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Transport of ADP/ATP Carrier into Mitochondria

PRECURSOR IMPORTED *IN VITRO* ACQUIRES FUNCTIONAL PROPERTIES OF THE MATURE PROTEIN*

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Precursor to ADP/ATP carrier synthesized *in vitro* is transferred into isolated mitochondria to a protease-resistant location. This process requires an electrical potential across the inner membrane. We show now that precursor imported in a cell-free system exhibits the same resistance to protease as the mature endogenous carrier. Furthermore, transferred protein, but not receptor-associated precursor, binds carboxyatractyloside, a specific inhibitor of the mature carrier and can be isolated by the purification procedure for the mature carrier. At least 70% of the precursor associated with mitochondria in the presence of a membrane potential acquires this functional characteristic. Finally, the binding of carboxyatractyloside can be modulated by treatment of the imported protein with sulfhydryl reagents in a manner indistinguishable from the authentic carrier protein. We conclude that import *in vitro* leads to correct assembly and orientation of the ADP/ATP carrier in the mitochondria.

The biogenesis of mitochondria requires the import of most of their constituent proteins from the cytoplasm (1). Uptake of precursor proteins into mitochondria occurs post-translationally and thus clearly differs from the cotranslational transfer of proteins through membranes described for the synthesis of secretory proteins (2, 3). Generally, two approaches are used to study post-translational transfer. The first entails pulse labeling of intact cells and analysis of the fate of newly synthesized proteins by cell fractionation (4). The second uses cell-free systems to synthesize radiolabeled precursors *in vitro* and follows subsequent uptake into isolated mitochondria (5, 6). The latter method allows the dissection of post-translational transfer into distinct steps *viz.* recognition and binding of precursors, transfer of precursors across membranes, and proteolytic processing (7).

An open question is whether the post-translational transfer observed *in vitro* truly reflects the process occurring in the living cell (see also Ref. 8). Evidence has been presented that in these systems precursor proteins are transferred to their correct intramitochondrial location, the matrix and the intermembrane space, respectively (8). Assembly of subunits of F_0F_1-ATPase *in vitro* has recently been demonstrated (9, 10). In order to further investigate the question of whether precursors achieve a functional form upon transport into mitochondria *in vitro*, we have examined in greater detail the *in vitro* import of mitochondrial ADP/ATP carrier. This protein is an integral protein of the inner mitochondrial membrane and mediates the electrogenic exchange of ATP and ADP. In this report, we demonstrate that ADP/ATP carrier imported into isolated *Neurospora crassa* mitochondria *in vitro* acquires structural and functional properties indistinguishable from those of the mature carrier.

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MATERIALS AND METHODS

Growth and Radioactive Labeling of Neurospora Cells—*N. crassa* wild type 74A was cultivated in Vogel's minimal medium as described (6). When indicated, cells were labeled with [^{3}H]leucine by adding 2 mCi/liter of [^{3}H]leucine (40–60 Ci/mmol; New England Nuclear) to the culture 1 h before harvesting.

Isolation of Mitochondria—*Neurospora* hyphae were harvested by filtration. For 1 g of hyphae, 1 g of washed quartz sand and 1 ml of SET (0.3 M sucrose, 1 mM EDTA, 30 mM Tris/HCl, pH 7.2) were added, and the homogenized paste was ground in a precooled mortar at 4 °C for 30 s. After addition of 3 ml of SET, mitochondria were isolated as described (6). The mitochondria were washed once with and finally resuspended in SET, at a concentration of 5–20 mg of protein/ml.

Protein Synthesis in Reticulocyte Lysate—Reticulocytes were isolated from anemic rabbits, and cell-free protein synthesis in the presence of [^{35}S]methionine (1000 Ci/mmol; New England Nuclear) was carried out according to Pelham and Jackson (11) as described (6). To the postribosomal supernatant, sucrose and methionine were added to concentrations of 300 and 1 mM, respectively.

Transfer in Vitro of Precursor to ADP/ATP Carrier—Mitochondria were added to the postribosomal supernatant to give a final concentration of 0.5–2 mg of protein/ml of reticulocyte lysate. Transfer was carried out by incubation at 25 °C for 30 min. Carboxyatractyloside was added to the transfer mixture to a final concentration of 10 μM 5 min before transfer was halted by cooling to 4 °C.

Treatment with Proteinase K—Proteinase K was dissolved in SET to 10 times the concentration finally used. After transfer, the reticulocyte lysate with the mitochondria was cooled to 4 °C, and 1/10 volume of proteinase K solution was added. Proteolysis was stopped after 30 min at 4 °C by addition of 0.1 M PMSF[1] in ethanol to a concentration of 1 mM.

Passage over Hydroxylapatite Columns—Mitochondria were reisolated from the reticulocyte lysate by centrifugation for 5 min in an Eppendorf centrifuge and dissolved in 100 μl of buffer containing 2.5% Triton X-100, 110 mM NaCl, 20 mM MOPS, pH 7.2 (high Triton buffer). The solution was centrifuged in an Eppendorf centrifuge for 5 min. Small columns containing 40 mg of hydroxylapatite (Bio-Gel HTP, Bio-Rad GmbH, Munich, West Germany) equilibrated in 0.5% Triton X-100, 50 mM NaCl, 10 mM MOPS, pH 7.2 (low Triton buffer) were poured in 1-ml Eppendorf pipette tips closed with glass wool. The supernatants of the mitochondrial extracts were then applied to these columns. When the sample had entered the hydroxylapatite bed, four times the column volume of low Triton buffer was added. From the eluate, ADP/ATP carrier was immunoprecipitated.

Determination of Radioactivity in Double Labeled Samples—Immunoprecipitates which contained both ^{3}H- and ^{35}S-labeled ADP/ATP carrier were subjected to SDS-gel electrophoresis, and the dried gels were fluorographed as described (12). Areas exhibiting the band

[1] The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; NEM, *N*-ethylmaleimide; SDS, sodium dodecyl sulfate; MOPS, 4-morpholinepropanesulfonic acid.

of ADP/ATP carrier and equal sized control areas were cut out of the gel and extracted overnight at room temperature by incubation in 0.5 ml of 1% SDS, 0.1 M Tris/Cl, pH 8.0. Scintillation fluid was added, and ^{3}H and ^{35}S radioactivity was measured in a Packard Tricarb 2425 scintillation counter as described (4). The activity found in the control area of each lane was subtracted from the radioactivity found in the area of the ADP/ATP carrier band, and the values were then corrected for the overspill of ^{35}S activity into the ^{3}H-channel.

Other Methods—Immunoprecipitation of ADP/ATP carrier employing *Staphylococcus aureus* was done as described (12). Dissociation of immunoprecipitates, SDS-polyacrylamide gel electrophoresis, and fluorography of gels was done as described (7). Protein was determined as before (7) except that SDS was omitted from the assay mixtures.

RESULTS

ADP/ATP Carrier Imported in Vitro Has the Same Resistance to Added Protease As the Mature Carrier—Precursor to the ADP/ATP carrier synthesized *in vitro* is transferred into mitochondria to a protease-resistant location in the presence of a mitochondrial membrane potential (6). We compared this "imported" precursor with the mature endogenous protein in regard to its resistance to added protease. Mitochondria labeled with [^{3}H]leucine were incubated in the postribosomal supernatant of a reticulocyte lysate to allow them to take up [^{35}S]methionine-labeled precursor. Proteinase K was then added to final concentrations of 10–400 μg/ml of transfer mixture and the samples incubated for 30 min at 4 °C after which the mitochondria were recovered by centrifugation. ADP/ATP carrier was immunoprecipitated, and the amount of ^{3}H and ^{35}S label was determined. Proteinase K reduced the recovery of ^{3}H- and ^{35}S-labeled ADP/ATP carrier to about the same extent (Fig. 1). This supports the idea that, upon import, the precursor gains the location and/or conformation typical of the mature pre-existing protein. Incubation in the transfer mixture leads to increased proteolytic sensitivity of the mature enzyme as compared to the protein in mitochondria similarly incubated in isolation medium. In the latter case, the ADP/ATP carrier is almost completely resistant to external protease (data not shown). Accordingly, the majority of the ADP/ATP carrier that associates with mitochondria is probably translocated into a position protected from added protease.

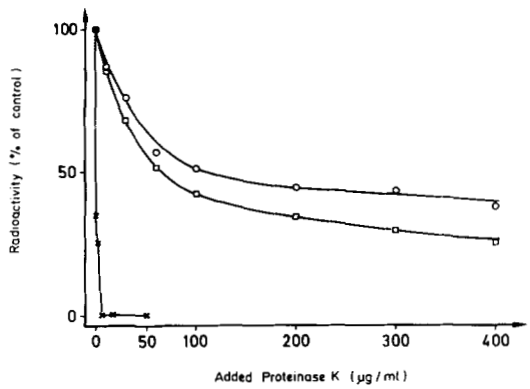

FIG. 1. **Sensitivity of various forms of ADP/ATP carrier to proteinase K treatment.** ^{3}H-labeled mitochondria were added to the postribosomal supernatant of a reticulocyte lysate containing [^{35}S]methionine-labeled *Neurospora* proteins. Transfer was started by incubation at 25 °C and stopped after 30 min by cooling to 4 °C. Proteinase K was added to aliquots of the transfer mixture to give the final concentrations shown on the *abscissa*. After 30 min, PMSF was added and mitochondria were reisolated. They were lysed with Triton X-100-containing buffer, and ADP/ATP carrier was immunoprecipitated. Dissociated immunoprecipitates were separated on SDS-polyacrylamide gel. The amount of ^{35}S and ^{3}H in the ADP/ATP carrier peak was determined as described under "Materials and Methods." □——□, imported precursor ^{35}S; ○——○, mature ADP/ATP carrier ^{3}H. Transfer of [^{35}S]methionine-labeled precursors was performed as above with the exception that 0.4 μM valinomycin was present. After cooling to 4 °C, proteinase K was added to final concentrations of 0–50 μg/ml of transfer mixture. Proteolysis was stopped after 30 min with PMSF, and ADP/ATP carrier was immunoprecipitated from reisolated mitochondria and analyzed on SDS-polyacrylamide gel. The gel was fluorographed, and the films were subjected to densitometry. ×——×, precursor bound to receptor sites on mitochondria.

In the absence of a membrane potential, a large amount of precursor to ADP/ATP carrier is bound by mitochondria to putative receptor sites (7). The sensitivity of this bound precursor was assessed by protease challenge using the conditions described above except the mitochondrial membrane potential was dissipated by the potassium ionophore valinomycin. Fig. 1 shows that bound precursor is much more sensitive to proteolytic attack than transferred or mature precursor, with about 2 μg of proteinase K/ml being sufficient to destroy half of the bound precursor. This indicates that the localization and/or conformation of bound precursor differs greatly from that of transferred precursor. The high protease sensitivity of the bound precursor suggests that it is bound to the outside of the outer mitochondrial membrane.

ADP/ATP Carrier Transferred in Vitro Binds Carboxyatractyloside—Carboxyatractyloside is a specific inhibitor of the ADP/ATP carrier which binds noncompetitively and with high affinity to the functional dimeric protein (13, 14). Upon solubilization of the mitochondrial membranes with detergent, it stabilizes the protein against denaturation and proteolysis (15, 16). The carboxyatractyloside carrier complex can be isolated by passage of detergent-solubilized mitochondria over a column of hydroxylapatite (14) being recovered in nearly pure form in the column eluate. The mature protein solubilized in the absence of carboxyatractyloside rapidly denatures and is bound by hydroxylapatite (16). If precursor to ADP/ATP carrier is transferred correctly into mitochondria, it should display the carboxyatractyloside-dependent passage typical for the mature enzyme. This was analyzed in the experiment shown in Fig. 2.

Isolated mitochondria were incubated with labeled precursor in the presence or absence of valinomycin. In both cases, precursor became associated with the mitochondria (Fig. 2, *lanes 1* and *2*). Only in the absence of valinomycin did the precursor become protease-resistant (Fig. 2, *lanes 3 versus 4*) in agreement with the data in Fig. 1. When mitochondria were reisolated from parallel reactions, dissolved with Triton X-100 in the absence of carboxyatractyloside, and applied to hydroxylapatite columns, no labeled ADP/ATP carrier eluted from the columns of any of the four reactions (*lanes 5–8*). If prior to reisolation of the mitochondria from the transfer mixture they were incubated with carboxyatractyloside, ADP/ATP carrier passed through the hyroxylapatite column (*lane 9*) if a membrane potential had been present during transfer. In contrast, precursor bound to mitochondria in the presence of valinomycin was retained on the column (*lane 10*). The precursor which resisted protease treatment of mitochondria had also acquired the ability to pass through hydroxylapatite (*lane 11*). ADP/ATP carrier transferred *in vitro* was not quantitatively recovered in the column eluates and in this respect behaves like the mature pre-existing protein (see also below). It is thus clear that ADP/ATP carrier transferred into mitochondria *in vitro* develops the ability to bind carboxyatractyloside.

Precursor in reticulocyte lysate was bound by hydroxylapatite whether or not carboxyatractyloside was present. Mature ADP/ATP carrier which was mixed with the reticulocyte

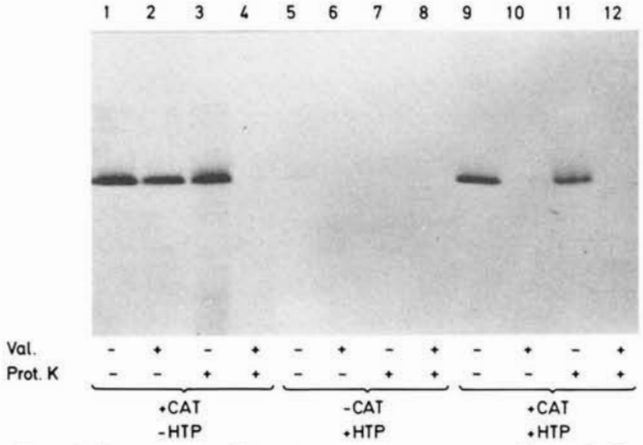

FIG. 2. **Protease-resistant precursor passes through hydroxylapatite in the presence of carboxyatractyloside, whereas protease-sensitive precursor is retained.** Mitochondria were added to a postribosomal supernatant of reticulocyte lysate, which contained ^{35}S-labeled precursors. To one-half of this mixture, valinomycin (*Val.*) was then added to a final concentration of 0.4 μM. After incubation at 25 °C for 30 min, each of the two portions received carboxyatractyloside (*CAT*) to a concentration of 10 μM and was again divided in two. From one-half, mitochondria were isolated immediately and lysed with Triton-containing buffer. The other half received proteinase K (*Prot. K*) to 50 μg/ml and was incubated at 4 °C for 30 min, and protease activity was stopped with PMSF. Then mitochondria were isolated and lysed. ADP/ATP carrier was immunoprecipitated from all samples (*lanes 1–4*). The same procedure was carried out in parallel with samples in *lanes 5–8* with the following modifications: no carboxyatractyloside was added, and the reisolated mitochondria were lysed and passed over columns of HTP as described under "Materials and Methods." From the column eluate, ADP/ATP carrier was immunoprecipitated. Otherwise, samples in *lanes 5–8* correspond to *lanes 1–4*. Samples in *lanes 9–12* were treated in parallel just as samples in *lanes 5–8*, except that to the reticulocyte lysate carboxyatractyloside was added at the end of the transfer period. Again, ADP/ATP carrier was immunoprecipitated from the eluate of the hydroxylapatite column. Otherwise, samples in *lanes 9–12* correspond to samples in *lanes 5–8*.

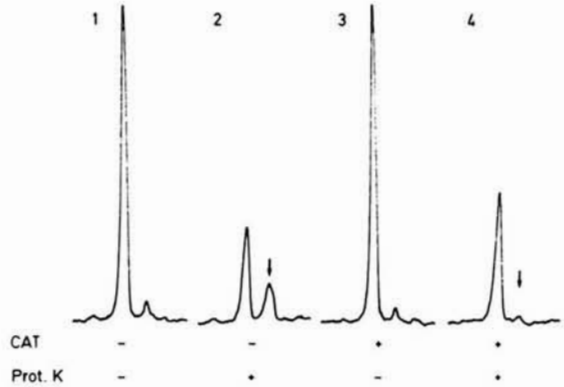

FIG. 3. **Carboxyatractyloside prevents proteolytic fragmentation of transferred ADP/ATP carrier by proteinase K.** Mitochondria were added to postribosomal supernatant of reticulocyte lysate as described. One portion served as a control, and the other portion received 10 μM carboxyatractyloside (*CAT*). After incubation, samples were cooled and halved again. From the first half, mitochondria were reisolated immediately, and to the second half, proteinase K (*Prot. K*) was added to 100 μg/ml of transfer mixture. Proteolysis was stopped after 30 min at 4 °C with 1 mM PMSF, and mitochondria were reisolated. Immunoprecipitation of ADP/ATP carrier and gel electrophoresis were done as described. The resulting fluorographs were subjected to densitometry. *Arrows* indicate position of proteolytic fragment.

lysate was not bound, indicating that binding was not due to the high amount of hemoglobin or some other component present in the reticulocyte lysate (data not shown).

It has been reported that carboxyatractyloside protects the ADP/ATP carrier against proteolysis. The uncomplexed protein in contrast is more sensitive to proteolysis (17). A typical breakdown product generated is a peptide of molecular weight 28,000. This fragment could sometimes be observed in the protease protection experiments similar to those described above if carboxyatractyloside was omitted from the digestion mixture. An example is shown in Fig. 3. In the experiment, mitochondria were allowed to import precursor proteins. From one portion of these mitochondria, the ADP/ATP carrier was immunoprecipitated without and with prior addition of carboxyatractyloside (*trace 1* and *3*). A second portion of mitochondria was treated with proteinase K in the absence (*trace 2*) or presence (*trace 4*) of carboxyatractyloside. Proteolysis was halted and ADP/ATP carrier immunoprecipitated. Densitometer tracings of the fluorograms show that in the presence of carboxyatractyloside the 28,000-kDa fragment was not generated. This result suggests that precursor imported *in vitro* shares the conformation of authentic ADP/ATP carrier as judged by the accessibility of protease-sensitive sites. Furthermore, it reinforces the result of Fig. 2, namely that carrier imported *in vitro* acquires the ability to bind carboxyatractyloside thereby changing its properties in a manner characteristic of the authentic protein. In agreement with the observation in Fig. 1, about half of the ADP/ATP carrier is completely degraded due to the exposure of mitochondria to the reticulocyte lysate.

ADP/ATP Carrier Transferred in Vitro Exhibits Reorientation of Nucleotide-binding Site—During translocation of nucleotides, the ADP/ATP carrier changes conformation. Two orientations of the protein have been described (18, 19): the c-state or "atractyloside conformation," in which the nucleotide-binding site is facing the cytoplasm and carboxyatractyloside can be bound, and the m-state or "bongkrekate conformation" where the nucleotide-binding site is oriented toward the matrix of mitochondria, and carboxyatractyloside cannot, but bongkrekate can bind. Furthermore, sulfhydryl groups are exposed in the m-state, which can react with NEM, thereby fixing the ADP/ATP carrier. The so modified protein is unable to reverse orientation, to bind carboxyatractyloside, or to pass through hydroxylapatite.

In order to analyze whether ADP/ATP carrier imported *in vitro* can reorient in the membrane, the ability of NEM to hold the carrier in the m-state was tested. Covalent binding of NEM should prevent binding of carboxyatractyloside and passage of the ADP/ATP carrier through hydroxylapatite. Table I gives a quantitative presentation of this phenomenon and a comparison with the behavior of the mature ADP/ATP carrier. Mitochondria were obtained from cells labeled with [^{3}H]leucine, and [^{35}S]methionine-labeled ADP/ATP carrier was transferred. The carboxyatractyloside-dependent passage of imported precursor through hydroxylapatite was reduced by pretreatment of the mitochondria with NEM. On the other hand, if carboxyatractyloside was added prior to NEM, the protein passed through hydroxylapatite. The imported precursor responded just as the mature protein responded. This demonstrates that the imported precursor can switch between the different orientations in the same way as the mature protein.

Transfer of ADP/ATP Carrier in Vitro Is Efficient—In earlier reports, we have shown that about 30% of mitochondria-associated precursor becomes protease-resistant after transfer *in vitro* (6). However, as demonstrated in Fig. 1, protease treatment can lead to considerable degradation of mature and transferred ADP/ATP carrier so that the actual

TABLE I

NEM inhibits binding of carboxyatractyloside to imported ADP/ATP carrier

[^{3}H]Leucine-labeled mitochondria were allowed to import [^{35}S] methionine-labeled precursor. Mitochondria were reisolated by centrifugation and resuspended with homogenization in the same volume of reticulocyte lysate, which did not contain labeled precursors. The sample was divided into four equal aliquots, and these were incubated for 10 min at 25 °C. Then carboxyatractyloside (CAT) or NEM, dissolved in SET, was added as indicated to final concentrations of 10 μM and 2 mM, respectively, and the incubation was continued for 10 min. CAT or NEM was then added to the samples as indicated in the table. After a further 10 min at 25 °C, mitochondria were reisolated, lysed, and applied to HTP columns. From the eluates, ADP/ATP carrier was immunoprecipitated and separated on SDS-polyacrylamide gel. Quantitation of [^{3}H]- and ^{35}S-ADP/ATP carrier was done as described under "Materials and Methods."

Additions		Pre-existing ADP/ATP carrier, ^{3}H label	Imported ADP/ATP carrier, ^{35}S label
First	Second		
		cpm	
SET	SET	59	56
SET	CAT	663	828
NEM	CAT	343	518
CAT	NEM	627	940

TABLE II

Quantitation of HTP passage of pre-existing and ADP/ATP carrier transferred in vitro

[^{3}H]Leucine-labeled mitochondria were allowed to bind in the presence of valinomycin (+Val) or import (−Val) [^{35}S]methionine-labeled precursors. After addition of carboxyatractyloside, each portion was halved and mitochondria were pelleted. The four pellets were lysed in Triton buffer, and the second one of each pair was passed over a column of HTP as described under "Materials and Methods." ADP/ATP carrier was immunoprecipitated from all samples, separated on an SDS gel, and fluorographed. The area of the ADP/ATP carrier band was cut out of the gel, and ^{3}H and ^{35}S radioactivity was determined as described under "Materials and Methods."

	Pre-existing, ADP/ATP carrier		Imported, ADP/ATP carrier	
	^{3}H label	Recovery after HTP	^{35}S label	Recovery after HTP
	cpm	%	*cpm*	%
−Val, −HTP	477	54	329	38
−Val, +HTP	258		125	
+Val, −HTP	449	59	210	1.4
+Val, +HTP	264		3	

efficiency of transfer may be underestimated. A nondestructive method is therefore desirable for judging the efficiency of the *in vitro* system. Carboxyatractyloside-dependent passage through hydroxylapatite offered a nondestructive yet selective method of measuring the efficiency of transfer. Accordingly, transfer of [^{35}S]methionine-labeled carrier was performed using mitochondria metabolically labeled with [^{3}H]leucine. This allowed the use of the assembled *in vivo* ADP/ATP carrier as an internal standard to estimate the yield of protein upon hydroxylapatite chromatography. As seen in Table II, pre-existing ^{3}H-labeled ADP/ATP carrier passed through hydroxylapatite whether valinomycin was present during the transfer time or not. About 50–60% of total immunoprecipitable ^{3}H-labeled ADP/ATP carrier applied appeared in the flow-through. This figure was not unexpected since chromatography on hydroxylapatite never results in complete recovery of the protein, a maximal yield of 70% being described for beef heart mitochondria (14). Passage of the *Neurospora* mitochondria through reticulocyte lysate may reduce the ability of ADP/ATP carrier to bind carboxyatractyloside and thus further reduce the yield. Elution of ^{35}S-labeled ADP/ATP carrier transferred *in vitro* was found only when valinomycin was absent during the transfer. The yield of ^{35}S-labeled precursor from the column was about 38%. Correction of this value for the observed loss of mature enzyme leads to an estimate of 70% for the efficiency with which precursor assembled into a functional form *in vitro*.

DISCUSSION

Several criteria indicate that precursor to ADP/ATP carrier transferred into mitochondria *in vitro* has been properly imported and assembled into a functional form. First, the imported precursor shows the same sensitivity to protease as authentic carrier assembled *in vivo*. Unassembled forms of precursor exhibited a much greater sensitivity. Second, the imported precursor developed the ability to bind carboxyatractyloside and in so doing acquired the chromatographic properties of authentic carrier. Binding of carboxyatractyloside is a specific and sensitive criterion for the functional ADP/ATP carrier. Carboxyatractyloside binds noncompetitively to the cytoplasmic side of the functional ADP/ATP carrier-dimer in the inner mitochondrial membrane (13). It thereby fixes the protein in one conformation so that it is disabled and cannot translocate nucleotides. Furthermore, this conformation protects the protein against proteolysis and, upon lysis of mitochondria, keeps it in a native form (15). The carboxyatractyloside-ADP/ATP carrier complex can thus be isolated by passage over hydroxylapatite (14). The uncomplexed protein is much more sensitive and upon lysis of mitochondria with detergent folds to a state unable to pass through hydroxylapatite. We oberved the same behavior by precursor transferred *in vitro*. Apparently, it became inserted into the membrane in such a way that the carboxyatractyloside-binding sites were on the correct side, namely the cytoplasmic side of the inner mitochondrial membrane. This membrane is impermeable to carboxyatractyloside, and thus, a precursor inserted in an inverted fashion could not be stabilized by carboxyatractyloside. Third, the mutually excluding interaction of imported precursor with NEM or carboxyatractyloside indicates that the imported carrier has gained the ability to reorient the nucleotide-binding sites in the inner membrane. These data taken together show clearly that in our *in vitro* system designed to study the import of proteins into mitochondria, the precursor to ADP/ADP carrier becomes assembled in a topologically correct fashion and gives rise to a functional dimeric complex in the membrane.

Other groups interested in biogenesis of mitochondrial proteins have investigated whether transfer of precursors *in vitro* leads to correctly processed and assembled enzymes. Mori *et al.* (20) observed that the precursor to ornithine transcarbamylase changes its $s_{20,w}$ value from 14 to 6.75 S after processing, with the $s_{20,w}$ value of the mature protein being 6.0 S. Recently, Gasser *et al.* (8) found about half the amount of precursors to cytochrome b_2 and isopropyl malate synthase transferred to the correct locations, the intermembrane space and the matrix of mitochondria, respectively, if the membrane potential had not been destroyed. Lewin and Norman (10) have shown that subunits of F_1-ATPase imported *in vitro* assemble into the F_1-ATPase complex. Schmidt *et al.* (9) demonstrated that the imported precursor to subunit 9 was assembled into the F_0F_1-ATPase in isolated mitochondria.

The results presented above represent a significant advance in the study of precursor import in that they present evidence that not only is the precursor imported *in vitro* but that it is also assembled into an active form with the same properties as the mature protein. This lends strong support to the idea that *in vitro* import systems can be employed to study indi-

vidual steps of the import process such as binding of precursors to receptors, translocation across membranes, and proteolytic processing.

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