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Proteinaceous Receptors for the Import of Mitochondrial Precursor Proteins*

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Craig Zwizinski‡, Manfred Schleyer, and Walter Neupert

From the Institut für Physiologische Chemie, Physikalische Biochemie und Zellbiologie der Universität München, Goethestrasse 33, D-8000 München, Federal Republic of Germany

Mild trypsin treatment of isolated *Neurospora* mitochondria strongly inhibits their ability to bind and import the precursors of several mitochondrial proteins. Evidence is presented for two proteins, the ADP/ATP carrier and the mitochondrial porin, that specific binding of the precursors to the outer surface of the mitochondria is affected by the protease treatment. We suggest that the receptors that mediate the import of these two precursors are proteinaceous. Treatment of mitochondria with elastase also inhibits the binding and import of the ADP/ATP carrier and the porin. In contrast the import of the precursors of subunits 2 and 9 of the mitochondrial proton-translocating ATPase was unaffected by elastase treatment at the concentrations used. We suggest that the import pathways of the latter two proteins are distinct from those of the ADP/ATP carrier and the porin.

Most mitochondrial proteins are synthesized in the cytoplasm and must be imported into the mitochondrion (1, 2). Numerous studies have indicated that these proteins exist, at least transiently, as soluble precursor forms in the cytosol. These extramitochondrial forms often possess NH_2-terminal peptide extensions of essentially unknown function. The cytoplasmic precursors bind to mitochondria and are then imported into the various mitochondrial compartments. This pathway raises several questions about the mechanisms by which precursors recognize the mitochondrion as their proper target while ignoring the other membranous organelles of a eukaryotic cell. Are there mitochondrial receptors for the precursors? If so, is there a single receptor for all imported proteins or are there many different receptors? Finally, if such receptors exist what is their chemical nature?

We have investigated these questions using a cell-free system in which the import of proteins into mitochondria occurs. Isolated mitochondria of the filamentous fungus *Neurospora crassa* are incubated with radiolabeled precursors synthesized *in vitro*. Under appropriate conditions, a variety of *Neurospora* precursors are imported into mitochondria in reactions which closely resemble the *in vivo* processes (1–4). We have observed that when the import of proteins is blocked in this system, some precursors bind to the surface of the mitochondria. This is true for the precursor of cytochrome *c* (termed apocytochrome *c*) (5, 6), an intermembrane space protein, the ADP/ATP exchange or carrier protein of the inner membrane (7–9), and for the precursor of the porin of the outer mitochondrial membrane (10). The data for all three proteins strongly suggests that the observed binding represents a step in the import pathway of these proteins.

We report here that the binding of the precursors of the ADP/ATP carrier and the mitochondrial porin to mitochondria is mediated by proteinaceous elements on the mitochondrial surface. Further, the receptors for these two proteins can be distinguished from those mediating the import of two other mitochondrial proteins, subunits 2 (β-subunit) and 9 (proteolipid, dicyclohexylcarbodiimide-binding protein) of the mitochondrial proton-translocating ATPase.

EXPERIMENTAL PROCEDURES

Materials—Trypsin (EC 3.4.21.4, 221 units/mg, treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone) was obtained from Worthington. Chromatographically purified elastase (EC 3.4.21.11, 95 units/mg), aprotinin, and valinomycin were from Sigma Chemie GmbH (Taufkirchen, FRG). PMSF[1] was from E. Merck (Darmstadt, FRG) and carboxyatractyloside and soybean trypsin inhibitor from Boehringer Mannheim GmbH (Mannheim, FRG).

Growth of Neurospora and Preparation of Mitochondria—Growth of *N. crassa* (wild type 74A) and preparation of mitochondria from lysed spheroplasts by differential centrifugation were as previously described (8). Before use, the mitochondria were washed once in SET buffer (0.3 M sucrose, 0.02 M Tris-HCl, 0.001 M EDTA, pH 7.2) containing 10^{-4} M PMSF, and then once with that same buffer without PMSF. The mitochondria were finally resuspended at 8–10 mg/ml in SET buffer.

Protease Treatment of Mitochondria—Mitochondria were adjusted to a protein concentration of 2 mg/ml, and aliquots (normally 0.5 ml) were placed in 1.5-ml microfuge tubes. The desired amount of trypsin or elastase was then added. Trypsin was freshly dissolved in and diluted with SET buffer for each experiment and used immediately. Elastase was prepared as a 5 mg/ml solution in 0.01 M Tris-HCl, pH 5.5, and stored in aliquots at −20 °C. For each experiment, a fresh aliquot was thawed by diluting to 0.5 mg/ml with SET buffer containing 0.1 mg/ml of aprotinin. The latter agent was included to inhibit any contaminating trypsin. After 10 min at 4 °C the elastase was further diluted with SET buffer containing aprotinin and used immediately. Controls without protease received an equal amount of the appropriate buffer instead of enzyme solution.

Mitochondria and protease were incubated for 15 min at 25 °C. Trypsin digestions were halted with soybean trypsin inhibitor. A 10-fold weight excess of inhibitor relative to the highest amount of protease used in a particular experiment was added to all samples. In some experiments PMSF was also added to a final concentration of 0.2 mM. Elastase digestions were halted by the addition of PMSF to a concentration of 0.5 mM. Samples were incubated for 3 min at 25 °C and then for 10 min at 4 °C following addition of the protease inhibitors.

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[1] The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; MOPS, 3-(*N*-morpholino)propanesulfonic acid; SDS, sodium dodecyl sulfate.

Protease-treated mitochondria were pelleted by centrifugation for 12 min at 17,000 × *g* at 4 °C in a Sorvall SS-34 or Beckman JA-20 rotor fitted with adaptors for 1.5-ml microfuge tubes. The mitochondria were washed once with 1 ml of SET buffer containing either 10^{-4} M PMSF (for elastase) or 0.1 mg/ml of soybean trypsin inhibitor plus 10^{-4} M PMSF (for trypsin) and then resuspended in a small volume of these buffers. At each stage of the washing, samples were transferred to fresh microfuge tubes. After determination of the protein recovered, the treated mitochondria were used in *in vitro* import experiments.

Binding and Import of Precursors Synthesized in Reticulocyte Lysate—Synthesis of [^{35}S]methionine-labeled *Neurospora* proteins in rabbit reticulocyte lysates and the preparation of osmotically buffered postribosomal supernatants from such lysates were as previously described (8). Binding or import was initiated by adding isolated *Neurospora* mitochondria to the supernatants at a final concentration of 0.15–0.30 mg of mitochondrial protein/ml. The reactions were then incubated for 20 min at 25 °C. Reactions were routinely performed in 1.5-ml microfuge tubes in total volumes of 0.1 to 0.2 ml. If only binding of precursors was to be examined, valinomycin (0.5 μM) was added to prevent energy-dependent import of precursors into the mitochondria (7, 8, 11). Mitochondria were recovered from the reactions by centrifugation for 12 min at 17,000 × *g* at 4 °C as described above. Residual fluid on the tube walls was pelleted by a brief centrifugation in a microfuge and removed by aspiration.

Assessment of ADP/ATP Carrier Import by Hydroxylapatite Chromatography—Recent work in this laboratory (3) has shown that ADP/ATP carrier protein imported into mitochondria *in vitro* acquires several properties of the authentic *in vivo* protein. One such property is the ADP/ATP carrier's characteristic behavior during chromatography on hydroxylapatite (12, 13). If authentic ADP/ATP carrier first binds the noncompetitive inhibitor carboxyatractyloside, it is stabilized against denaturation when solubilized from the mitochondrial membrane with Triton X-100 and passes through hydroxylapatite. Without carboxyatractyloside the solubilized protein rapidly denatures and is bound by hydroxylapatite. ADP/ATP carrier imported *in vitro* also passes through hydroxylapatite if carboxyatractyloside is present during solubilization whereas the unimported precursor form binds. This behavior serves as a convenient nondestructive measure of carrier import into mitochondria.

To assess import of ADP/ATP carrier, mitochondria pelleted from the *in vitro* import reactions described above were solubilized in 2.5% (v/v) Triton X-100, 0.11 M NaCl, 0.02 M MOPS, 0.005 M EDTA, pH 6.8, containing 2 μM carboxyatractyloside. Inclusion of carboxyatractyloside in the solubilization buffer was seen to be effective in stabilizing the carrier.[2] After the mitochondrial pellet dissolved, the extract was incubated for 20 min at 25 °C and then clarified by centrifugation for 10 min at 27,000 × *g* at 4 °C. A 300-μl portion of the extract was then applied to a small column containing 30 mg (dry weight) of hydroxylapatite (Bio-Gel HTP; Bio-Rad) equilibrated at 4 °C with 0.5% (v/v) Triton X-100, 0.05 M NaCl, 0.01 M MOPS, pH 7.2 (HTP buffer), and the passthrough collected. The column was washed with 300 μl of HTP buffer, and this wash was added to the passthrough fraction. The amount of carrier present was determined by immunoprecipitation and was taken to reflect imported carrier. Total carrier associated with mitochondria was determined by adding 300 μl of HTP buffer to 300 μl of clarified extract that had not been passed over hydroxylapatite and immunoprecipitating for carrier.

Immunoprecipitation—ADP/ATP carrier and the mitochondrial porin were precipitated from Triton X-100-solubilized mitochondria as previously described for the ADP/ATP carrier (8, 9). In the experiments reported here, the immune complexes were harvested with *Staphylococcus* protein A bound to agarose beads (protein A-agarose, Sigma).

ATPase subunit 2 was precipitated from SDS-solubilized mitochondria as previously described (11) with the following exceptions: β-mercaptoethanol was omitted from the SDS solution, no preabsorption was performed, and the immune complexes were harvested with protein A-agarose. Specific antibody to ATPase subunit 2 was the generous gift of Dr. Walter Sebald (Gesellschaft für Biotechnologische Forschung, Braunschweig-Stöckheim, FRG).

Other Methods—Mitochondrial protein was determined using the dye-binding assay of Bradford (14) (Bio-Rad) with bovine γ-globulin as the standard. Polyacrylamide gel electrophoresis, visualization of radioactive bands by fluorography, and densitometric analysis were as before (7, 11). Visualization of proteins in polyacrylamide gels using Coomassie brilliant blue was performed as described in Ref. 15. Immunoreplica analysis using lactoperoxidase linked to sheep anti-rabbit antibodies was performed essentially as described in Ref. 16.

[2] C. Zwizinski, unpublished observations.

RESULTS

Trypsin Treatment of Mitochondria Abolishes Their Ability to Bind and Import ADP/ATP Carrier Precursor—When import *in vitro* of the ADP/ATP carrier is blocked by dissipating the membrane potential, the precursor binds to isolated *Neurospora* mitochondria (Fig. 1 and Refs. 7–9). In a previous report (9) we demonstrated that this bound precursor could be imported into mitochondria if the membrane potential was re-established and that this import occurred directly from the binding site. We concluded that the binding of ADP/ATP carrier precursor to mitochondria in the absence of a mem-

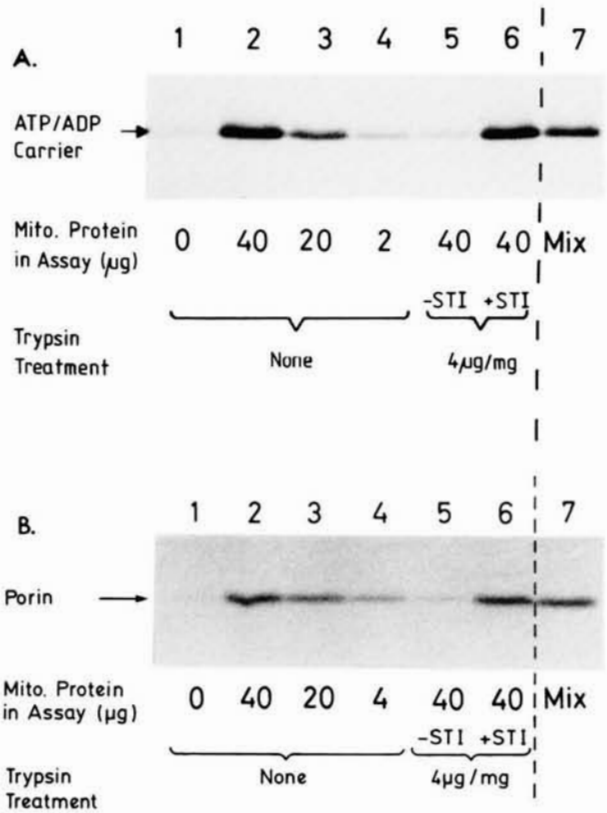

FIG. 1. **Effect of trypsin pretreatment on the ability of *Neurospora* mitochondria to bind the precursors of the ADP/ATP carrier and the porin.** *A*, freshly isolated *Neurospora* mitochondria were treated with trypsin as described under "Experimental Procedures." The pretreatment reactions contained either no trypsin, 4 μg of trypsin/mg of mitochondrial protein, or 4 μg of trypsin/mg of protein where the trypsin was preincubated with a 10-fold weight excess of soybean trypsin inhibitor (*STI*). Proteolysis was stopped by the addition of soybean trypsin inhibitor to those reactions not already containing it and the mitochondria reisolated and washed. Treated mitochondria were then mixed with ^{35}S-labeled precursors synthesized in rabbit reticulocyte lysate. Reactions contained: no mitochondria (*lane 1*); 40 μg (as protein, *lane 2*), 20 μg (*lane 3*), or 2 μg (*lane 4*) of untreated mitochondria; 40 μg of trypsin-treated mitochondria (*lane 5*); or 40 μg of mitochondria treated with trypsin and soybean trypsin inhibitor (*lane 6*). In *lane 7*, the binding reaction contained 20 μg of untreated mitochondria and 40 μg of trypsinized mitochondria. All reactions contained valinomycin. After incubation, the mitochondria were reisolated, solubilized in Triton X-100 containing buffer, and immunoprecipitated for ADP/ATP carrier. (± *STI*, with or without preincubation with soybean trypsin inhibitor.) *B*, the experiment was identical to that of *A* except that the samples were precipitated for the mitochondrial porin.

brane potential represented an interaction with functional receptor sites.

Fig. 1*A* shows that trypsin pretreatment of *Neurospora* mitochondria destroys their ability to bind the ADP/ATP carrier precursor. This inhibition is clearly due to the action of the protease as the effect can be prevented by soybean trypsin inhibitor (*lane 5 versus 6*). The inhibition is not the result of generation of an inhibitor or due to residual trypsin in the treated mitochondria since mixing of treated and untreated mitochondria has no effect on the ability of the latter to bind precursor (*lane 7*). Trypsin treatment routinely reduced the binding of carrier to 10% or less of control values (*e.g.* Figs. 2 and 5).

The minimum amount of trypsin required to abolish carrier binding was determined (Fig. 2). Using a 15-min incubation at 25 °C, 0.4 μg of trypsin per mg of mitochondrial protein produced nearly the full extent of inhibition. This curve was very reproducible (see Fig. 5) with 0.5 μg/mg always producing an 80 to 90% decrease in the ability of the treated mitochondria to bind precursor. The crucial parameter appeared to be the ratio of protease used to mitochondrial protein treated since curves identical to those in Figs. 2 and 5 were obtained when 10-fold higher concentrations of mitochondria and protease were used (not shown). The approximately 10% protease-resistant binding always observed was unaffected even by very high levels of protease (0.5 mg/mg of mitochondria) and probably represents nonspecific binding of the precursor to mitochondria. This level of nonspecific binding is consistent with our previous estimates (9). We expected that loss of binding should be accompanied by the loss of the ability to import the carrier precursor. Fig. 5 shows that the ability of the treated mitochondria to import carrier indeed declined in parallel with their ability to bind it.

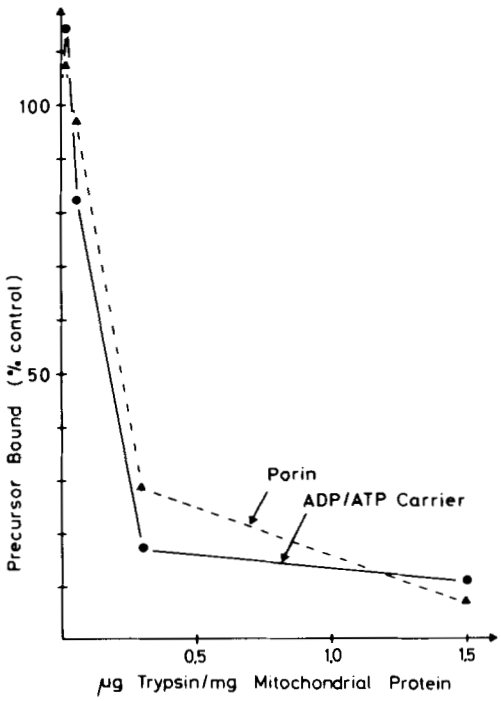

FIG. 2. **Titration of the amount of trypsin required to abolish binding of ADP/ATP carrier and porin precursors by mitochondria.** Mitochondria were treated with the indicated amounts of trypsin and then assayed for their ability to bind precursors in the presence of valinomycin. Mitochondria recovered from the binding reactions were solubilized and then sequentially immunoprecipitated for porin and ADP/ATP carrier. Immunoprecipitates were analyzed by SDS-gel electrophoresis and fluorography. The amount of ADP/ATP carrier and porin was estimated by densitometric scanning of appropriately exposed fluorograms. The results are plotted as the percentage of precursor bound by 40 μg of untreated mitochondria.

It is important for assessing the effect of protease treatment on the binding and import of precursors by mitochondria that the conditions used are such that a decrease in the amount of mitochondria (*i.e.* of binding sites) used in the reaction results in a decrease in the amount of bound or imported protein. In other words, the assays must be in the linear range. In all the experiments reported in this paper, controls were performed to ensure that this was the case (see for example Fig. 1) even if they are not shown.

Trypsin Treatment Abolishes the Ability of Mitochondria to Bind and Import the Precursor of the Outer Membrane Porin—The mitochondrial porin of *Neurospora*, like porin from yeast (17, 18), assembles very rapidly into the outer membrane of isolated mitochondria (10). This process appears to be independent of the mitochondrial membrane potential and is accompanied by the development of extreme resistance to proteolytic digestion of the assembled porin. At low temperature another form of mitochondrially associated porin is detectable (10). This porin is protease sensitive and appears to represent porin bound to receptor sites since it is quantitatively inserted into the outer membrane upon further incubation (10).

Trypsin treatment of mitochondria also led to the abolition of porin precursor binding and import (Fig. 1*B*, *lane 5*). As with the carrier, this effect was prevented by soybean trypsin inhibitor (*lane 6*) and was not the result of an inhibitor generated by trypsin or of residual trypsin (*lane 7*). Porin binding and import displayed the same high degree of protease sensitivity as carrier binding (Fig. 2).

Effect of Trypsin Treatment on the Structural Integrity of Mitochondria—At the levels of trypsin used in these experiments, minimal effects on mitochondrial integrity were noted. Fig. 3 shows the polypeptide profiles of the mitochondria used in the experiment of Fig. 5. It is clear that minimal changes occurred even at the highest levels of trypsin used. In fact, only a single band was observed to be altered in the profiles of whole mitochondria (Fig. 3, *double asterisk*). Further evidence of the intactness of these mitochondria was obtained by examining the content of the outer membrane porin (19) and of the matrix enzyme citrate synthase (EC 4.1.3.7) (20, 21). Treatment with relatively high levels of trypsin (4 μg/mg) had no effect on the mitochondrial content of these two proteins (Fig. 4). Thus the trypsin-treated mitochondria neither lysed nor lost their outer membranes. Examination of the recovery of porin and citrate synthase showed that trypsin treatment had no effect on the recovery of mitochondria from the binding reactions (Fig. 4, *lanes 6* and *7*). Other work from this laboratory has shown that trypsinization of mitochondria at the levels used in this study has a minimal effect on the ability of treated mitochondria to bind and import apocytochrome *c*, the precursor of cytochrome *c*, *in vitro*.[3] This reaction is known to be very sensitive to structural damage to the mitochondria (22).

We conclude that the effects of trypsin on the binding and import of the ADP/ATP carrier and the mitochondrial porin are the result of the destruction of proteinaceous receptors on the mitochondrial surface necessary for this process.

Effect of Trypsin Treatment on the Binding and Import of Other Mitochondrial Precursors—We examined the effect of trypsin treatment on the import of the precursors of subunits 2 and 9 of the mitochondrial proton-translocating ATPase

[3] H. Koehler, B. Hennig, and W. Neupert, manuscript in preparation.

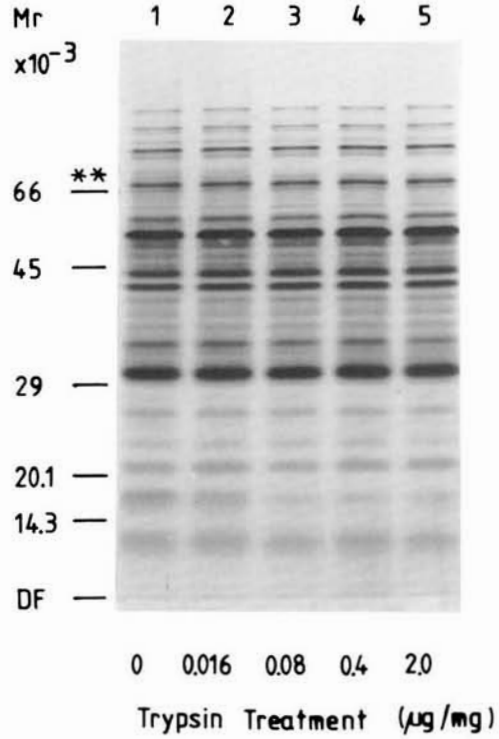

FIG. 3. **Polypeptide pattern of untreated and trypsin-treated mitochondria.** The mitochondria used in the experiment shown in Fig. 5 (30 μg of mitochondrial protein) were subjected to SDS-gel electrophoresis. Protein bands were visualized by staining with Coomassie brilliant blue. ** indicates the position of the only visible changes in the polypeptide profile. The mobilities and molecular weights of standard proteins are indicated at the *left side* of the figure. *DF*, dye front.

(23). Import of both precursors was strongly inhibited by trypsin treatment of the mitochondria (Fig. 5 and data not shown). We were unable to determine whether the effect of trypsin in these two cases represented an effect on binding or on import. As previously described (4), we are unable to efficiently visualize the precursor for subunit 9 in order to accurately assess its binding. The problem with subunit 2 was the observation that the precursor did not bind to mitochondria in the absence of import.

The latter interesting effect is illustrated in Fig. 6 (*upper*). In this experiment precursor was allowed to interact with mitochondria in the presence or absence of the potassium ionophore valinomycin. This agent collapses the membrane potential and prevents the import of presubunit 2 (8, 11). Under these conditions, essentially no precursor associated with mitochondria (Fig. 6, compare *lanes 1*, *4*, and *7* with *lanes 2*, *5*, and *8*). This was in contrast to the large amount of subunit polypeptide (as imported mature) associated with mitochondria when there was a potential (*lanes 3*, *6*, and *9*). This effect was not due to degradation of the unimported precursor since at each of the first two time points in Fig. 6 sufficient importable precursor was available to result in an increase of mature subunit at the next time point. The antibody and the immunoprecipitation procedure used here have previously been shown to result in efficient precipitation of presubunit 2 (11).

The behavior of subunit 2 precursor contrasted sharply with the behavior of the precursor of the ADP/ATP carrier in the same experiment (Fig. 6, *lower*). As previously observed (7–9), carrier precursor bound avidly to mitochondria in the absence of import.

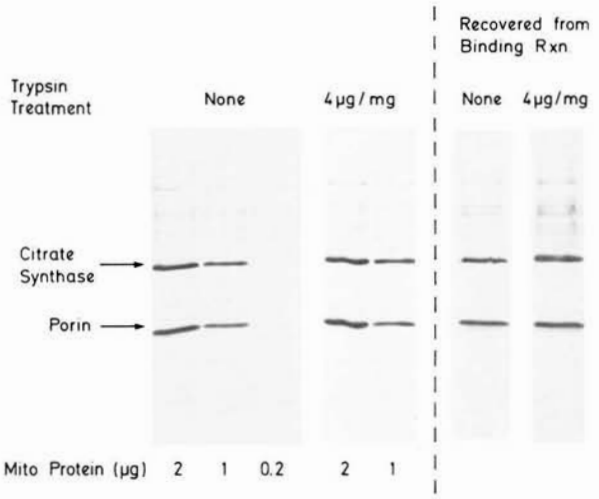

FIG. 4. **Trypsin-treated mitochondria retain their outer membranes and are efficiently recovered from the binding reactions.** Mitochondria were treated with either trypsin (4 μg/mg of mitochondrial protein) or in parallel with no trypsin. Trypsin-treated mitochondria exhibited a 90% decrease relative to the controls in their ability to bind the precursors of the ADP/ATP carrier and porin (not shown). After proteolysis, the indicated amounts of mitochondrial protein were subjected to SDS-gel electrophoresis. The separated proteins were transferred to nitrocellulose paper and porin and citrate synthase visualized by immunoreplica analysis (*left side*). Aliquots of treated and untreated mitochondria were also mixed with reticulocyte lysate and incubated as normal for binding. The mitochondria were reisolated, dissolved in sample buffer, and aliquots were assayed for porin and citrate synthase by immunoreplica analysis. The size of the aliquots was such that they should have contained 2 μg of mitochondrial protein if recovery from the binding reactions (*Rxn.*) was quantitative (*right side*).

Effect of Elastase Pretreatment on the Ability of Mitochondria to Bind and Import Precursors—Treatment of *Neurospora* mitochondria with the protease elastase had a distinctly different effect on the binding and import of precursors. Fig. 7 shows that treatment with very low levels of elastase strongly inhibited the ability of the mitochondria to bind and import the precursor of the ADP/ATP carrier. Import of the mitochondrial porin was affected in an identical manner (data not shown). Controls (not shown here) similar to those described earlier for trypsin indicated that elastase neither generated an inhibitor nor was present in the mitochondria used in the assays. Inclusion of PMSF, an inhibitor of elastase, in the protease treatments prevented the inhibition. The only obvious change in the polypeptide pattern of the treated mitochondria was the disappearance of the band previously seen to be degraded by trypsin. Examination of the specific contents of porin and citrate synthase showed that no change had occurred and, as with trypsin-treated mitochondria, the efficiency of mitochondria recovery from the binding reactions was unchanged.

As can be seen in Fig. 7, elastase did not produce quite as extensive a loss of binding and import of the carrier as compared with trypsin (Figs. 2 and 5). This was also true for the mitochondrial porin (data not shown). The maximum inhibition produced by elastase treatment varied between 50 and 80% with different preparations of mitochondria, with the average level being about 70%. It should be emphasized that the variation was in the maximum extent of inhibition and not in the concentration of elastase required to produce the maximum effect. Thus an experiment identical to that shown in Fig. 7 produced similar curves except that binding and import of carrier were reduced by only 50 to 60% relative

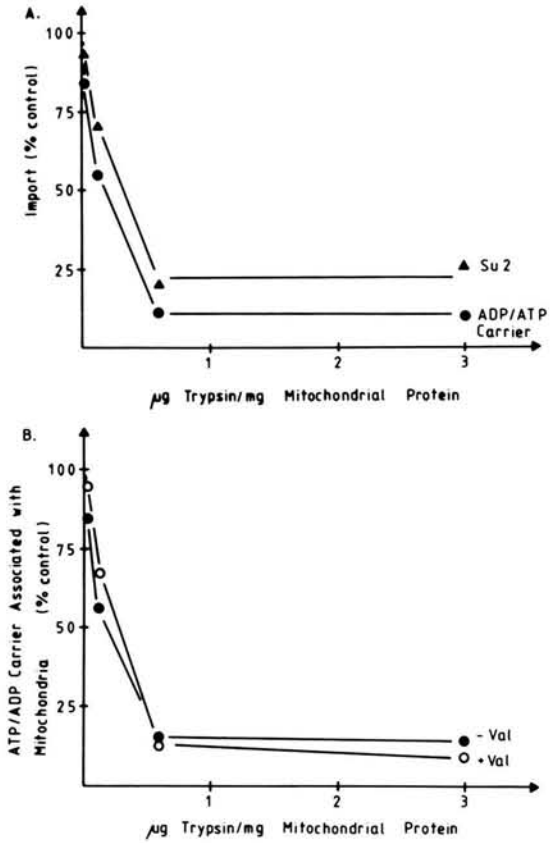

FIG. 5. **Trypsin treatment of mitochondria inhibits the import of ADP/ATP carrier and ATPase subunit 2.** Mitochondria were treated with the amounts of trypsin indicated in the figure. They were then incubated with radiolabeled precursors in the presence and absence of valinomycin. After incubation, the reactions were divided in two and the mitochondria in each portion recovered by centrifugation. One portion of mitochondria was solubilized and immunoprecipitated for ATPase subunit 2. Import was taken to be the amount of mature subunit 2 formed (*A*, ▲——▲). The second portion was solubilized as for hydroxylapatite chromatography as described under "Experimental Procedures" and then divided into two aliquots. One aliquot was immunoprecipitated for ADP/ATP carrier to yield the total amount of carrier (bound and/or imported) associated with the mitochondria in the presence (*B*, ○——○) or absence (*B*, ●——●) of valinomycin. The other aliquot was subjected to hydroxylapatite chromatography and the passthrough fraction immunoprecipitated for ADP/ATP carrier. This carrier was taken to reflect ADP/ATP carrier imported into mitochondria (*A*, ●——●). For both ADP/ATP carrier and ATPase subunit 2, no import was observed in the presence of valinomycin. *Su 2*, ATPase subunit 2; *Val*, valinomycin.

to control values (not shown). We consider this phenomenon further under "Discussion."

Concentrations of elastase sufficient to severely inhibit import of the carrier and porin precursors had little effect on the import of ATPase subunits 2 (Fig. 7) and 9 (not shown). We interpret this result to indicate that elastase does not attack the surface receptors active in the import of the latter two proteins and further that elastase does not generally decrease the ability of mitochondria to import proteins. Since elastase does affect the binding and import of porin and ADP/ATP carrier, we suggest that the receptor(s) for these two proteins are distinct from the receptors for ATPase subunits 2 and 9.

DISCUSSION

Mild proteolytic treatment of *Neurospora* mitochondria renders them unable to efficiently bind and import the pre-

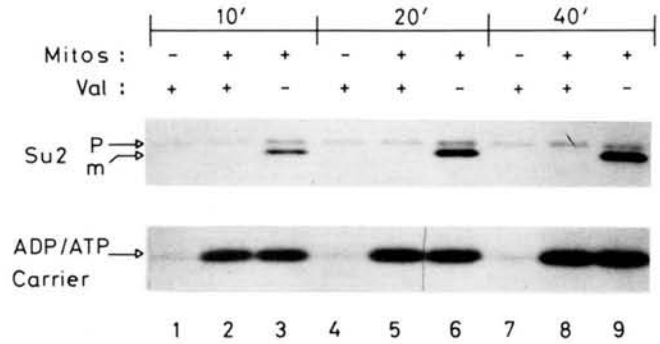

FIG. 6. **ATPase subunit 2 does not bind to mitochondria when its import is blocked.** Three import reactions (0.35 ml) were constituted as follows: no mitochondria, plus valinomycin (*lanes1*, *4*, and *7*); plus mitochondria, plus valinomycin (*lanes 2*, *5*, and *8*); plus mitochondria, no valinomycin (*lanes 3*, *6*, and *9*). Reactions with mitochondria (*Mitos*) contained 75 μg of mitochondrial protein and all reactions contained ^{35}S-labeled precursors in rabbit reticulocyte lysate. After incubation for the times indicated in the figure, 100-μl aliquots of each reaction were removed and the mitochondria pelleted by centrifugation for 5 min at 10,000 × *g* in a microfuge. The isolated mitochondria were then sequentially immunoprecipitated for ATPase subunit 2 (*upper*) and ADP/ATP carrier (*lower*). Immunoprecipitates were subjected to SDS-gel electrophoresis and the radioactive proteins visualized by fluorography. *Su2*, ATPase subunit 2; *P*, precursor; *m*, mature; *Val*, valinomycin.

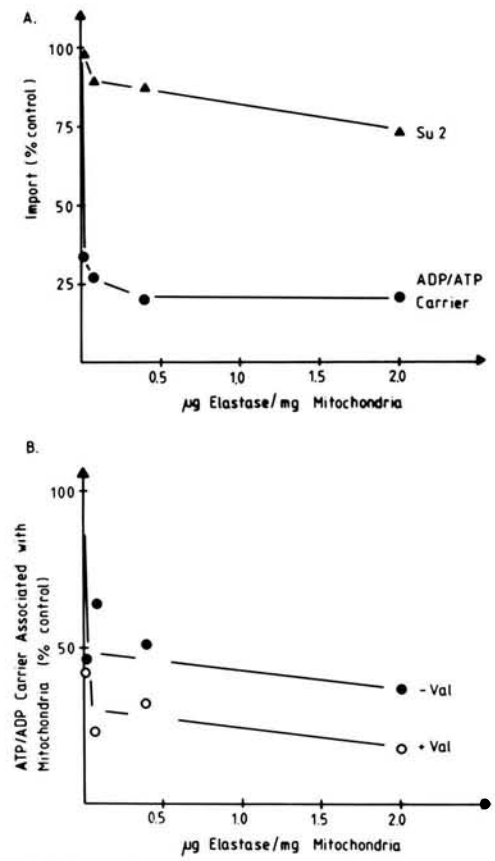

FIG. 7. **Differential effect of elastase on the ability of mitochondria to import the precursors of the ADP/ATP carrier and ATPase subunit 2.** Mitochondria were pretreated with various amounts of elastase and the ability of the treated mitochondria to bind and import precursors was assessed as in the experiment of Fig. 5. *A*, import of ATPase subunit 2 (▲——▲) and ADP/ATP carrier (●——●). *B*, carrier associated with mitochondria in the presence (○——○) and absence (●——●) of valinomycin. *Su2*, ATPase subunit 2; *Val*, valinomycin.

cursors of the ADP/ATP carrier and the mitochondrial porin. This did not appear to be the result of gross physical disruption or increased lability of the mitochondria. We interpret these results to indicate that the functional binding of the above precursors to isolated mitochondria, previously documented by this laboratory (9, 10), is mediated by proteins on the mitochondrial surface.

We also report that mild trypsin treatment abolishes the ability of mitochondria to import the precursors of subunits 2 and 9 of the mitochondrial proton-translocating ATPase. We were unable to decide whether binding or import of these two precursors was affected by the protease. For subunit 9, this was due to a technical problem that prevented visualization of the larger precursor. The case of presubunit 2 was more interesting. Here we were unable to evaluate binding in the absence of import because the precursor is not bound to a significant extent by mitochondria in the absence of a membrane potential (Fig. 6). This result illustrates that detection of precursor binding in *in vitro* import systems cannot be taken for granted. Further, it suggests that what is often taken as binding in such systems may, in fact, not be. For example, in Fig. 6 a small amount of presubunit 2 is visible in *lanes 2, 5,* and *8*. This material, however, clearly represents precursor bound to tube walls and not to mitochondria. In experiments where import is low, this small amount of precursor can appear quite impressive; however, the normal case is that the amount of subunit 2 radioactivity apparently associated with mitochondria in the absence of import is much less than with import.

Examination of several recent papers indicates that the precursors to yeast ATPase subunit 2 (24) and cytochrome b_2 (25, 26) bind poorly to mitochondria in the absence of import. In the case of the latter protein, however, some precursor can be shown to be bound to authentic receptor sites (27). In this laboratory, low binding of precursor in the absence of import has been observed for subunits I and VII of the *Neurospora* cytochrome bc_1 complex (28). The low precursor binding may have a trivial explanation (*e.g.* relatively low affinity of the receptor) but could reflect such interesting possibilities as efficient binding requiring the mitochondrial membrane potential.

Treatment of *Neurospora* mitochondria with elastase resulted in a decrease in their ability to bind and import the precursors of the ADP/ATP carrier and the mitochondrial porin. The same mitochondria, however, showed unimpaired import of ATPase subunits 2 and 9. These data support the notion that binding of carrier and porin is mediated by proteins on the mitochondrial surface. Further, we interpret these results as indicating that the receptors for porin and carrier are distinct from those of the ATPase subunits. Previous work in this laboratory has shown that receptors for the import of the precursor of cytochrome *c* are different from those used in the import of ATPase subunit 9 and the ADP/ATP carrier (29). The data presented here suggest that the receptors for the latter two proteins are also different implying at least three separate import pathways in *Neurospora* mitochondria.

An intriguing feature of elastase action is its variable and incomplete inhibition of the binding and import of ATP/ADP carrier and porin. One possibility is that *Neurospora* contains two distinct types of mitochondria, one possessing elastase-sensitive receptors, the other not. Alternatively, each mitochondrion might possess two distinct types of receptors for porin and carrier. Perhaps the most interesting possibility is that the receptors exist in two states (*e.g.* active and inactive), only one of which is elastase sensitive. The currently available data allow no satisfactory explanation for the phenomenon, however.

Several reports have recently appeared concerning the effect of protease treatment on the ability of isolated mitochondria to bind and import precursor proteins. Argan *et al.* (30) showed that treatment of rat liver mitochondria with trypsin and chymotrypsin abolished import of ornithine carbamyltransferase. These authors did not, however, convincingly demonstrate that precursor binding was the affected step. Schatz and co-workers (18, 27) have shown that the *in vitro* import of ATPase subunit 2 of the yeast *Saccharomyces cerevisiae* can be abolished by trypsin treatment as can the import of cytochrome b_2. In the case of cytochrome b_2, convincing data was obtained indicating that the binding of the precursor was abolished by the protease treatment. It should be noted that these authors also reported that import of the major 29-kilodalton protein of the yeast outer mitochondrial membrane, which appears to correspond to *Neurospora* porin, was not affected by trypsin treatment of the mitochondria (18). This contrasts with the data presented in this report where porin import was seen to be inhibited by both trypsin and elastase pretreatment.

Proteinaceous surface receptors have now been strongly implicated in the import of four mitochondrial proteins. Cytochrome b_2 of yeast appears to require a surface receptor for its import (18, 27). We feel that the results presented here support the existence of proteinaceous receptors for the import of porin and the ADP/ATP carrier into *Neurospora* mitochondria. Finally, the high affinity binding sites detectable on the surface of *Neurospora* mitochondria that mediate the import of apocytochrome *c* (5, 6) have recently been purified to homogeneity in this laboratory and found to be proteins.[3] The next step in the analysis of protein import into mitochondria clearly must be the isolation and detailed characterization of these receptor proteins.

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