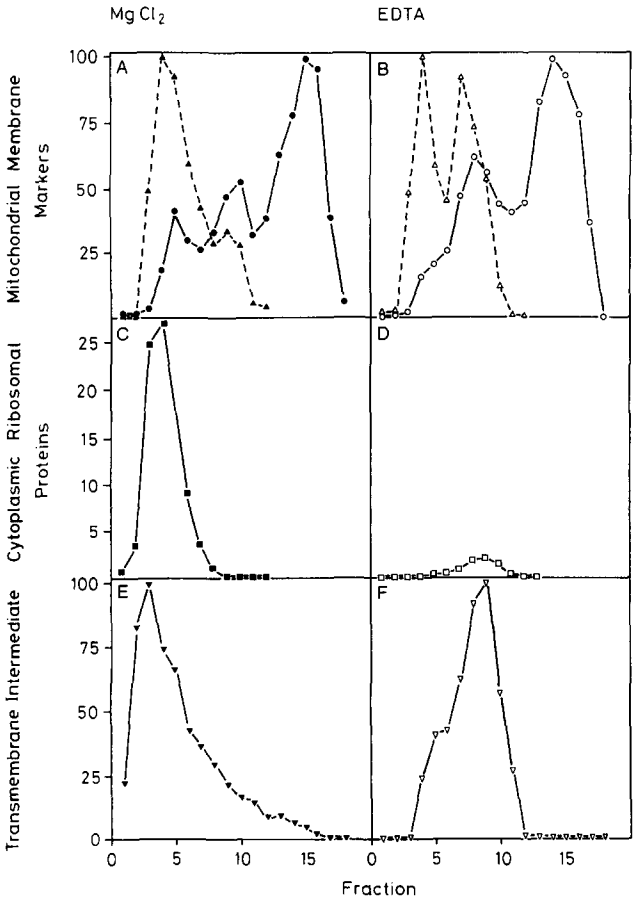

Figure 2. Distribution of inner and outer membrane marker proteins (*A* and *B*), of cytoplasmic ribosomes (*C* and *D*), and of a contact site labeling translocation intermediate (*E* and *F*) in submitochondrial membrane vesicles produced in the presence of EDTA or $MgCl_2$. Mitochondria were prepared either in the presence of 10 mM EDTA or 2 mM $MgCl_2$ and incubated with the chimeric precursor protein as described in Fig. 3. *A* and *B* show the distribution of (△) cytochrome oxidase subunit II (Poyton and Schatz, 1975) and the outer membrane porin (○) from $MgCl_2$-treated (*A*; *closed symbols*) and EDTA-treated (*B*; *open symbols*) samples as determined by immunoblotting, decoration with specific antisera and scanning densitometry. For *C* and *D*, sucrose gradient fractions from $MgCl_2$-treated (*C*) and EDTA-treated (*D*) samples were analyzed by immunoblotting with antisera raised against cytoplasmic ribosomes. The distribution of the transmembraneous, cleaved translocation intermediate (□) was determined by SDS PAGE and fluorography, and quantified by scanning densitometry for $MgCl_2$-treated (*E*) and EDTA-treated (*F*) samples. Ordinates are given in arbitrary units.

hydrated in an ethanol series, and embedded in white resin (London Resin Company, Limited) according to the manufacturer's instructions. Embedded samples were sectioned with a Reichert microtome equipped with a diamond knife. Sections were contrasted for 5 min in a 3:1 mixture of $KMnO_4$/uranyl acetate, jet-washed with water, incubated with lead acetate for 1.5 min (Millonig, 1961), jet-washed with water, and viewed in a transmission electron microscope (model EM109; Carl Zeiss, Inc., Thornwood, NY).

For immunoelectron microscopy studies, isolated yeast mitochondria were incubated in buffer S (1.2 M sorbitol buffered with 20 mM Hepes-KOH, pH 7.4) for 10 min at 4°C, and fixed by incubation in buffer S containing 0.5% glutaraldehyde and 3% paraformaldehyde for 2 h at 0°C. After several washes, samples were immobilized in agarose, dehydrated in an ethanol series at progressively lower temperatures (−20−−40°C), and embedded in Lowicryl K4M resin at −40°C according to the manufacturer's instructions. Postembedding immunogold decorations using commercially available goat anti-rabbit immunoglobulins coupled to colloidal gold (Janssen Life Sciences, Beerse, Belgium) were carried out according to the manufacturer's instructions: grids containing ultrathin sections were preadsorbed on a drop of 1% ovalbumin and 1% BSA in TBS (10 mM Tris-HCl pH 8.2, 150 mM NaCl, 20 mM NaN_3, and 0.1% BSA) for 10 min. Thereafter, samples were incubated with affinity-purified antibodies in TBS supplemented with 1% ovalbumin and 1% BSA for 1 h. After three washes with TBS containing 0.1% Tween 20 and 0.1% Triton X-100, grids were incubated with gold-coupled second antibody, as described above. Unbound material was removed by washes, as above, and sections were contrasted with 4% uranyl acetate for 10 min and lead acetate for 1.5 min.

Miscellaneous

Protein concentration was assayed by the BCA-procedure described in a

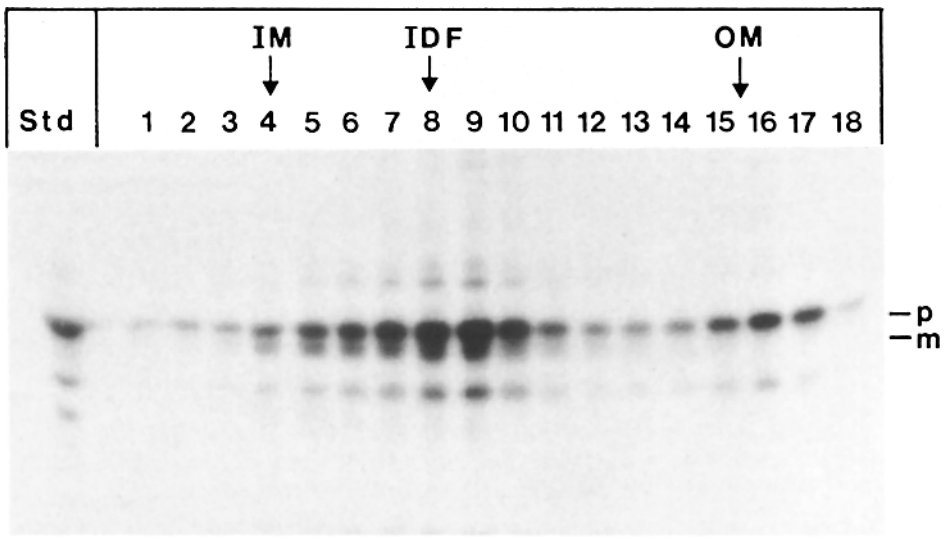

Figure 3. A membrane-spanning translocation intermediate generated from a COXIV-DHFR derivative COOH-terminally coupled to bovine trypsin inhibitor accumulates exclusively in intermediate-density submitochondrial particles. Mitochondria (6 mg) were incubated with 7.5 μg of the chimeric protein (6–7 × 10^5 dpm/μg) in the presence of an ATP-generating system for 20 min at 30°C as described in Materials and Methods. They were separated from unbound material by centrifugation, mixed with 12 mg of carrier mitochondria, and converted to submitochondrial particles. These particles were separated in a sucrose gradient and each gradient fraction was analyzed for radiolabeled uncleaved (*p*) and cleaved (*m*) chimeric protein by SDS-PAGE and fluorography. The peaks of inner membrane (*IM*), intermediate-density fraction (*IDF*), and outer membrane fraction (*OM*) (see Fig. 2) are indicated on the top. Std., 24 ng of radiolabeled chimeric protein.

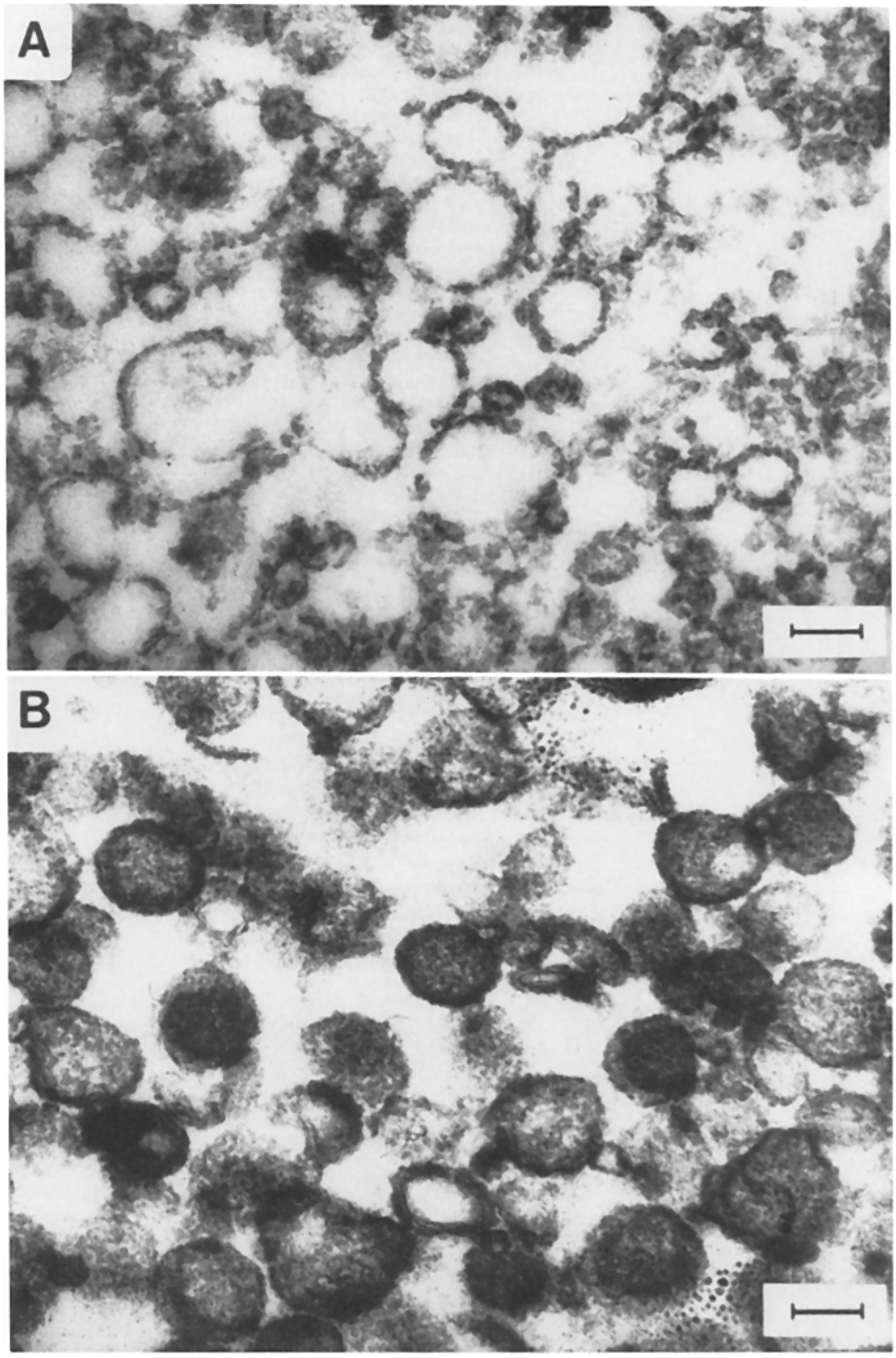

Figure 4. The intermediate-density fraction is composed of attached inner and outer membrane vesicles. Submitochondrial membrane vesicles were stained for cytochrome *c* oxidase using 3,3′-diaminobenzidine and examined by transmission EM. *A*, *B*, and *C*: outer, inner, and intermediate density membrane vesicles, respectively. *D*, vesicles from the intermediate density fraction at higher magnification. Bars, 100 nm.

company brochure by Pierce Chemical Co. (Rockford, IL). SDS-PAGE and immune blotting were described by Haid and Suissa (1983). Radiolabeled bands on dried polyacrylamide gels were visualized by fluorometry (Chamberlain, 1979) and quantified by scanning of the exposed x-ray films with a CAMAG TLC scanner coupled to an integrator. Antisera against mitochondrial proteins were prepared according to Knudsen (1985). Briefly, proteins were separated by preparative SDS-PAGE, transferred to nitrocellulose and visualized using Ponceau S stain. Bands containing ~0.05–0.1 mg of protein were excised, dried, solubilized in DMSO and used for antibody production. Antisera raised against cytoplasmic ribosomes were prepared according to Daum et al. (1982). Subcellular fractionation and isolation of ribosomes were carried out as described by Hurt et al. (1984) and Suissa and Schatz (1982).

Results

Preparation of a Submitochondrial Membrane Vesicle Fraction that Contains Marker Proteins from the Inner and Outer Mitochondrial Membranes and Is Associated with Cytoplasmic Ribosomes

Our aim was to use different import intermediates to identify and to characterize a submitochondrial membrane fraction with import activity. Subfractionation of mitochondria was strongly influenced by $MgCl_2$. When isolated yeast mito-

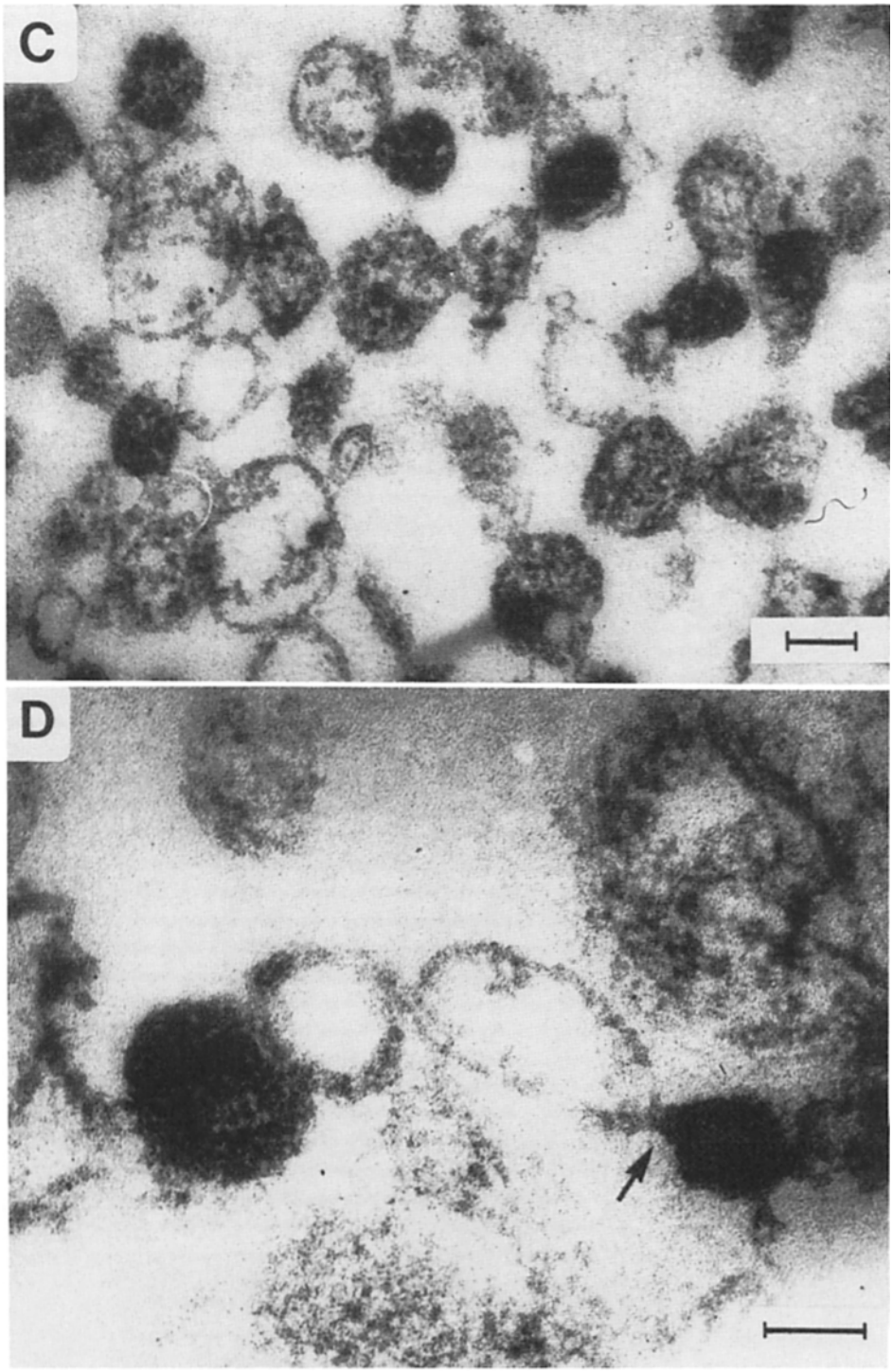

Figure 4.

chondria were osmotically shocked, further disrupted by sonication and the resulting vesicles were separated by centrifugation through a sucrose density gradient, submitochondrial vesicles produced in the presence of $MgCl_2$ separated into two bands, whereas those produced from EDTA-washed mitochondria in $MgCl_2$-free buffers separated into three bands (Fig. 1).

When $MgCl_2$ was present throughout the preparation, the densest vesicle band contained an inner membrane marker protein, low, but detectable levels of an outer membrane marker protein, as well as proteins found in cytoplasmic ribosomes. In contrast, the less dense band was ribosome free and contained an outer membrane marker protein (Fig. 2, *A* and *C*). When the mitochondria were washed with EDTA and subfractionated in the absence of $MgCl_2$, the dense band was enriched in inner membrane markers, the light band was enriched in an outer membrane marker, and the intermediate-density band contained both inner and outer membrane markers (fractions 8–11, Fig. 2 *B*). Moreover, both the relative amounts and localization of cytoplasmic

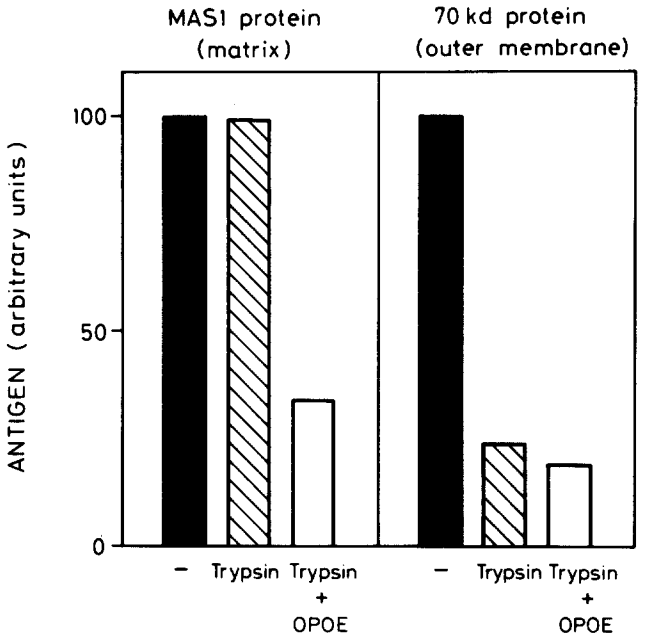

Figure 5. The intermediate-density fraction is composed of right-side-out, sealed inner membrane vesicles and leaky outer membrane fragments. Intermediate-density vesicles were incubated with 50 μg/ml trypsin for 10 min at 0°C in the presence or absence of 0.5% (vol/vol) octylpolyoxyethylene (OPOE). Trypsin was inactivated by addition of a 10-fold excess of soybean trypsin inhibitor and 1 mM PMSF and all samples were analyzed by immunoblotting using antisera against the *MAS1*-encoded subunit of the matrix protease and the 70-kD outer membrane protein. Antigens were quantified by densitometric scanning and are given in arbitrary units.

ribosomes were affected by preparation in the presence or absence of $MgCl_2$: EDTA treatment resulted in a loss of membrane-associated cytoplasmic ribosomes from the densest membrane band; those that were still associated with the submitochondrial particles cofractionated with the intermediate-density fraction (Fig. 2, *C* and *D*). This suggests the existence of a mitochondrial membrane fraction that contains both inner and outer membrane markers, associates with cytoplasmic ribosomes, and becomes lighter if attached cytoplasmic ribosomes are removed.

A Transmembraneous Intermediate Arrested at a Late Step of Translocation Cofractionates with the Intermediate-density Vesicles and with Mitochondrial-associated Cytoplasmic Ribosomes

Kellems et al. (1975) had observed that cytoplasmic ribosomes are associated with mitochondria at membrane contact sites. Our findings that the intermediate-density fraction contains proteins from both mitochondrial membranes as well as associated cytoplasmic ribosomes are consistent with the possibility that this membrane fraction may be derived from mitochondrial membrane contact sites. To determine the submitochondrial localization of this membrane fraction, we used a translocation intermediate which labels contact sites as well as import sites. This intermediate was produced using a COXIV-DHFR derivative covalently coupled to BPTI (Vestweber and Schatz, 1988*b*). When EDTA-treated mitochondria that had accumulated this intermediate were subfractionated in the absence of $MgCl_2$, both the cleaved and uncleaved forms of the chimeric protein were recovered with the submitochondrial particles. The uncleaved forms of both the BPTI-coupled fusion protein and of residual free (uncoupled) COXIV-DHFR were detected in both outer membranes and in intermediate-density vesicles. In contrast, the cleaved form (which must span both mitochondrial membranes) was detected almost exclusively in the intermediate-density fraction (Fig. 3).

The buoyant density of this contact site marker was influenced by the presence of $MgCl_2$ (Fig. 2, *E* and *F*): in EDTA-treated samples, this marker was found in the intermediate-density fraction; in vesicles prepared with $MgCl_2$, it was found in the densest band. In all cases, this marker for protein import sites cofractionated with the mitochondria-bound cytoplasmic ribosomes. We conclude that mitochondria-bound cytoplasmic ribosomes are associated with the same submitochondrial fraction that also contains a partly translocated precursor protein, and that this fraction contains sites of close contact between the two mitochondrial membranes.

The Intermediate-density Fraction Contains Inner and Outer Membrane Vesicles Attached to Each Other

To determine the morphology of different submitochondrial vesicles, samples from inner, outer, and intermediate-density vesicles were stained for cytochrome *c* oxidase by 3,3′-diamino-benzidine, and visualized as thin sections by EM. Inner membrane vesicles were identified by being filled with the electron dense cytochrome *c* oxidase reaction product (Fig. 4 *B*); they were clearly distinct from outer membrane vesicles that were not appreciably stained by this reagent (Fig. 4 *A*). The intermediate-density fraction was composed of unstained and stained vesicles (Fig. 4, *C* and *D*). Identification of a membrane fraction containing associated inner and outer membranes was not particularly surprising: our interest was the nature of association of these vesicles. In contrast to reports from Ohlendieck et al. (1986), we did not detect any vesicles encapsulated within other vesicles, but inner and outer membrane vesicles attached side-by-side. Although the frequency of attached vesicles within a given section varied with the orientation of the sample relative to the plane of sectioning, the morphology of the attachment site between some vesicles (*arrow* in Fig. 4 *D*) suggested that the vesicles were in fact attached to each other rather than simply in close proximity.

Orientation and Integrity of the Membranes in the Intermediate Density Vesicles

We determined the sidedness and integrity of the inner and outer membrane in the intermediate-density vesicles by checking the accessibility of several marker proteins to externally added trypsin (Fig. 5). The markers were the *MAS1*-encoded subunit of the matrix protease (Yaffe and Schatz, 1984; Witte et al., 1988; Yang et al., 1988), and the 70-kD protein which protrudes from the outer face of the outer membrane (Reizman et al., 1983). The matrix marker was trypsin inaccessible in the absence of detergent, and accessible in the presence of detergent, suggesting that it is encapsulated within intact inner membrane vesicles (Fig. 5 *A*). In studies analogous to those reported previously (Hwang et al., 1989), we

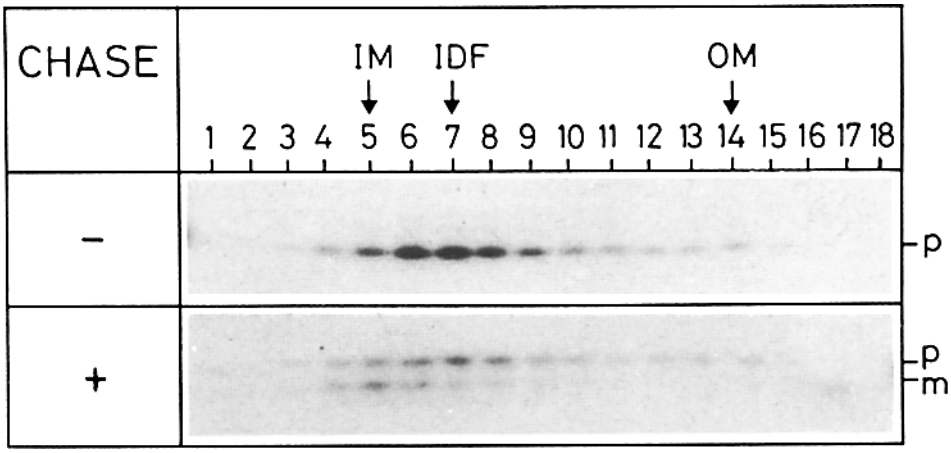

Figure 6. The ATP-depletion intermediate specifically accumulates in an intermediate-density submitochondrial membrane fraction. The intermediate was produced by incubating 12 mg of mitochondria in import buffer in the absence of ATP (see Materials and Methods), but in the presence of respiratory substrates and with purified, radiolabeled COXIV-DHFR precursor protein (0.018 mg; 3×10^8 cpm/mg) at 30°C for 10 min. After separation of mitochondria from unbound precursor by centrifugation, half of the sample was chased by incubation in import buffer containing ATP and an ATP-generating system at 30°C for 10 min. Samples containing nonchased or chased COXIV-DHFR protein were then mixed with 12 mg of carrier mitochondria and converted to submitochondrial particles. These were separated by centrifugation on sucrose density gradients. The distribution of the precursor (*p*) and mature (*m*) forms of the COXIV-DHFR fusion protein relative to the peaks of mitochondrial outer membrane (*OM*), inner membrane (*IM*), and intermediate-density fraction (*IDF*) (determined as described in Fig. 2 and 3) are shown for samples produced from mitochondria containing the bound precursor protein (*top*) and from mitochondria whose bound precursor had been chased by incubation with ATP before vesicle preparation (*bottom*).

established a membrane potential in the isolated intermediate-density vesicles by incubating them with respiratory substrates (succinate or ascorbate) in the presence of cytochrome *c*. Because generation of a potential was absolutely dependent upon addition of cytochrome *c*, an intermembrane space protein (not shown), the inner membrane component of the intermediate density fraction must be right-side-out. In contrast, 77% of the 70-kD outer membrane marker was trypsin-sensitive under all conditions (Fig. 5 *B*). Thus, three-fourths of the outer membranes were either right-side-out, or inside-out and leaky. As this vesicle fraction appears to contain inner and outer membranes attached side-to-side (Fig. 4), we favor (but have no direct evidence for) the possibility that the vesicles contain rightside-out, sealed inner membranes attached to inside-out, outer membranes that are mostly leaky. The data reported below support this view.

The ATP-depletion Intermediate Cofractionates with the Intermediate-density Membrane Fraction

In previous studies from this laboratory, Eilers et al. (1988) observed that ATP-depleted mitochondria bound the COXIV-DHFR fusion protein at their surface; a potential across the inner membrane increased binding fourfold. Upon addition of ATP, 73% of the precursor that had bound to energized mitochondria was translocated across both membranes and converted to mature form by the matrix-located processing protease. Because this chase occurred even with uncoupled mitochondria, the ATP-depletion intermediate is a true import intermediate. To characterize further the intermediate-density fraction, we generated submitochondrial membrane vesicles from mitochondria that had accumulated the ATP depletion intermediate. This intermediate cofractionated almost exclusively with the intermediate-density vesicle fraction (Fig. 6, *top*; Fig. 7 *B*).

To confirm that the precursor found in the intermediate-density fraction was derived from the ATP-depletion intermediate which had been productively bound to mitochondria, mitochondria containing the intermediate were chased with ATP before being converted to submitochondrial particles. This chase, which releases the intermediate from its surface-bound state and transports it into the mitochondrial matrix in whole mitochondria, decreased the amount of precursor in the intermediate density fraction by 60% (Fig. 6, *bottom*; Fig. 7 *B*). The low amount of mature COXIV-DHFR retained by the vesicles cofractionated with matrix- and inner membrane markers (Fig. 7 *A*) and presumably represented imported, cleaved protein that had been encapsulated along with some matrix components within inner membrane vesicles.

Isolated Intermediate-density Vesicles Internalize Bound ATP-depletion Intermediate upon Addition of ATP

When intermediate-density vesicles were isolated from mitochondria that had bound the radioactive ATP-depletion intermediate, ~70% of the intermediate was recovered with the vesicles accessible to externally added trypsin (Fig. 8 and Table I). This agreed with the observation that, in these vesicles, 77% of the 70-kD mitochondrial surface protein was trypsin accessible. The ATP-depletion intermediate associated with the intermediate-density vesicles was thus still outside the inner membrane. The trypsin-inaccessible fraction of the intermediate, like that of the 70-kD outer membrane protein, was probably encapsulated in inside-out, closed outer membrane vesicles.

When the intermediate density vesicles were incubated with ATP, ~40% of their bound, trypsin-accessible ATP-depletion intermediate was chased into a trypsin-resistant compartment (Table I). The protease-protected COXIV-DHFR protein retained its presequence. Our interpretation of this observation is that protease protection resulted from ATP-dependent translocation of the intermediate across an

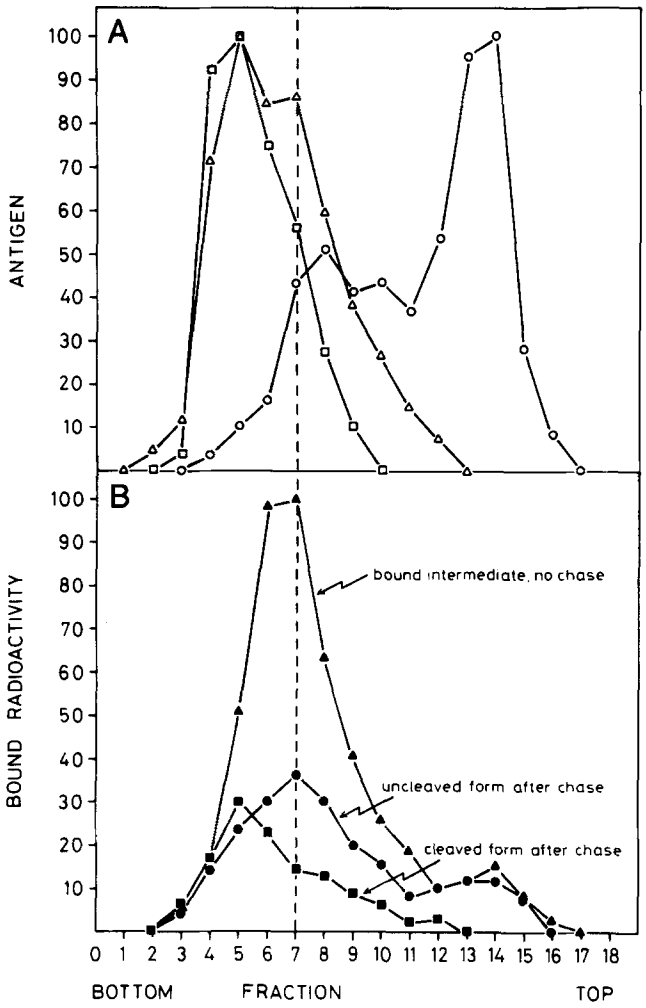

Figure 7. Quantitation of the distribution of membrane marker proteins and of bound and chased ATP depletion intermediate in submitochondrial membrane vesicles. Mitochondria that had bound the ATP-depletion intermediate were incubated without additions, or chased by addition of ATP before vesicle preparation and separation of vesicles by sucrose density gradients as described in Fig. 6. Sucrose gradients were separated into 17 fractions and each fraction was analyzed for marker proteins cytochrome oxidase subunit IV of the inner membrane (△), citrate synthase of the mitochondrial matrix (□), and the 70-kD outer membrane protein (○) as described in Fig. 2 (Fig. 7 *A*). The distribution of the ATP-depletion intermediate (▲), and of residual precursor (●) and mature (■) forms of the COXIV-DHFR fusion protein recovered from mitochondria that had been treated with ATP before vesicle preparation were determined as described in Fig. 3 (Fig. 7 *B*).

intact vesicle membrane. Translocation of the uncleaved precursor form suggests that vesicles did not catalyze the proteolytic removal of the presequence, most probably because the matrix protease had been inactivated. The intermediate density vesicles are thus deficient in presequence processing activity, but contain an active machinery for translocation of prebound ATP-depletion intermediate.

Using this system, we also observed that ATP induced release of the bound import intermediate from the vesicle membrane. When vesicles containing bound intermediate were incubated with ATP and then permeabilized (but not solubilized) with low levels of a nonionic detergent (Vestweber and Schatz, 1988*a*) ~30% of the prebound intermediate was released into the soluble phase. No such release was seen if incubation was in the absence of ATP, or in the presence of a nonhydrolyzable ATP analogue (Fig. 9). This result, coupled with our previous finding, suggests that ATP causes prebound ATP-depletion intermediate to be translocated across the vesicle membrane, and to be released from the vesicle membrane.

The Intermediate Density Fraction Is Enriched in Specific Mitochondrial Proteins

We were interested in identifying components specific to the intermediate-density membrane fraction. Comparison of the proteins present in the inner membrane, outer membrane, and intermediate-density fraction revealed a number of proteins which were present in all three fractions, but enriched in the intermediate-density fraction (Fig. 10). For the studies described below, we focused primarily on two bands, which we refer to as contact site proteins 1 and 2 (CSP-1 and CSP-2). The apparent molecular masses of these proteins are 100 and 64 kD, respectively. Polyclonal antibodies were made against each of these proteins. As shown in Fig. 11 *A*, antisera against each contact site protein recognized a single protein band that was similar in apparent molecular weight to the original antigen. Using these antibodies, we also observed that the distribution of each of the putative contact site proteins in sucrose gradient fractions of submitochondrial

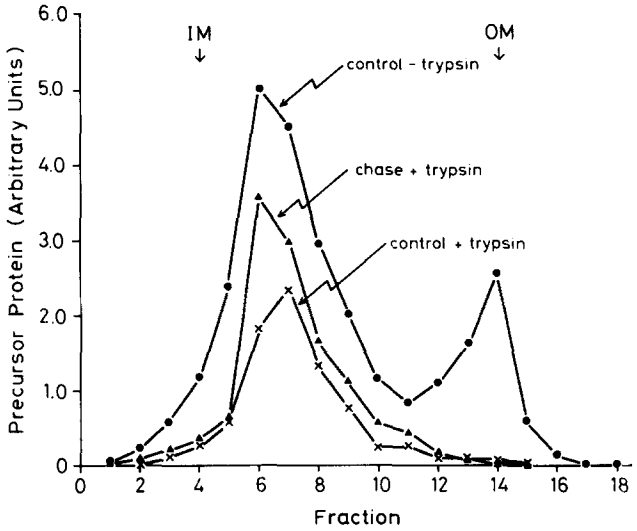

Figure 8. The intermediate-density fraction has translocation activity. Submitochondrial vesicles containing bound ATP-depletion intermediate were prepared and fractionated on a sucrose gradient. Each of the 18 vesicle fractions collected from the gradient were incubated in the absence or presence of an ATP-generating system at 15°C for 45 min and then treated with 50 μg trypsin/ml at 0°C for 10 min. Inactivation of trypsin, separation of proteins by SDS-PAGE, and analysis and quantification of the amount of radiolabeled protein were all carried out as described in Fig. 5. The amount of precursor protein in each fraction was quantified by scanning densitometry: (●) total precursor in unchased control vesicles; (x) trypsin-inaccessible precursor in unchased control vesicles; and (▲) trypsin-inaccessible precursor in vesicles chased with ATP. The peaks of inner membranes (*IM*) and outer membranes (*OM*) in the sucrose gradient are indicated on the top.

Table I. Treatment of Vesicles

Treatment of vesicles	Amount of radiolabeled protein associated with vesicles	
		%
No chase		
Untreated	2.0	100
+ Trypsin	0.61	30
+ Trypsin + OPOE	0.27	13
Chase with ATP		
Untreated	1.9	100
+ Trypsin	1.1	58
+ Trypsin + OPOE	0.24	13

The ATP-depletion intermediate associated with intermediate-density vesicles is partly rendered trypsin inaccessible by incubation with ATP. Mitochondria containing bound ATP-depletion intermediate were fractionated into inner membranes, outer membranes, and intermediate-density vesicles. The intermediate-density vesicles were incubated in the absence or presence of ATP as in Fig. 5 and analyzed directly, or first incubated with trypsin in the absence or presence of octylpolyoxyethylene (OPOE) as in Fig. 5. Values are expressed as arbitrary units derived from densitometric scans. To estimate chase efficiencies, the amount of radiolabeled proteins in untreated controls was defined as 100%.

membrane vesicles was quite different from that of either inner or outer membrane marker protein: CSP-1 and CSP-2 were present in the inner membrane fraction, detectable in low levels in the outer membrane fraction, and enriched in the intermediate density fraction (Fig. 11 *B*). Quantitation of the enrichment of each of these proteins and of inner and outer membrane marker proteins is shown in Table II.

The vesicle populations originally used to identify and characterize proteins enriched in the intermediate-density fraction were produced from EDTA-treated mitochondria and were therefore depleted of cytoplasmic ribosomes. Fig. 11 shows that CSP-1 and CSP-2 are mitochondrial proteins rather than proteins of attached cytoplasmic ribosomes: antisera monospecific for these proteins detected the proteins in mitochondria, but not in a ribosomal fraction (Fig. 12). CSP-1 and CSP-2 are then mitochondrial proteins that are enriched in the intermediate-density fraction.

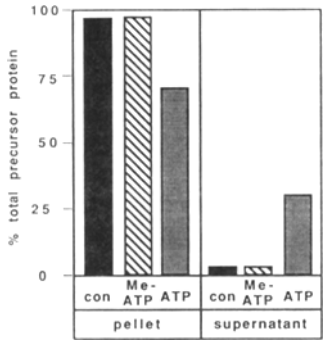

Figure 9. ATP-dependent translocation of the prebound ATP-depletion intermediate causes release of the intermediate from the mitochondrial membrane. Submitochondrial membrane vesicles containing prebound ATP-depletion intermediate were prepared as described in Fig. 6. The intermediate density fraction was divided into three aliquots which were incubated for 1 h at room temperature without additions (*con*), with 0.1 mM ATP and an ATP-generating system (*ATP*), or with 0.1 mM beta, gamma-methylene-ATP (*MeATP*). Samples were then treated with 0.5% (vol/vol) octyl polyoxyethylene, and separated into a membrane pellet (*p*) and a soluble fraction (*s*) by centrifugation in an airfuge (Beckman Instruments, Fullerton, CA) for 30 min at 30 psi at 4°C. The amount of precursor was determined as in Fig. 3.

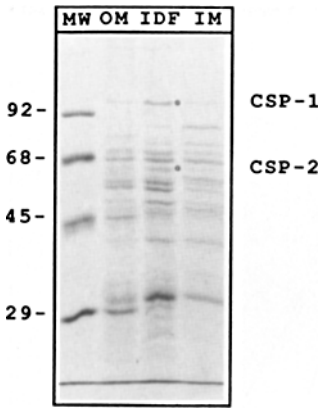

Figure 10. Analysis of the protein composition of submitochondrial membrane vesicles by SDS-PAGE. Equal amounts of protein from outer membrane (*OM*), intermediate-density fraction (*IDF*), and inner membrane (*IM*) were separated by SDS-8% PAGE and visualized using Coomassie blue stain. Numbers on the left are sizes (in kilodaltons) of the marker proteins (*MW*). Marks beside the sample from the intermediate density fraction identify proteins which appeared to be enriched in that fraction. The enriched bands labeled "CSP-1" and "CSP-2" were selected for further studies.

Localization of CSP-1 and CSP-2 by Immune Electron Microscopy

The biochemical evidence suggests that we have identified mitochondrial proteins that are present in all three submitochondrial membrane fractions but are enriched in the intermediate-density fraction. Morphological studies using EM further support this view.

To enhance the visualization of the different mitochondrial compartments, isolated mitochondria were incubated in hypertonic medium before fixation and embedding. This treatment condensed the matrix, separated the inner and outer membranes, and differentiated two distinct regions of the mitochondrial surface: "free" outer membrane (areas where the boundary membrane was visualized as a single membrane), and areas where the inner membrane and highly condensed matrix appeared to be in contact with the outer membrane (contact sites). Approximately 25% of the outer membrane was present in such contact sites (Table III).

In these "shrunken" mitochondria, antisera against the outer membrane marker porin (Fig. 13 *A*, *top*, and Table IV) decorated the mitochondrial surface. Conversely, antisera against holocytochrome oxidase (Fig. 13 *A*, *bottom*, and Table IV) labeled the inner membrane, at the interface between the electron dense matrix and the electron translucent intermembrane space. In both cases, membranes were uniformly labeled: within the limits of the error, the percentage of each marker at contact sites reflected the relative contribution of each membrane to the contact site (Tables III and IV). These two markers are then neither enriched in, nor excluded from the contact site.

With respect to the putative contact site proteins, preimmune sera gave negligible labeling, and antisera against CSP-1 and CSP-2 gave significant labeling of mitochondria (Fig. 13, *B* and *C*). Moreover, the distribution of these proteins appeared to be distinct from that of either porin or cytochrome oxidase since contact sites contained as many gold particles as the "free" inner membrane (Table IV). Because only 9% of the inner membrane was present in contact sites (Table III), the density of both CSP-1 and CSP-2 in the contact site must be an order of magnitude higher than in the inner membrane. These findings agree with the biochemical evidence described above.

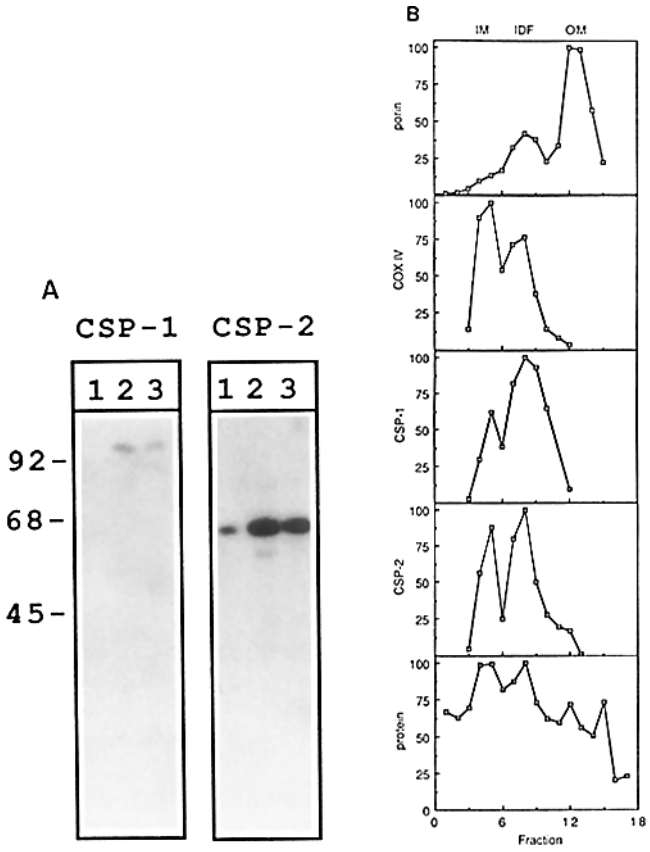

Figure 11. Polyclonal antisera raised against CSP-1 or CSP-2 recognize proteins that are enriched in the intermediate density fraction. For *A*, equal amounts of protein from the outer membrane (lane *1*), intermediate-density fraction (lane *2*), and inner membrane (lane *3*) were analyzed by immune blotting with polyclonal antisera raised against CSP-1 and CSP-2, and antibody-antigen complexes were detected using ^{125}I-protein A and fluorography. Apparent molecular weights of marker proteins are shown on the left. For *B*, the distribution of outer and inner membrane markers, porin, and COX IV, and of the contact site proteins in sucrose density gradient fractions of submitochondrial membrane vesicles were analyzed by immunoblotting and quantified by densitometry. Total protein content in each of the gradient fractions was determined as described in Materials and Methods. With each ordinate, 100 is defined as the amount of a component in the gradient fraction which contains the peak of the component(s). The positions of inner membrane (*IM*), intermediate-density fraction (*IDF*), and outer membrane (*OM*) are shown above.

Table II. Two Mitochondrial Proteins Are Enriched in Contact Site Vesicles

	Specific content			
Membrane	Porin	COX IV	CSP-1	CSP-2
OM	27.6	1.9	1.9	2.4
IDF	9.8	28.4	14.0	12.7
IM	1.9	36.8	8.7	11.1

The distribution of an outer membrane protein (porin), of the inner membrane marker (cytochrome oxidase subunit IV, COXIV), and of CSP-1 and CSP-2 in sucrose density gradient fractions of submitochondrial membrane vesicles were determined and quantified as described in Fig. 11 *B*. The amount of each protein (in arbitrary units) was analyzed in each of the three peak gradient fractions. Specific content in each of these fractions is given as amount per milligram total protein.

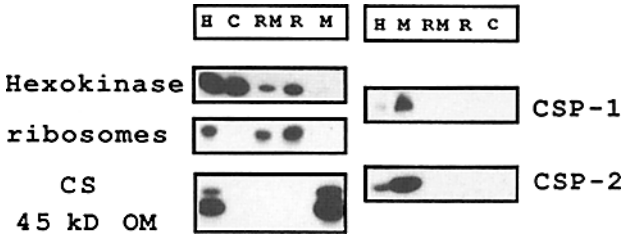

Figure 12. Subcellular localization of the two contact site proteins. Yeast spheroplasts were homogenized and subcellular fractions were separated by differential centrifugation as described previously (Hurt et al., 1984). To isolate cytoplasmic ribosomes, the rough microsomal fraction was extracted with 1% Triton X-100 and ribosomes in the particulate fraction were separated from soluble components by ultracentrifugation (150,000 *g*, for 1 h at 4°C) according to Suissa and Schatz (1982). Equal amounts of protein were separated by SDS-8% PAGE, and analyzed by immunoblots with antisera against hexokinase (a cytosolic protein), cytoplasmic ribosomal proteins (*ribosomes*), a major 45-kD protein of the outer membrane (*45 kD OM*), citrate synthase (*CS*, a matrix protein) as well as with antisera against CSP-1 and CSP-2. *H*, *C*, *RM*, *R*, and *M*, homogenate, cytosol, rough microsomes, cytoplasmic ribosomes and mitochondria, respectively.

Discussion

In this study we specifically tagged the contact sites between the two mitochondrial membranes with two different partly translocated precursor proteins; we separated these sites from the bulk of inner and outer membrane, determined the morphology of the isolated contact sites, and showed that they contain translocation activity and that they are enriched in specific proteins. These studies confirm previous findings that suggest that protein import into mitochondria (Schleyer and Neupert, 1985; Schwaiger et al., 1987) and into chloroplasts (Pain et al., 1988) occurs at membrane contact sites. Moreover, our observation, correlating ribosome binding sites with mitochondrial protein import sites, is in agreement with previous reports (Kellems et al., 1975; Ades and Butwo, 1980; Suissa and Schatz, 1982) and suggests that both nascent chains and full-length translation products are imported through the same region on the mitochondrial surface. Our finding that an import intermediate that binds to mitochondria in an energy-dependent manner cofractionates with contact sites suggests that membrane potential–dependent processes within the import pathway may occur at these membrane junctions. Finally, we provide evidence for protein translocation in a membrane fraction which contains in-

Table III. Percentage of Outer and Inner Membrane Present in Contact Sites

Membrane type	Total length of membrane	Length of membrane not in contact site	Membrane in contact site
		μm	%
Outer membrane	142	107	25
Inner membrane	384	349	9

Isolated mitochondria were incubated in hypertonic media, fixed, and embedded in Lowicryl resins using the progressive low-temperature procedure. Total lengths of outer membrane, inner membrane, and contact site were measured in 53 mitochondrial profiles using a polarimeter coupled to a microprocessor.

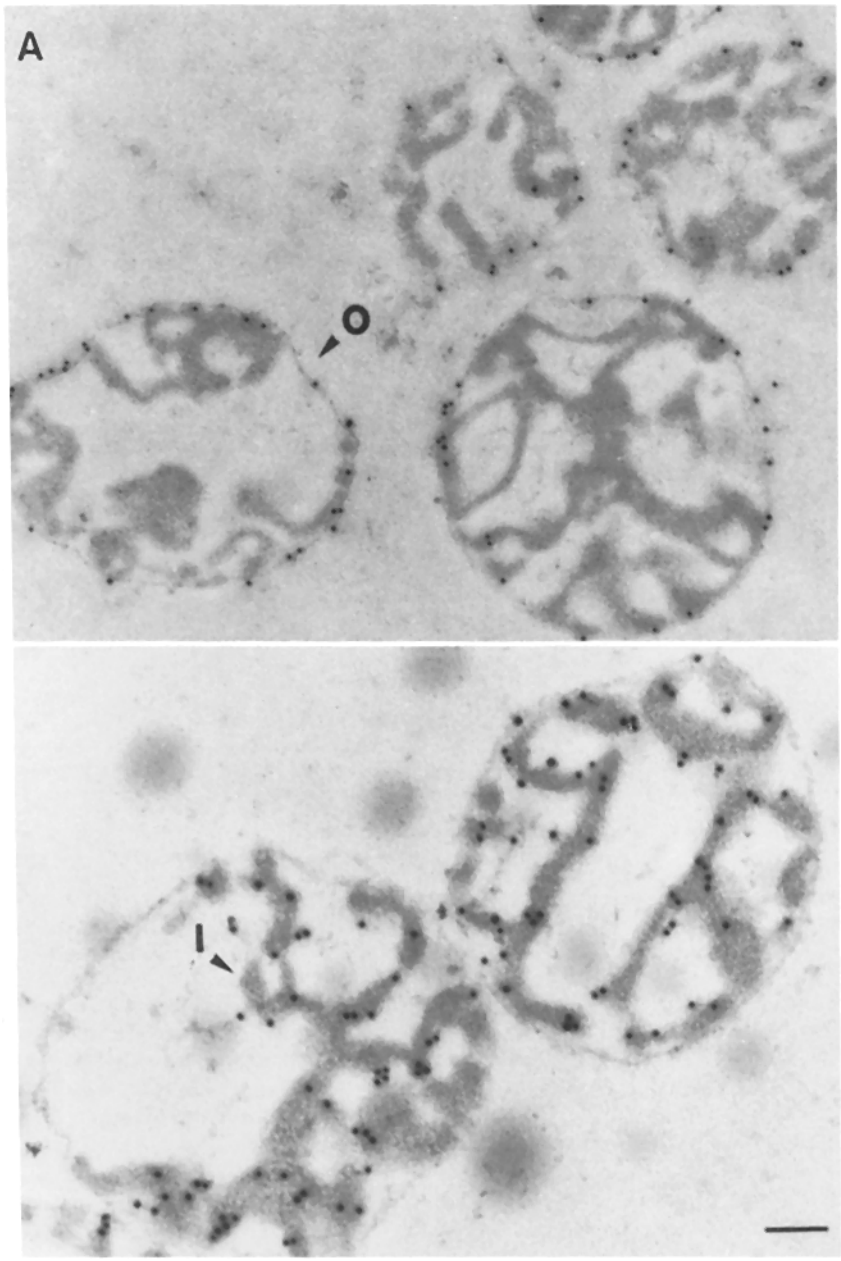

Figure 13. Submitochondrial distribution of proteins enriched in the intermediate-density fraction. Samples of mitochondria were dehydrated and embedded in Lowicryl resin using the progressive low-temperature technique described in Materials and Methods. Ultrathin sections were decorated with immunoglobulins raised against porin (*A*, *top*), holocytochrome oxidase (*A*, *bottom*), and CSP-1 and CSP-2 (*B* and *C*, *top* and *bottom*); antibody-antigen interactions were detected using a gold-coupled second antibody and electron microscopy. *O*, outer membrane; *I*, inner membrane; *CS*, membrane junctions. Bars, 100 nm in *A* and *C*, and 200 nM for *B*.

ner and outer membrane junctions and describe two putative contact site proteins.

Recently, we reported that isolated inner membrane vesicles which are essentially free of outer membrane carry out ATP- and membrane potential–dependent protein translocation (Hwang et al., 1989). Several lines of evidence suggest that the translocation activity observed in the intermediate-density vesicles is distinct from that in inner membrane vesicles. First, mitochondria, whose import sites have been "jammed" with the BPTI-coupled fusion protein become import competent if the inner membrane is rendered accessible (Hwang et al., 1989). Secondly, neither of the import intermediates that "tag" the contact site cofractionates with the translocation-active inner membrane fraction (Figs. 3, 6, and 7). Finally, import across inner membrane vesicles is mem-

Table IV. Distribution of Gold Particles in Immune Electron Microscopy Studies

Antibody against	No. of mitochondria studied	No. of particles on: Inner membrane	Outer membrane	Contact sites	Particles at contact sites
			n		%
Porin	50	25	331	186	34
COX	64	790	37	153	16
CSP-1	76	118	9	149	54
CSP-2	50	70	4	86	54

Porin (an outer membrane marker), holocytochrome oxidase (COX; an inner membrane marker), and both CSP-1 and CSP-2 were visualized in embedded thin sections with the corresponding gold-labeled immunoglobulins (Materials and Methods) and the distribution of gold particles at contact sites, at contact site–free inner membrane and contact site–free outer membrane was quantified.

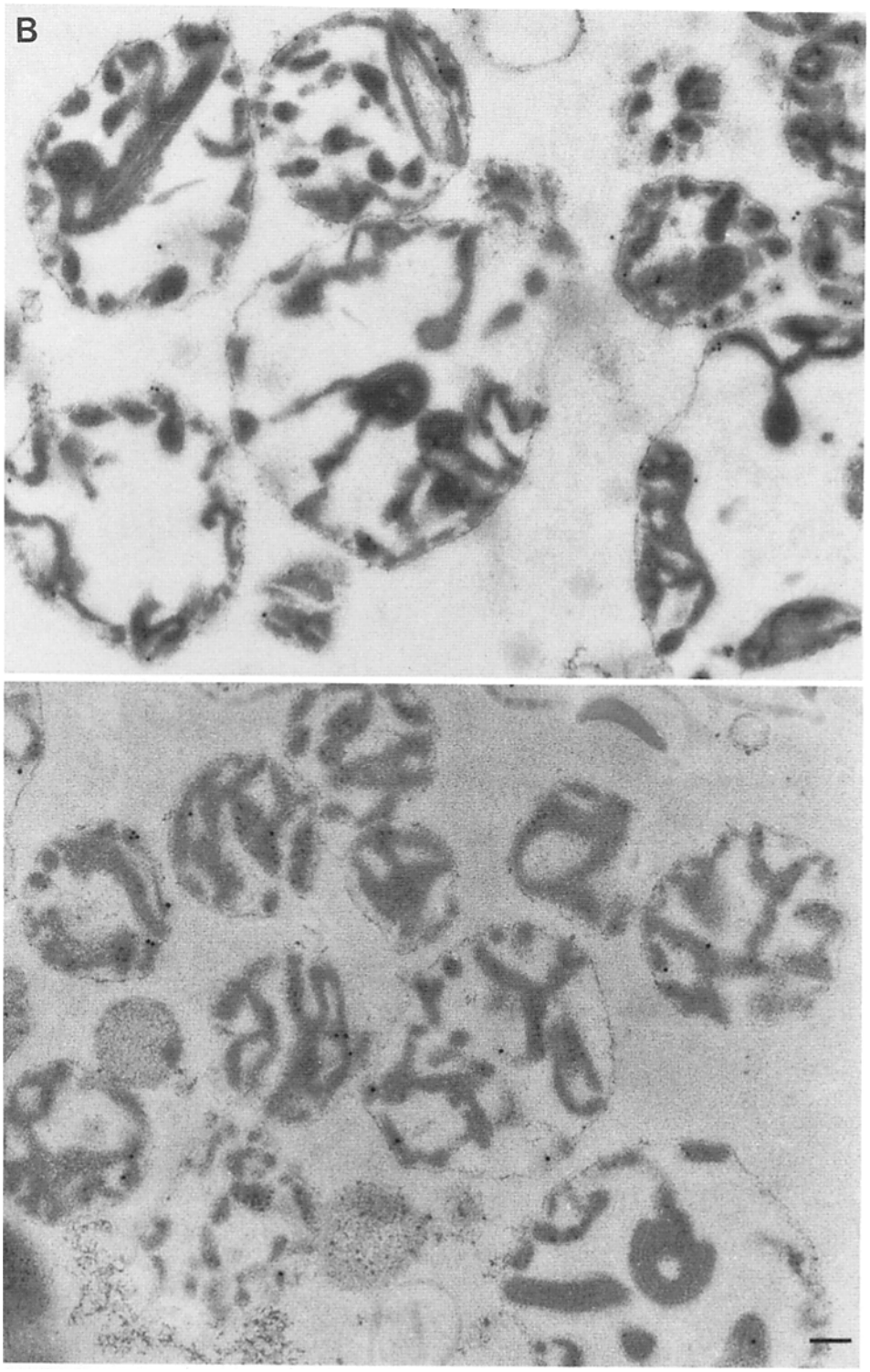

Figure 13.

brane potential dependent, whereas translocation of a prebound import intermediate in intact mitochondria and in the contact site fraction does not require a membrane potential. These findings suggest that there is translocation activity at contact sites (the physiological import site) and within the inner membrane (a nonphysiological system).

The contact site fraction appears to be deficient in matrix-localized processing protease. Because the contact site vesicles are also depleted of citrate synthase (another matrix marker) and since the vesicles are prepared in the presence of EDTA (an inhibitor of the matrix protease), this lack of processing activity was not unexpected. However, the contact site vesicles still maintain productive association to the prebound import intermediate, translocate the intermediate upon addition of ATP, release the precursor protein from the membrane, and preserve attachment of the two mitochondrial membranes.

Ohlendieck et al. (1986), who worked with rat liver mito-

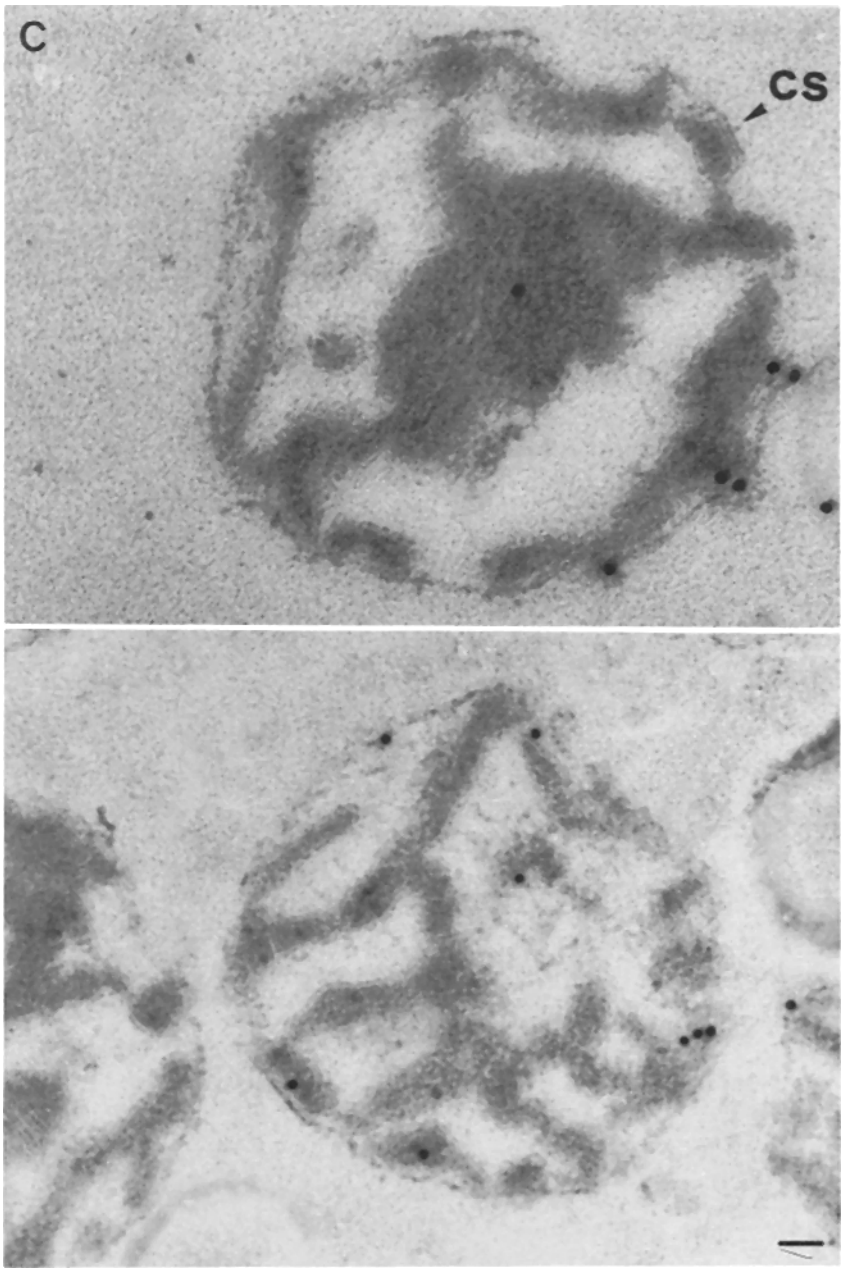

Figure 13.

chondria, suggested that many metabolic processes occur at inner and outer membrane junctions and that the formation of membrane contacts is controlled by the metabolic state of the mitochondria. Although we have identified two proteins which may be part of the contact site, the function of these proteins is unknown. Our finding that these proteins are present in the inner membrane, but enriched in the contact site is consistent with the view that contact sites are dynamic structures. The detection of "cryptic" import sites in inner membrane vesicles (Hwang et al., 1989) further support this possibility. However, this model will remain speculative until the components of the contact sites have been characterized in greater detail.

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