Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 258, No. 7, Issue of April 10, pp. 4071–4074, 1983
Printed in U.S.A.

# Transfer of Proteins into Mitochondria

## PRECURSOR TO THE ADP/ATP CARRIER BINDS TO RECEPTOR SITES ON ISOLATED MITOCHONDRIA*

(Received for publication, November 30, 1982)

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**The precursor form of *Neurospora crassa* mitochondrial ADP/ATP carrier synthesized in a cell-free protein-synthesizing system can be imported into isolated mitochondria. If the mitochondrial transmembrane potential is abolished, import does not occur but the precursor binds to the mitochondrial surface. Upon reestablishment of the membrane potential, the bound precursor is imported. This occurs without dissociation of the bound precursor from the mitochondrial surface. We conclude that the binding observed represents an interaction with receptor sites and thus is an early step in the import pathway.**

Most mitochondrial proteins are coded for by nuclear genes and are synthesized outside the mitochondria on cytoplasmic polysomes. Studies using a number of proteins and organisms support a pathway for import in which mitochondrial proteins are synthesized as extramitochondrial precursor forms and are then imported into mitochondria post-translationally (1, 2).

One example, studied in detail in our laboratory, is the ADP/ATP exchange protein (ADP/ATP carrier) of the fungus *Neurospora crassa*. This protein mediates the exchange of ADP and ATP across the inner mitochondrial membrane allowing mitochondrially synthesized ATP to reach the cytosol (3, 4). This abundant protein (6% of the mitochondrial protein) is synthesized on free polysomes as an apparently soluble precursor and is then imported into mitochondria (5–7). In contrast to most other mitochondrial proteins, the precursor of the ADP/ATP carrier does not appear to differ in molecular weight as compared to the mature protein (8).

The import of ADP/ATP carrier has been reproduced *in vitro* using radiolabeled ADP/ATP carrier synthesized in a reticulocyte cell-free translation system (7). When isolated *N. crassa* mitochondria were added to the protein synthetic mixture, ADP/ATP carrier became associated with mitochondria and became resistant to externally added protease. The carrier so imported develops the ability to bind carboxyatractyloside, a specific inhibitor of the ADP/ATP carrier[1] (4). Thus, this *in vitro* assembly reaction appears to reflect uptake and assembly of ADP/ATP carrier into a functional state reminiscent of that observed *in vivo*.

We have previously observed (9) that agents which dissipate the mitochondrial membrane potential abolish the import of radiolabeled ADP/ATP carrier into the mitochondria. Under these conditions, the precursor instead binds to the mitochondria. This bound form is very sensitive to degradation by externally added protease and does not appear to interact with carboxyatractyloside.[1]

We wish to identify intermediates in the import process and to identify components of the mitochondrial machinery required for this process. It was thus of interest whether the binding of *in vitro*-synthesized ADP/ATP carrier precursor to mitochondria represented binding to authentic receptor sites. In this communication, we show that *in vitro*-synthesized ADP/ATP carrier bound to mitochondria in the absence of a membrane potential can be efficiently imported upon the establishment of such a potential. Further, evidence is presented that this import occurs directly from the bound state. We conclude that the binding of *in vitro*-synthesized ADP/ATP carrier is almost exclusively to receptor sites that are on the import pathway.

### MATERIALS AND METHODS

*Synthesis of Precursor to ADP/ATP Carrier and Preparation of Mitochondria*—Precursor to ADP/ATP carrier was synthesized in rabbit reticulocyte lysate as previously described (9). Where indicated, [$^{35}$S]methionine and *N. crassa* nucleic acids were omitted from otherwise normally treated lysates to produce "no synthesis" lysates lacking mitochondrial precursor proteins. Treatment of lysates following synthesis and the preparation of postribosomal supernatants were as previously described (9). Before use, lysates were made 100 μM in PMSF[2] by the addition of 1 μl/ml of lysate of a 0.1 M stock solution in ethanol. The inhibitors antimycin A and oligomycin were present in all lysates at final concentrations of 10 and 5 μM, respectively. Antimycin and oligomycin were added as a stock solution in ethanol (2.5 μl/ml of lysate) containing 4 mM antimycin A and 2 mM oligomycin.

Mitochondria from wild type *N. crassa* (strain 74A) were prepared from spheroplasts as previously described (9). The final mitochondrial pellet was suspended by homogenization in 300 mM sucrose, 10 mM Tris-HCl, pH 7.2, 0.1 mM EDTA and 0.1 mM PMSF. Mitochondria were used within 1 h of preparation.

*Binding and Import of ADP/ATP Carrier*—*In vitro*-synthesized ADP/ATP carrier was bound to mitochondria by incubating labeled reticulocyte lysate with isolated *Neurospora* mitochondria for 15 min at 25 °C in a 1.5-ml plastic microfuge tube. The volume of reticulocyte lysate and amount of mitochondria used in each experiment are given in the figure legends. After chilling to 4 °C, mitochondria were recovered from the reaction by centrifugation in a Sorvall SS-34 rotor fitted with adaptors for 1.5-ml microfuge tubes. Centrifugation was performed at 4 °C for 12 min at 15,000 × *g*. The tube containing the mitochondrial pellets was recentrifuged at 4 °C for 1 min in a microfuge to pellet residual fluid from the tube wall and the fluid was removed by aspiration. This centrifugation protocol was routinely used to reisolate mitochondria.

The mitochondria with bound precursor were resuspended in no synthesis lysate and used in the import experiments. In some cases,

* This work was supported by Deutsche Forschungsgemeinschaft Grant 101/18-9 and the Fonds der Dchemischen Industrie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a postdoctoral fellowship from the Alexander von Humboldt Stiftung.

[1] Schleyer, M., and Neupert, W., manuscript in preparation.

[2] The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TMPD, *N,N,N′,N′*-tetramethylphenylenediamine.

mitochondria were washed with no synthesis lysate prior to use in the import reactions. Centrifugation was as above with the mitochondria being transferred to new microfuge tubes with each wash.

Import of the bound carrier was carried out in a second incubation as described in the figure legends. As before, reactions were performed in 1.5-ml plastic microfuge tubes. After this second incubation, the mitochondria were assayed for total and imported ADP/ATP carrier.

Total ADP/ATP carrier was assessed by immediately reisolating mitochondria from a portion of the import reaction following the incubation. The mitochondrial pellet was dissolved and carrier immunoprecipitated as described below. Import was assayed as the amount of protease-resistant carrier associated with the mitochondria following the second incubation. The portion of the reaction to be assayed was made 50 μg/ml in Proteinase K (Boehringer Mannheim) by the addition of the appropriate amount of a 1-mg/ml stock solution. The sample was incubated for 60 min at 4 °C. The sample was then made 1 mM in PMSF by the addition of an aliquot of a 0.1 M stock solution in ethanol and the incubation continued for another 10 min. Mitochondria were then reisolated and ADP/ATP carrier immunoprecipitated.

*Immunoprecipitation of ADP/ATP Carrier*—ADP/ATP carrier was immunoprecipitated from Triton X-100 solubilized mitochondria essentially as previously described (9). In the present work, mitochondria were dissolved in 0.3 ml of Triton X-100/KCl buffer without the addition of *p*-chloromercuribenzoate. Immune complexes were isolated using Protein A-Agarose (Sigma). The times for interaction of antibody with antigen, and for the binding of immune complexes to Protein A-Agarose were 60 and 30 min, respectively. In all cases, conditions were such that immunoprecipitation was quantitative.

*Electrophoretic Analysis of Immunoprecipitates*—Immune complexes were dissociated by heating at 95 °C in a buffer containing 2% (w/v) SDS, 60 mM Tris-HCl, pH 6.8, 5% (v/v) 2-mercaptoethanol, and 10% (v/v) glycerol. Samples were then subjected to SDS-polyacrylamide gel electrophoresis as previously described (9). Gels were impregnated with sodium salicylate by the method of Chamberlin (10), dried, and radioactive bands visualized by exposing the dried gel to Kodak X AR-5 x-ray film.

Quantitation of the immunoprecipitated carrier was achieved by densitometry of the fluorograms. In all cases, multiple exposures of the fluorograms were examined to assure that the exposure analyzed was in the linear response range of the film.

*Other Methods*—Mitochondrial protein was determined by the dye-binding method of Bradford (11) using IgG as a standard. The procedure was as described by the commercial supplier (Bio-Rad). Mitochondria were disrupted with SDS prior to assay. Standards were constituted such that they reflected the buffer composition of the samples.

## RESULTS AND DISCUSSION

The demonstration that the binding of *in vitro*-synthesized ADP/ATP carrier to mitochondria represented binding to specific receptor sites was complicated by the nature of the precursor available for such experiments. As synthesized in reticulocyte lysate, the precursor ADP/ATP carrier is present in very low amounts. Further, ADP/ATP carrier precursor is just one of many precursors present since unfractionated mRNA was used to direct protein synthesis. These factors combined to make extremely difficult the application of many of the established criteria for identifying a specific receptor-ligand interaction such as the binding being of high affinity, reversible, and occurring at a limited number of sites (12). Therefore, we chose to take another approach to this problem *i.e.* to demonstrate that the binding of *in vitro*-synthesized ADP/ATP carrier is on the assembly pathway.

*Precursor to ADP/ATP Carrier Bound to Mitochondria in the Absence of a Membrane Potential Is Imported If Such a Potential Is Subsequently Established*—Mitochondria were incubated with reticulocyte lysate containing radiolabeled precursor in the presence of antimycin A and oligomycin. The former inhibitor prevents the formation of a mitochondrial membrane potential via the electron transport chain, while the latter prevents the formation of such a potential by the mitochondrial, proton-translocating ATPase (9, 13). At the end of the incubation, the mitochondria were recovered by centrifugation and resuspended in no synthesis lysate (lysate not containing labeled precursor, see "Materials and Methods") that contained antimycin A and oligomycin. Aliquots of this mitochondrial suspension were then reincubated with and without the establishment of a membrane potential (see below). Following this second incubation, the total amount of carrier and the amount of protease-resistant (imported) carrier associated with the mitochondria were determined.

As previously observed (7, 9), mitochondria incubated with radiolabeled ADP/ATP carrier in the absence of a membrane potential have ADP/ATP carrier associated with them upon reisolation (Fig. 1, *Reactions 1*, *2*, and *3*, *PROT K* (−)). In the continued absence of a membrane potential, this carrier was completely degraded by added protease (Fig. 1, *Reaction 1*, *PROT K* (+)), indicating that it had not been imported into the mitochondria. In contrast, when ascorbate and TMPD were included in the second incubation, appearance of protease-resistant carrier was observed (Fig. 1, *Reaction 2*, *PROT K* (+)). Ascorbate and TMPD act by injecting electrons into the electron transport chain at the level of complex IV, generating a membrane potential during the subsequent transfer of electrons to oxygen (13). That the membrane potential so generated was indeed responsible for the import of the bound ADP/ATP carrier was strongly suggested by the observation that cyanide, which prevents electron transfer through complex IV, completely abolished the import (Fig. 1, *Reaction 3*, *PROT K* (+)).

*Precursor to ADP/ATP Carrier Is Firmly Bound to the Mitochondria and Import from the Bound State Is Efficient*—The ADP/ATP carrier that associated with mitochondria in the absence of a membrane potential was resistant to repeated washing (Fig. 2, *Reactions 1* and *2 versus 3* and *4*). In the experiment shown, 80 to 90% of the carrier initially associated with mitochondria remained after two washes. Similar resistance to washing was seen in a number of separate

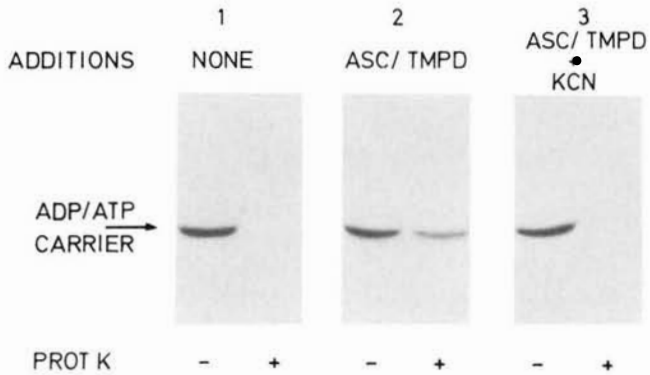


FIG. 1. **Precursor to ADP/ATP carrier binds to mitochondria in the absence of a membrane potential, and bound precursor is imported when a potential is established.** Precursor to ADP/ATP carrier was bound to mitochondria in a 525-μl reaction containing 25 μl of a suspension of isolated *Neurospora* mitochondria (200 μg of protein) and 500 μl of radiolabeled reticulocyte lysate. Mitochondria were recovered from the binding reaction and resuspended in 650 μl of no synthesis lysate. Aliquots (200 μl) of the mitochondrial suspension were transferred to new tubes (*Reactions 1–3*), and 2 μl of distilled water (*Reactions 1* and *2*) or 100 mM potassium cyanide (*Reaction 3*) were added. This was followed by the addition at 20-s intervals of either 4 μl of a solution containing 200 mM sodium ascorbate and 10 mM TMPD (*Reactions 2* and *3*) or 4 μl of water (*Reaction 1*). Samples were incubated at 25 °C for 10 min following this last addition. At the end of this incubation, all reactions were made chemically identical by the timed addition of the appropriate amounts of water, cyanide stock, and ascorbate/TMPD stock and the incubation continued for an additional 5 min. Total carrier (*Reactions 1–3*, *PROT K* (−)) and protease-resistant carrier (*Reactions 1–3*, *PROT K* (+)) associated with mitochondria were then detemined for each reaction.

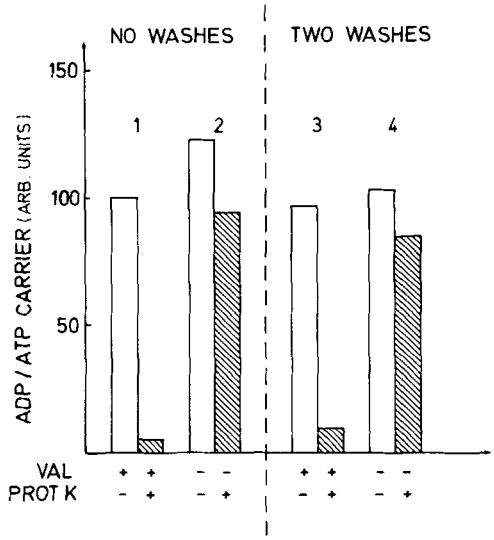


FIG. 2. **Precursor to ADP/ATP carrier binds tightly to mitochondria, and the import of the bound carrier is efficient.** Precursor to the ADP/ATP carrier was bound to mitochondria in a 1.1-ml reaction containing 1 ml of labeled reticulocyte lysate and 100 μl of mitochondrial suspension (700 μg of protein). The mitochondria with bound precursor were resuspended in 1 ml of no synthesis lysate after reisolation. One portion (480 μl) of this suspension was placed in a new tube and held on ice ("unwashed" mitochondria). A second 480-μl aliquot was placed in a new tube and the mitochondria pelleted by centrifugation. These mitochondria were then washed once more with, and finally resuspended in, 480 μl of no synthesis lysate ("twice washed" mitochondria). Import of the bound carrier was carried out in reactions constituted as follows: 220 μl of unwashed (*Reactions 1* and *2*) or twice washed (*Reactions 3* and *4*) mitochondria; 1.1 μl of 0.2 mM valinomycin in ethanol (*Reactions 1* and *3*) or 1.1 μl of ethanol (*Reactions 2* and *4*); and 4.4 μl of a solution containing 200 mM sodium ascorbate and 10 mM TMPD (all reactions). Ascorbate TMPD solution was added last at 15-s intervals and the reaction incubated for 10 min at 25 °C. The reactions were then made chemically identical by the timed addition of ethanol or valinomycin stock and the incubation continued for a further 2 min. Portions (105 μl) were then assessed for total (*PROT K* (−)) and protease-resistant (*PROT K* (+)) carrier associated with the mitochondria. Results are expressed in arbitrary units such that the amount of total carrier in *Reaction 1* equals 100.

experiments (not shown). The precursor thus seems to bind tightly to the mitochondria. Washing did not result in the acquisition of protease resistance by the precursor, indicating import had not occurred under these conditions (Fig. 2, *Reaction 3*).

In both the washed and unwashed mitochondria, bound carrier was efficiently imported if a membrane potential was allowed to develop during the second incubation (Fig. 2, *Reactions 2* and *4*). In the experiment shown, approximately 70% of the bound carrier became protease resistant. In other experiments (not shown), this value varied from 40 to 90%. Experiments with mitochondria labeled *in vivo* have revealed that authentic, mature ADP/ATP carrier displays incomplete (30 to 90%) resistance to protease digestion under the conditions used here.[1] This appears to be a function both of the protease concentrations employed and the degree of integrity of the mitochondria following isolation and experimental manipulation. We feel, therefore, that the bulk of the bound ADP/ATP carrier can be imported and that the binding of the radiolabeled precursor is almost exclusively to sites from which import can occur.

*Import of Bound Precursor Upon Energization of Mitochondria Occurs Without Release from the Binding Sites*—The stability of the binding of the ADP/ATP carrier precursor to the mitochondria and the efficiency of its subsequent import suggested that this import occurred directly from the binding site. It remained a possibility, however, that the precursor was bound to "nonfunctional" or "nonspecific" sites in the absence of a membrane potential and that, when the potential was re-established, the precursor dissociated from these sites and was then bound to and imported from different, "authentic" sites.

In order to differentiate between these alternatives, advantage was taken of the observation that, all other conditions being equal, the import of unbound carrier was decreased by dilution of the import reaction mixture with lysate containing no precursors. Examination of this effect revealed that it was the binding of the precursor that was decreased by the dilution (not shown). It seemed clear that if the bound precursor first dissociated from the mitochondria, its subsequent rebinding and import would be sensitive to dilution of the reaction mixture. In contrast, if import was occurring directly from the bound state, this reaction would be expected to be independent of the volume of the reaction.

In the experiment shown in Fig. 3, the effect of dilution on the import of bound and unbound carrier was examined. The import of unbound ADP/ATP carrier was strongly reduced

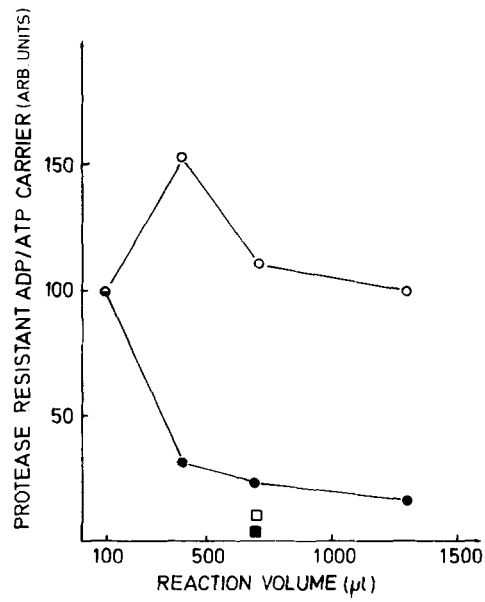


FIG. 3. **Import of bound precursor to ADP/ATP carrier is unaffected by dilution of the reaction mixture.** Mitochondria (*36* μl of suspension, 300 μg of protein) were incubated in two parallel reactions with either 600 μl of labeled reticulocyte lysate or with 600 μl of no synthesis lysate as appropriate for binding. The former reaction served as a source of mitochondria with bound precursor. The mitochondria were reisolated, washed once with 800 μl of no synthesis lysate, and were then resuspended in 600 μl of no synthesis lysate. Aliquots (100 μl) of the two mitochondrial suspensions (with and without bound precursor) were transferred to new microfuge tubes containing 0 μl, 300 μl, 600 μl, or 1200 μl of no synthesis lysate. 50 μl of labeled reticulocyte lysate was added to those samples containing mitochondria without bound precursor while those reactions containing bound precursor received 50 μl of no synthesis lysate. Import was immediately initiated by the addition of ascorbate and TMPD to final concentrations of 4 and 0.1 mM, respectively, and the reactions incubated for 30 min at 25 °C. Import of ADP/ATP carrier was assessed as protease resistance as before. The amount of carrier imported at each dilution is expressed in arbitrary units, with the amount of carrier imported in the undiluted reaction of each dilution series being taken as 100. ●——●, import of unbound precursor to ADP/ATP carrier; ○——○, import of bound precursor. The reactions containing 600 μl of added no synthesis lysate were run in duplicate with the duplicate reaction containing 1 μM valinomycin. ■, import of unbound precursor in the presence of valinomycin; □, import of bound precursor in the presence of valinomycin.

as the volume of the import reaction was increased (Fig. 3, *lower curve*). In sharp contrast, the import of bound ADP/ATP carrier into the mitochondria is virtually unaffected over the volume range employed. The continued validity of the protease assay under the conditions used as indicated by the fact that the abolition of the membrane potential by the potassium ionophore valinomycin completely prevented the appearance of protease-resistant ADP/ATP carrier (Fig. 3). This result strongly suggests that import of the bound precursor occurs without intermediate release of the precursor from the mitochondria.

The experiment described in Fig. 3 does not rule out possibilities such as release of the bound carrier into a "special" compartment not in equilibrium with the bulk, extramitochondrial solution or that some "activation" of the released precursor occurs, rendering its import resistant to dilution. On the other hand, the most straightforward interpretation of all the data presented here is that transfer of bound carrier occurs directly from the bound state. It thus appears that the binding exhibits one of the most important characteristics of a specific receptor-ligand interaction; it is connected to the biological process in question. We suggest therefore, that the binding of *in vitro*-synthesized precursor of the ADP/ATP carrier protein to isolated mitochondria represents binding to authentic receptor sites.

This report represents the second instance in which the binding of a precursor protein to mitochondria has been directly related to its import. We have previously described (14) conditions where apocytochrome *c*, the precursor to cytochrome *c*, may be bound to mitochondria and then imported. The import process of cytochrome *c* is different from that of the ADP/ATP carrier as it is independent of the mitochondrial membrane potential (15). Furtermore, the binding sites for apocytochrome *c* are distinct from those of the ADP/ATP carrier and a number of other proteins whose import is energy-dependent (15). This was shown by competition experiments employing very high concentrations of chemically produced apocytochrome *c*. The specific binding sites identified in the present work thus represent another type of binding site, one involved in the import of a mitochondrial precursor whose import is dependent upon the mitochondrial membrane potential.

*Acknowledgments*—We are grateful to Hannelore Strulik for expert and tireless technical assistance.

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