THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 258, No. 21, Issue of November 10, pp. 13340–13346, 1983
Printed in U.S.A.

Precursor Proteins Are Transported into Mitochondria in the Absence of Proteolytic Cleavage of the Additional Sequences*

(Received for publication, May 6, 1983)

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Many nuclear-coded mitochondrial proteins are synthesized as larger precursor polypeptides that are proteolytically processed during import into the mitochondrion. This processing appears to be catalyzed by a soluble, metal-dependent protease localized in the mitochondrial matrix. In this report we employ an *in vitro* system to investigate the role of processing in protein import. Intact *Neurospora crassa* mitochondria were incubated with radiolabeled precursors in the presence of the chelator *o*-phenanthroline. Under these conditions, the processing of the precursors of the β-subunit of F_1-ATPase ($F_1\beta$) and subunit 9 of the F_0F_1-ATPase was strongly inhibited. Protease-mapping studies indicated that import of the precursor proteins into the mitochondria continued in the absence of processing. Upon readdition of divalent metal to the treated mitochondria, the imported precursors were quantitatively converted to their mature forms. This processing of imported precursors occurred in the absence of a mitochondrial membrane potential and was extremely rapid even at 0 °C. This suggests that all or part of the polypeptide chain of the imported precursors had been translocated into the matrix location of the processing enzyme. Localization experiments suggested that the precursor to $F_1\beta$ is peripherally associated with the mitochondrial membrane while the precursor to subunit 9 appeared to be tightly bound to the membrane. We conclude that proteolytic processing is not necessary for the translocation of precursor proteins across mitochondrial membranes, but rather occurs subsequent to this event. On the basis of these and other results, a hypothetical pathway for the import of $F_1\beta$ and subunit 9 is proposed.

The import of newly synthesized mitochondrial proteins from their site of synthesis in the cytosol into the mitochondrion is a multistep process (1, 2). These proteins are released from the ribosomes as soluble cytosolic precursors. In many cases these precursor forms contain an NH_2-terminal peptide extension not found in the mature protein. The free precursors bind to the surface of the mitochondrion probably via specific receptor sites. They are then imported into the mitochondria by a process involving translocation into or through one or both of the mitochondrial membranes. In the case of those proteins which cross the inner membrane or are permanently inserted into it, this translocation is dependent on an electrical potential across the inner membrane.

During or shortly after the translocation step the NH_2-terminal peptide extensions present on many precursors are removed (1, 2). The role of this proteolytic processing in the assembly process and the molecular mechanism by which it is accomplished are presently unclear. Recently, proteolytic activities which can remove part or all of the peptide extensions have been detected in extracts of mitochondria from rat liver and heart (1, 3–6), the yeast *Saccharomyces cerevisiae* (3, 7), and the filamentous fungus *Neurospora crassa*.[1] The activities detected show striking similarities: all are metal-dependent, requiring the presence of divalent metal ions such as manganese or zinc ions for full activity. Accordingly, they are all inhibited by chelating agents capable of complexing these metals. In general these activities are unaffected by a wide variety of other protease inhibitors. It has been shown that the activities from yeast (3), rat (3–5), and *Neurospora*[1] are apparently soluble constituents of the mitochondrial matrix. In contrast to this, analogous processing enzymes in bacteria and endoplasmic reticulum (8) are integral membrane proteins. Some purification of the various mitochondrial processing activities has been achieved (6, 7),[2] however, they are not currently available in pure form.

We have sought to elucidate the role of proteolytic processing in the import process. In particular, we have tried to determine whether processing is an obligate step or whether import into the correct mitochondrial compartment can occur without removal of the additional sequences. We have used the cell-free precursor transport system developed in this laboratory (see Ref. 1 for review) to examine the individual steps of the import process. In this system, precursors to *Neurospora* mitochondrial proteins are synthesized in a reticulocyte cell-free protein-synthesizing system. When these precursors are incubated with isolated *Neurospora* mitochondria, binding, translocation, and processing of precursors occur.

The effects of chelating agents known to inhibit the soluble processing activity extractable from *Neurospora* mitochondria on the cell-free transport system were examined. We find that in the presence of EDTA, micromolar concentrations of the hydrophobic chelating agent *o*-phenanthroline (1,10-phenanthroline) inhibit the processing of the precursors to subunit 9[3] (proteolipid, dicyclohexylcarbodiimide-binding protein) and the β-subunit (subunit 2) of the mitochondrial proton-translocating ATPase. Under the conditions employed the

* This work was supported by the Deutsche Forschungsgemeinschaft (Ne/101/18-9) and by the Fonds der Chemischen 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 Stiftang.

[1] B. Schmidt and W. Neupert, manuscript in preparation.

[2] B. Schmidt, unpublished data.

[3] The abbreviations used are: subunit 9, subunit 9 of F_0F_1-ATPase; $F_1\beta$, subunit 2 of F_0F_1-ATPase; F_0F_1-ATPase, mitochondrial proton-translocating ATPase; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride.

uncleaved precursors are imported into mitochondria in an energy-dependent manner. Localization studies suggest that the imported precursors are attached to mitochondrial membranes. The precursor to $F_1\beta$ appears to be peripherally bound to the membrane as a considerable portion is extractable by salt, while that of subunit 9 is more tightly associated, possibly as an integral membrane protein. The results indicate that import and correct localization of mitochondrial precursors can occur in the absence of proteolytic processing. Therefore the transmembrane movements of protein into mitochondria appear not to be obligatorily coupled to precursor processing. This result is discussed in relation to current models of protein import into mitochondria.

MATERIALS AND METHODS

Growth of Neurospora and Preparation of Mitochondria—*N. crassa* wild type 74A was grown in Vogel's minimal medium for 14–15 h as previously described (9). Following harvest by filtration, the cells were converted to spheroplasts and mitochondria isolated as previously described (9). The final mitochondrial pellet was suspended at 2–6 mg of protein/ml in SET buffer (0.3 M sucrose, 0.01 M Tris-HCl, pH 7.2, 0.001 M EDTA, 10^{-4} M PMSF). Mitochondria were used within 90 min of preparation.

Synthesis of Neurospora Proteins in Reticulocyte Lysate—[^{35}S]Methionine-labeled *Neurospora* proteins were synthesized in rabbit reticulocyte lysate as described in Ref. 9 except that the synthesis time was reduced to 30 min. A postribosomal supernatant was then prepared (9) and used in the transfer reaction (see below).

Transfer of Proteins from Reticulocyte Lysate into Mitochondria—For *in vitro* transfer of proteins into mitochondria, isolated *Neurospora* mitochondria were added to reticulocyte lysate to a concentration of 0.2–0.3 mg/ml. The mitochondrial suspension (in SET buffer) typically constituted 1/10 to 1/20 of the total reaction volume. Transfer was performed for 20–40 min at 25 °C. EDTA was added to reactions such that the final concentration was 5 mM. Where indicated, valinomycin, *o*-phenanthroline, antimycin A, and oligomycin (all from Sigma) were added such that their final concentrations were 0.5 μM, 50–100 μM, 10 μM, and 5 μM, respectively. These agents were added as concentrated stocks in ethanol such that the ethanol concentration during transfer was 0.5% (v/v) or less. To reactions not containing these agents was added an equal volume of ethanol. At the end of the transfer incubation, energy inhibitors and *o*-phenanthroline were added to those reactions that did not contain those agents and the incubation continued for 3 min. Transfer reactions were then chilled on ice.

Mitochondria were recovered from the transfer reaction mixture by centrifugation for 12 min, 20,000 × *g* at 4 °C. This centrifugation was always performed using 1.5-ml plastic microfuge tubes centrifuged in a Sorvall SS-34 rotor fitted with adaptors. Residual fluid on the tube wall was pelleted by a brief centrifugation in a microfuge and removed by aspiration.

Immunoprecipitation of ATPase Subunit 9 and F_1-ATPase Subunit—ATPase subunit 9 was immunoprecipitated using antisera prepared as previously described (9). Rabbit IgG specific for the β-subunit of *N. crassa* F_1-ATPase was the generous gift of Dr. Walter Sebald (Gesellschaft fur Biotechnologische Forschung, Braunschweig-Stoeckheim). This antibody was raised to purified β-subunit isolated from F_1-ATPase (14) essentially according to the method of Tzagaloff (15).

Mitochondria were dissociated in SDS prior to immunoprecipitation. When mitochondrial pellets were analyzed they were dissolved in 25 to 50 μl of 2% SDS (w/v), 60 mM Tris-HCl, pH 6.8, 5% (v/v) 2-mercaptoethanol, and heated at 95 °C for 3 min. The samples were then diluted with 1 ml of 1% (v/v) Triton X-100, 0.3 M KCl, 1 mM PMSF, 1 μM *o*-phenanthroline. When a suspension of mitochondria was analyzed, the sample (usually 100–200 μl) was made 1% SDS (w/v), 30 μM Tris-Cl, pH 6.8, 2.5% 2-mercaptoethanol, and then heated. Triton X-100/KCl buffer was added as above. Prior to immunoprecipitation, samples were clarified by centrifugation at 20,000 × *g* for 10 min at 4 °C.

Subunit 9 was precipitated from solubilized mitochondria using antibody bound to protein A-agarose (Sigma) as previously described (9). $F_1\beta$ subunit was precipitated following preabsorption of the mitochondrial extract with one-half the amount of heat-killed formalin-fixed *Staphylococcus aureus* cells (10) eventually used to harvest immune complexes. To the preasorbed sample was added anti-$F_1\beta$ IgG in an amount seen in preliminary experiments to be sufficient for complete precipitation from the quantity of mitochondria used in the experiment. After 90 min of incubation at 4 °C, immune complexes were harvested and washed as previously described (9).

Subunit 9 immunoprecipitates were dissociated as previously described (9) in 2% SDS (w/v), 60 M Tris HCl, pH 6.8, 5% (v/v) 2-mercaptoethanol. Precipitates of $F_1\beta$ were dissociated in the same buffer by heating at 95 °C for 3 min. Electrophoretic analysis of the precipitates was performed using gels containing 16% acrylamide (9).

Other Methods—Mitochondrial protein was determined using the dye-binding method of Bradford (11) (Bio-Rad). Fluorography of the SDS gels and visualization of radioactive bands was as before (12). Quantitation of fluorograms by densitometry was as before (12).

RESULTS

Transport and Processing of ATPase Subunit 9 and β-Subunit of F_1-ATPase in Vitro—Incubation of isolated *Neurospora* mitochondria with radiolabeled reticulocyte lysate leads to the energy-dependent uptake and processing of the precursor to ATPase subunit 9 (13) (Fig. 1*B*, *lane 1 versus lane 5*). This processing has been shown to produce the correct NH_2 terminus and the *in vitro* imported protein is associated with the proton-translocating ATPase (13).

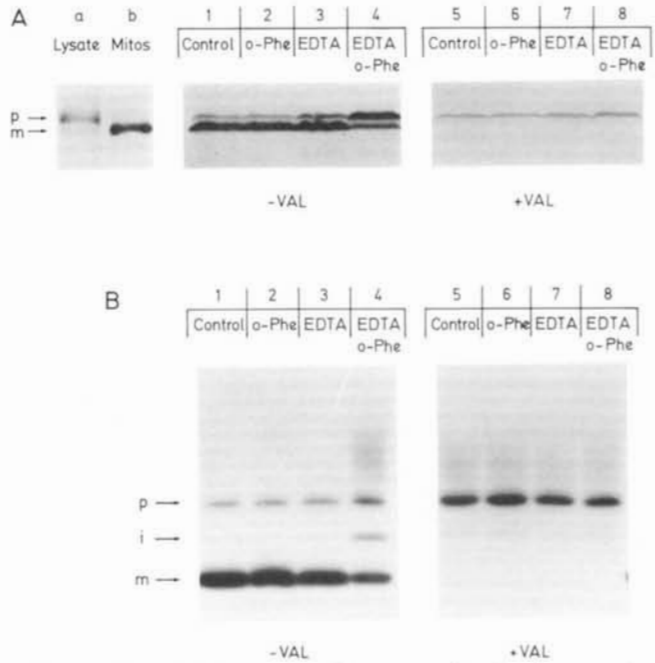

FIG. 1. **Metal chelators inhibit processing by intact mitochondria.** *A*, β-subunit of F_1-ATPase. Isolated *Neurospora* mitochondria were incubated with [^{35}S]methionine-labeled reticulocyte lysate as described under "Materials and Methods." Reactions contained: *lanes 1* and *5*, no additions; *lanes 2* and *6*, 100 μM *o*-phenanthroline; *lanes 3* and *7*, 5 μM EDTA; *lanes 4* and *8*, 5 μM EDTA and 100 μM *o*-phenanthroline. Transfer was performed in the presence (*lanes 5* through *8*) or absence (*lanes 1* through *4*) of 0.5 μM valinomycin (*VAL*). After completion of transfer the mitochondria were reisolated by centrifugation and the β-subunit of *Neurospora* F_1-ATPase was immunoprecipitated. *Tracks a* and *b* show β-subunit of F_1-ATPase immunoprecipitated from reticulocyte lysate (*track a*) and from [^{35}S]sulfate-labeled *Neurospora* mitochondria (*track b*). Dissociation of these precipitates was performed at 4 °C. Under these conditions the heavy chain of IgG migrated at an apparent molecular weight of 70,000 rather than 55,000. In the latter case a small influence on the migration of precursor of $F_1\beta$ and mature $F_1\beta$ was noticeable. *B*, subunit 9 of F_1F_0-ATPase (proteolipid, dicyclohexylcarbodiimide-binding protein). Transfer reactions were constituted as in *A* with the exception that the *o*-phenanthroline (*o-Phe*) concentration was 50 μM. Mitochondria reisolated after transfer were precipitated with antibody to ATPase subunit 9. *p*, precursor; *i*, intermediate; *m*, nature.

In the present work we were also interested in the β-subunit of *Neurospora* F_1-ATPase. As shown in Fig. 1*A* (*track a*) antibody specific for *Neurospora* $F_1\beta$ precipitated a radiolabeled polypeptide from reticulocyte lysate with an apparent molecular weight of 58,000. The same antibody precipitated a polypeptide from metabolically labeled *Neurospora* mitochondria of 56,000 apparent molecular weight (Fig. 1*A*, *track b*). This corresponds to the reported molecular weight for the β-subunit of the F_1-ATPase in *Neurospora* (16). We assume that the polypeptide precipitated from reticulocyte lysate represents the precursor form of *Neurospora* $F_1\beta$ analogous to the precursors detected for numerous mitochondrial proteins including the β-subunit of yeast F_1-ATPase (1, 2, 17, 18).

Incubation of radiolabeled reticulocyte lysate containing precursor of $F_1\beta$ with isolated mitochondria led to the appearance of a labeled polypeptide with the electrophoretic mobility of mature $F_1\beta$ (Fig. 1*A*, *lane 1*). The formation of mature $F_1\beta$ was inhibited by valinomycin *lane 5*) and by a combination of antimycin A and oligomycin (not shown), indicating the reaction requires the mitochondrial membrane potential (9). The mature $F_1\beta$ produced in this reaction was associated with mitochondria and was resistant to added protease if the mitochondrial permeability barrier was intact (Fig. 3, see below). We conclude that isolated *Neurospora* mitochondria import and process the precursor to $F_1\beta$ in a manner similar to other precursors studied in this system (9, 13, 18, 19).

o-Phenanthroline Inhibits the Processing of Precursor of Subunit 9 and Precursor $F_1\beta$ by Intact Mitochondria—Experiments with extracts of *Neurospora* mitochondria have revealed the presence of an activity capable of converting the precursors of subunit 9 and $F_1\beta$ to their mature sizes.[1] This activity is sensitive to chelating agents such as *o*-phenanthroline and EDTA as are similar activities described by others. To examine the effect of chelating agents on the processing and import of precursors by mitochondria, isolated mitochondria were incubated in reticulocyte lysate containing EDTA and *o*-phenanthroline. The chelating agents when applied together inhibited the appearance of mature sized $F_1\beta$ (Fig. 1*A*, *lane 1 versus lane 4*) and subunit 9 (Fig. 1*B*, *lane 1 versus lane 4*) associated with mitochondria. This inhibition was accompanied by an accumulation of the precursor forms. In the case of $F_1\beta$ the amount of accumulated precursor equaled the decrease in the amount of mature form. As previously noted (13), antisera to subunit 9 used in this laboratory only poorly recognizes the precursor to subunit 9 making a similar comparison for subunit 9 difficult. In the case of subunit 9 there also consistently appeared a prominent labeled polypeptide with a mobility intermediate between precursor and mature subunit 9 upon SDS-gel electrophoresis (Fig. 1*B*, *lane 4*). For convenience this polypeptide will be referred to as an intermediate subunit 9 although no claim is made here that it represents a true intermediate in subunit 9 import.

The inhibition of precursor processing by EDTA and *o*-phenanthroline required the presence of both chelators, each agent alone being ineffective (Fig. 1, *A* and *B*, *lanes 2* and *3 versus 4*). Titration of the amount of each chelator required indicated that 50 μM *o*-phenanthroline and 3 μM EDTA were just sufficient to observe inhibition. Increasing the concentrations of the chelators beyond these levels did not significantly improve the extent of inhibition over that seen in Fig. 1. Preincubation of the mitochondria with a mixture of EDTA and *o*-phenanthroline also failed to improve the extent of inhibition.

The differential effects of EDTA and *o*-phenanthroline are apparently due to different accessibilities to the matrix processing enzyme. Thus EDTA, a charged molecule, is ineffective since it cannot penetrate the inner membrane and enter the matrix. The relatively hydrophobic and uncharged *o*-phenanthroline molecule would be expected to penetrate the membrane and thus reach the matrix processing enzyme. This interpretation is strengthened by the observation that the charged phenanthroline derivative, bathophenanthroline disulfonic acid, cannot replace *o*-phenanthroline (not shown). Bathophenanthroline disulfonic acid is an effective inhibitor of processing in extracts.[1]

We assume that EDTA acts to chelate extramitochondrial metal in the transfer reactions which could otherwise complex the small amounts of *o*-phenanthroline used here. Attempts to circumvent this problem by simply increasing the amount of *o*-phenanthroline were unsuccessful. At levels of phenanthroline sufficient to affect processing (4–6 mM), very little precursor associated with the mitochondria suggesting that the mitochondria had been severely damaged (data not shown).

EDTA and *o*-phenanthroline, as well as the combination of the two, did not affect the binding of the precursors as indicated by the number of the precursors associated with mitochondria in the absence of a membrane potential (Fig. 1, *A* and *B*, *lanes 5–8*). This suggests that the chelators do not seriously interfere with the interaction of the precursors with the receptors.

Transfer of Proteins into Mitochondria Continues in the Presence of EDTA and o-Phenanthroline—The possibility was considered that the combination of *o*-phenanthroline and EDTA damaged the mitochondria in a nonspecific manner. We thus assessed the ability of mitochondria treated with EDTA and *o*-phenanthroline to import the precursor of the mitochondrial ATP/ADP carrier protein. Import of the carrier does not involve the proteolytic removal of a leader sequence (20, 21). If EDTA and phenanthroline act specifically by inhibition of the matrix protease, the import of carrier should be unaffected by these agents.

When the reactions of Fig. 1*B* were tested for carrier import, as defined by energy-dependent appearance of protease-resistant carrier (21), no effect of EDTA plus *o*-phenanthroline *versus* EDTA alone was observed (Fig. 2, *lane 5 versus 6*). The processing of precursor of subunit 9 in the same reactions (Fig. 1*B*, *lane 3 versus 4*) was strongly depressed in the presence of *o*-phenanthroline. In another experiment (not shown) the transfer of carrier was seen to be resistant to *o*-phenanthroline at concentrations up to 450 μM in the presence

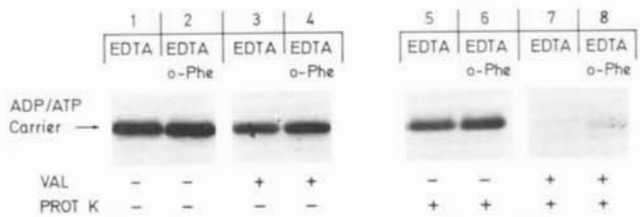

FIG. 2. **Transfer of the ADP/ATP carrier protein into mitochondria is not affected by *o*-phenanthroline.** Aliquots of the transfer reactions in Fig. 1*B* containing either EDTA or EDTA plus *o*-phenanthroline (*o-Phe*) in the absence of valinomycin (*VAL*) were removed. These aliquots were then divided in two. Mitochondria were immediately recovered from one portion of each reaction (*lanes 1–4*), dissolved, and immunoprecipitated for ATP/ADP carrier. To the second portion of each reaction (*lanes 5–8*) were added proteinase K (*PROT K*) (100 μg/ml) and the sample incubated for 60 min at 4 °C. Proteolysis was halted by addition of PMSF to 1 mM and incubation continued for 10 min at 4 °C. Mitochondria were then reisolated and ATP/ADP carrier precipitated. *Lanes 1–5*, transfer in the presence of 5 μM EDTA; *lanes 2* and *6*, 5 μM EDTA and 50 μM *o*-phenanthroline; *lanes 3* and *7*, 5 μM EDTA and 0.5 μM valinomycin; *lanes 4* and *8*; 5 μM EDTA, 50 μM *o*-phenanthroline, 0.5 μM valinomycin.

of EDTA. Thus the effect of EDTA and *o*-phenanthroline appears to be specific for the processing step.

Precursors Accumulated in the Presence of EDTA and o-Phenanthroline Are Imported into Mitochondria—To determine whether the precursor forms seen above were transferred into mitochondria, we applied the generally accepted criterion of protease resistance. As seen in Fig. 3*A* incubation of mitochondria with labeled reticulocyte lysate in the presence of EDTA and phenanthroline led to the accumulation of precursor of $F_1\beta$ as before (*lane 7 versus 1*). When mitochondria recovered from the reaction were treated with proteinase K, a considerable portion of the accumulated precursor of $F_1\beta$ was seen to be protease-resistant (*lane 10*). If the mitochondrial permeability barrier was disrupted by Triton X-100 (*lane 11*) or sonication (*lane 12*) in the presence of protease, this resistant material was virtually eliminated. A similar pattern of protease sensitivity was observed for mature $F_1\beta$ in reactions lacking *o*-phenanthroline (Fig. 3*A*, *lane 4*). Protease-resistant precursor of $F_1\beta$ was consistently observed in the absence of *o*-phenanthroline albeit in greatly reduced amounts. This material was also digested in the presence of detergent or upon sonication and possibly reflects inefficient processing of imported precursor of $F_1\beta$. In all conditions of Fig. 3*A*, no protease-resistant material was associated with mitochondria from reactions containing valinomycin (not shown).

Similar results were obtained for precursor of subunit 9 accumulated in the presence of EDTA and *o*-phenanthroline. When the reactions of Fig. 3*A* were precipitated for subunit 9 both precursor of subunit 9 and intermediate subunit 9 were seen to be proteinase K-resistant (Fig. 3*B*, *lane 7 versus 10*). Disruption of the mitochondria with detergent (*lane 8 versus 11*) or sonication (*lane 9 versus 12*) led to the complete loss of proteinase-resistant precursor subunit 9 and intermediate subunit 9. The diffuse band of labeled material with mobility slightly greater than mature proteolipid visible in some of the protease-treated samples (Fig. 3*B*, *lanes 5, 6, 11,* and *12*) represents fragments of precursor of subunit 9 and mature subunit 9 (13). Precursor of subunit 9 that associated with mitochondria in the absence of a membrane potential was protease-sensitive regardless of whether *o*-phenanthroline was present during the transfer reaction or not (not shown).

We conclude that, by the criterion of protease resistance, transfer of precursor of $F_1\beta$ and precursor subunit 9 into mitochondria continue in the absence of proteolytic processing.

Mode of Association of Imported Precursors with the Mitochondria—Subunit 9 is a very hydrophobic, integral protein of the mitochondrial inner membrane (16). $F_1\beta$, on the other hand, is a subunit of the large, essentially soluble F_1-ATPase complex which is peripherally associated with the inner membrane (16). We were thus interested in determining whether

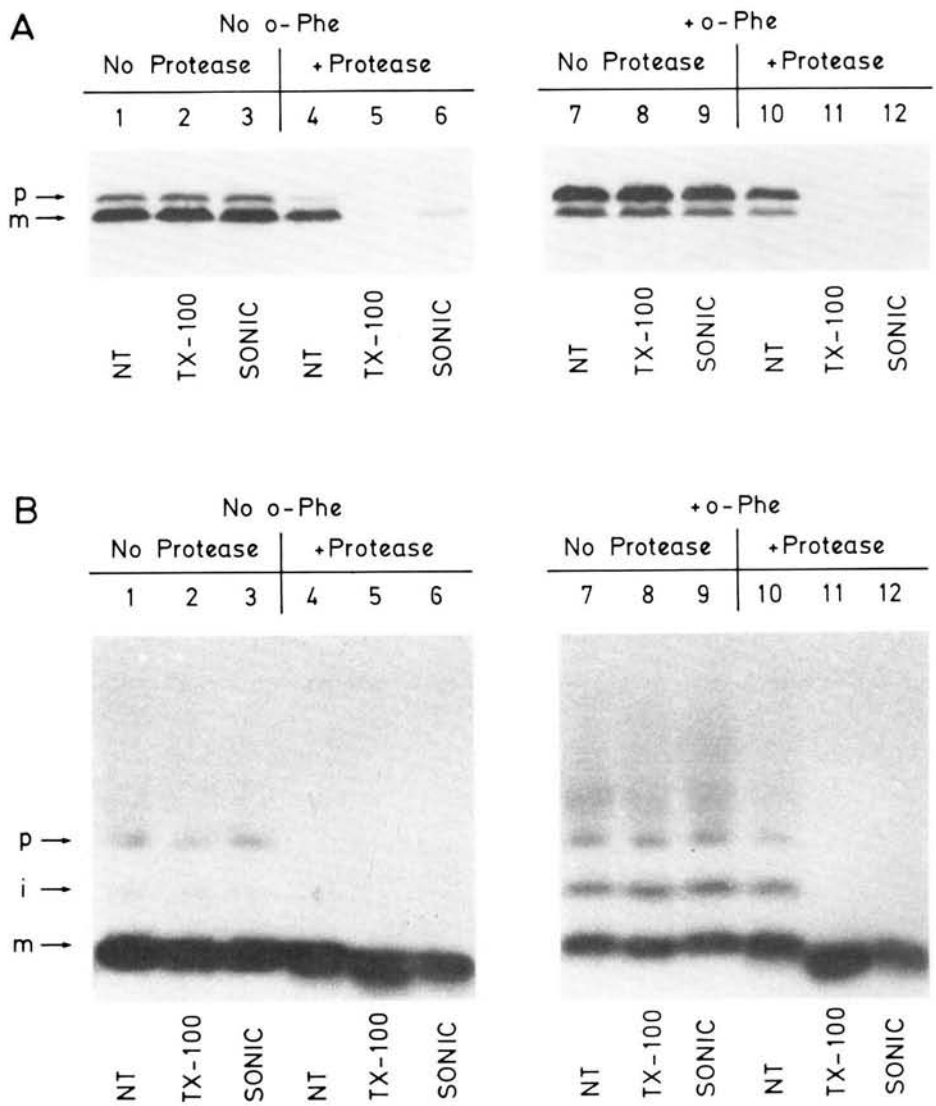

FIG. 3. **Precursor proteins transferred into mitochondria in the presence of *o*-phenthroline are in a protease-protected location.** *A*, β-subunit of F_1-ATPase. Transfers without (*lanes 1–6*) and with (*lanes 7–12*) 100 μM *o*-phenanthroline (*o-Phe*) were performed as described under "Materials and Methods." Reactions were stopped by addition of valinomycin and the mitochondria pelleted by centrifugation. Mitochondria were resuspended at 108 μg/ml in SET buffer containing 100 μM *o*-phenanthroline and 100 μM dithioerythritol. Each of the suspensions was divided into two portions one of which received proteinase K (20 μg/ml, Boehringer Mannheim GmbH, FRG) in SET buffer, while the other received only SET buffer. The portions were then further divided into three 200-μl aliquots. One aliquot was kept as control, the second was lysed with Triton X-100, and the third was subjected to sonication. All 12 samples were then incubated for 50 min on ice. Following this incubation, PMSF (2 mM) was added and the samples kept for 10 min on ice to stop proteolysis. Triton X-100 (0.25%) was then added to those samples not containing Triton X-100. Immunoprecipitation for $F_1\beta$ was then performed. *Lanes 1, 4, 7,* and *10*, no treatment and no additions (*NT*); *lanes 2, 5, 8,* and *11*, plus 0.25% Triton X-100 (*TX-100*); *lanes 3, 6, 9,* and *12*, 30-s sonication with a Model 220-F Sonicator with microtip (Heat Systems-Ultrasonics Inc., Plainview, NY) at an output of 40 W (SONIC). *Lanes 1–3, 7,* and *8*, no protease treatment; *lanes 4–6* and *10–12*, treatment with proteinase K. *B*, subunit 9 of F_oF_1-ATPase. The reactions of *A* were immunoprecipitated for ATPase subunit 9 following precipitation of $F_1\beta$. *p*, precursor; *i*, intermediate; *m*, mature.

the transferred precursors represented membrane-bound or soluble forms.

In the experiment of Fig. 4, mitochondria were allowed to import proteins in the presence of *o*-phenanthroline. The mitochondria were reisolated and suspended in buffer containing 0.15 M NaCl, EDTA, and *o*-phenanthroline. Nonimported proteins were removed by proteolysis and one portion of the suspension adjusted to 1 M NaCl. The mitochondria were disrupted by sonication and the sonic extracts were separated into soluble and pelletable material by ultracentrifugation. The efficiency of removal of mitochondrial membranes was monitored by following the removal of the porin (22), the major protein of the outer membrane, and the ATP/ADP carrier (23, 24), the major protein of the inner membrane. Immunoreplica analysis (25) indicated that in either low or high salt the supernatant fractions contained 10% or less of the porin and ATP/ADP carrier present in the unfractionated sonic extract (not shown). In the same samples, more than 90% of the soluble matrix enzyme citrate synthase (26, 27) (EC 4.1.3.7) was found in the high speed supernatant.

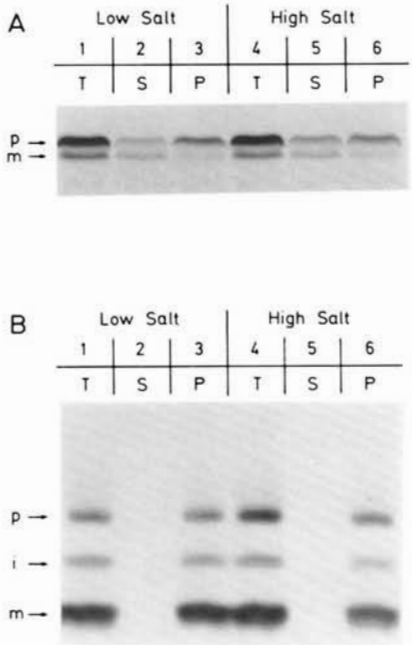

FIG. 4. **Association of imported precursors with mitochondrial membranes.** *A*, β-subunit of F_1-ATPase. Mitochondria were incubated with reticulocyte lysate supernatant containing EDTA and *o*-phenanthroline (100 μM). Transfer was topped by the addition of antimycin A and oligomycin to 10 and 5 μM, respectively. The mitochondria were reisolated and suspended at a protein concentration of approximately 1 mg/ml in NTP buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.2, 0.1 mM EDTA, 0.5 mM *o*-phenanthroline, 10 mM antimycin A, 5 μM oligomycin). Proteinase K was added to 10 μg/ml and the samples incubated for 30 min at 4 °C. Proteolysis was stopped by the addition of 0.5 mM PMSF. Parallel digestion of an aliquot of the mitochondrial suspension in the presence of 0.1% (v/v) Triton X-100 showed no protease-resistant material. The proteolyzed mitochondria were washed once with NTP buffer plus 0.1 mM PMSF and then resuspended in the original volume of that buffer. The sample was halved and one-half was adjusted to 1 M NaCl by the addition of one-third volume of NTP buffer containing 3 M NaCl. The other received normal NTP buffer. The two portions were then sonicated twice for 30 s at an output of 40 W. Samples were cooled in ice water and 2 min were allowed between sonications. Immediately following sonication, dithioerythritol was added to 0.5 mM. The sonic extracts were then divided into two equal portions. One was precipitated for radiolabeled $F_1\beta$ polypeptides (*T*; *lanes 1* and *4*) and served as total $F_1\beta$. The other portion was centrifuged for 60 min, 144,000 × *g* at 4 °C. The supernatant was removed and analyzed for radiolabeled $F_1\beta$ and for various mitochondrial markers (*S*; *lanes 2* and *5*). The pellet was resuspended in the original volume of NTP buffer plus 1% (w/v) SDS and analyzed as was the supernatant (*P*; *lanes 3* and *6*). *B*, ATPase subunit 9 was precipitated from the total sonic extract, supernatant, and pellet samples after they had been precipitated for $F_1\beta$. *p*, precursor; *i*, intermediate; *m*, mature.

When sonication was performed in the presence of 0.15 M NaCl the majority of the precursor of $F_1\beta$ was found in the high speed pellet (Fig. 4*A*, *lanes 1–3*). Quantitation of appropriately exposed fluorograms indicated that 70% of the recovered precursor of $F_1\beta$ (85% of total) was pelletable, while 30% was found in the supernatant. Mature $F_1\beta$ partitioned predominantly into the supernatant, with about 60% of the recovered material (84% of total) being soluble. The same result was obtained with mature $F_1\beta$ produced in the absence of *o*-phenanthroline (not shown). This behavior resembled that of pre-existing $F_1\beta$ as immunoreplica analysis (not shown) indicated that at least 50% of the pre-existing $F_1\beta$ was found in the supernatant fraction. This result was as expected given the reported (15, 16) tendency of *Neurospora* F_1-ATPase to dissociate from the membrane and dissociate into subunits under conditions similar to those used here. In the presence of 1 M NaCl 48% of the recovered precursor of $F_1\beta$ (81% of total) was found in the supernatant with the remainder being pelletable (Fig. 4*A*, *lanes 4–6*). This increase of precursor of $F_1\beta$ in the supernatant was not accompanied by an increase in membrane markers or precursor of subunit 9 (below). We suggest these data are consistent with imported precursor of $F_1\beta$ being peripherally bound to mitochondrial membranes.

In contrast to precursor of $F_1\beta$, almost all of the recovered (75–100%) precursor and intermediate subunit 9 were found in the pellet fractions (Fig. 4*B*). Increasing the salt concentration to 1 M had no effect on the distribution of precursor and intermediate subunit 9. Thus, unlike precursor of $F_1\beta$, the accumulated precursor and intermediate subunit 9 appears strongly associated with the mitochondrial membrane.

Precursors Imported in the Presence of o-Phenanthroline Can Be Processed to Mature Size in Intact Mitochondria—An important question raised by the apparent import of precursor of $F_1\beta$ and precursor of subunit 9 is whether the imported precursors represent true intermediates in the import process. One alternative, for example, is that transfer in the presence of EDTA and *o*-phenanthroline leads to the accumulation of precursor in a denatured form or in a compartment that does not contain processing activity (*e.g.* intermembrane space). Experiments with extracts from *Neurospora* mitochondria had shown that the inhibition of the processing activity by chelating agents was fully reversible upon addition of excess metal ions.[2] We therefore investigated this question by observing the effects of adding divalent metal ions to mitochondria containing imported precursors.

Precursor of $F_1\beta$ (Fig. 5*A*) and precursor of subunit 9 (Fig. 5*B*) were accumulated by transfer in the presence of *o*-phenanthroline. Following reisolation, the mitochondria were incubated with (*lane 2*) and without (*lane 1*) excess $MnCl_2$. Nearly complete conversion of precursor of $F_1\beta$ occurred in the presence of manganese ions. When subunit 9 was precipitated from the same reactions (Fig. 5*B*) precursor and intermediate subunit 9 were seen to disappear and the amount of label with the mobility of mature subunit 9 increased. Identical results were obtained when following transfer, the mitochondria were treated with protease prior to addition of metal to ensure that only imported molecules contributed to the data (Fig. 5, *A* and *B*, *lanes 3* and *4*). When protease-treated mitochondria incubated with manganese ions were reisolated by centrifugation, all of the mature size $F_1\beta$ pelleted indicating the mitochondria were still intact. Since the buffer used was the same as that used in the localization experiments of Fig. 4, considerable mature $F_1\beta$ would have been expected

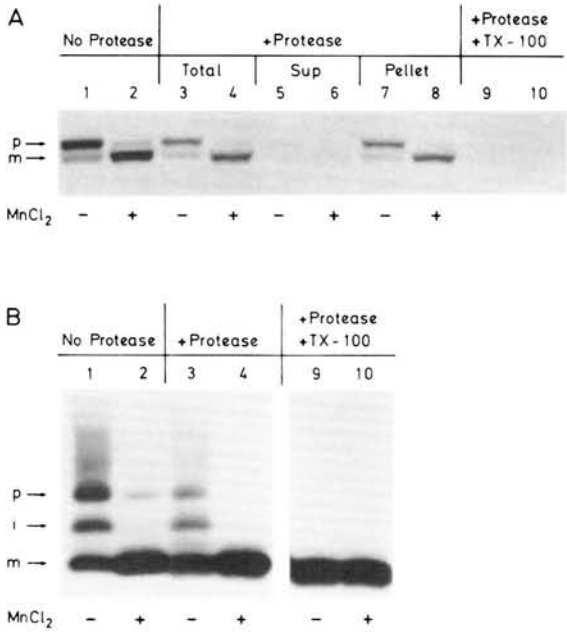

FIG. 5. **Precursors imported in the presence of *o*-phenanthroline can be processed to mature size in intact mitochondria.** *A*, β-subunit of F_1-ATPase. Mitochondria were incubated in reticulocyte lysate supernatant in the presence of EDTA and *o*-phenanthroline. Import was stopped by the addition of antimycin A and oligomycin. The mitochondria were then reisolated and suspended at 0.73 mg/ml in NTP buffer conaining antimycin A (10 μM) and oligomycin (5 μM). One portion (250 μl) of this suspension was removed, received NTP buffer, and was incubated for 40 min at 4 °C (*total; lanes 1* and 2). A second aliquot (500 μl) was treated identically except it received 20 μg/ml of proteinase K in NTP buffer. (*+protease; lanes 3–8*). A third aliquot (250 μl) also received proteinase K and, in addition, 0.1% (v/v) Triton X-100 (*+protease, +TX-100; lanes 9* and *10*). Proteolysis was halted by the addition of 0.5 mM PMSF. Each of the samples above was halved. One-half received 5 μl of 25 mM $MnCl_2$ (*lanes 2, 4, 6, 8,* and *10*) while the other received 5 μl of water (*lanes 1, 3, 5, 7,* and *9*). Samples were incubated for 20 min at 25 °C at which time EDTA was added to 5 mM. The unproteolyzed samples (*lanes 1* and *2*), the samples proteolyzed in the presence of Triton (*lanes 9* and *10*) and half of the samples proteolyzed without Triton (*lanes 3* and *4*) were then immunoprecipitated with antibody to $F_1\beta$. The other half of the sample proteolyzed in the absence of detergent was centrifuged for 12 min, 20,000 × g at 4 °C. The supernatant (*Sup*) (*lanes 5* and *6*) was removed and precipitated for $F_1\beta$. The pellet (*lanes 7* and *8*) was resuspended in the original volume of NTP buffer and then precipitated with anti-$F_1\beta$. *B*, subunit 9 of ATPase. After precipitation with anti-$F_1\beta$ selected samples were precipitated for ATPase subunit 9. *p*, precursor; *i*, intermediate; *m*, mature.

in the supernatant in the event of significant lysis or leakage of the mitochondria. In another experiment (not shown) mitochondria were allowed to accumulate precursor of $F_1\beta$ and were then reincubated with and without added manganese. The protease resistance of the precursor and mature $F_1\beta$ was then assessed following this incubation. Considerable protease-resistant precursor of $F_1\beta$ (no metal) and mature $F_1\beta$ (plus metal) were observed. This again indicates that the metal-dependent conversion is occurring in substantially intact mitochondria.

Precursor of $F_1\beta$ and precursor of subunit 9 thus accumulate in a processing-competent form either in the matrix upon readdition of metal. Two observations favor the former possibility. First, processing of imported precursors upon readdition of metal occurred in the presence of antimycin A and oligomycin (Fig. 5) or valinomycin (not shown), agents which prevent the formation of the mitochondrial membrane potential (9). Thus a membrane potential does not appear to be required for the processing of imported precursors. Such a potential is required when precursors must be transported to the processing enzyme (Fig. 1 and Ref 9). Second, processing upon readdition of metal was seen to be a very rapid process (data not shown). At 0 °C, under conditions otherwise similar to those in Fig. 5, the conversion of precursors of subunit 9 and $F_1\beta$ to their mature forms after the addition of metal was complete within 2 min. This rapid processing at 0 °C makes it very unlikely that the imported precursors must be translocated across a membrane(s) to the matrix space, location of the processing activity.

DISCUSSION

The results presented in this report show that the proteolytic processing of mitochondrial precursor proteins is apparently not an obligate step in their import into mitochondria. When the processing of the precursors to ATPase subunits 9 $F_1\beta$ by intact *Neurospora* mitochondria *in vitro* was inhibited by metal chelators, the precursor forms of these proteins continued to be imported. This import was characterized by the acquisition of resistance to protease added to intact mitochondria. As observed for the import of subunit 9 and $F_1\beta$ in untreated mitochondria, the import of the precursors was dependent on the mitochondrial membrane potential.

Imported precursor of subunit 9 and the intermediate form appeared to be firmly bound to mitochondrial membranes. Precursor of $F_1\beta$, in contrast, appeared to be more loosely bound to the mitochondrial membranes. These differing relationships to membranes may reflect those of the mature proteins, subunit 9 being a transmembrane integral component of the inner membrane whereas $F_1\beta$ is a component of the peripherally bound F_1-ATPase complex.

The intramitochondrial precursors detected here appear to be authentic intermediates in the assembly process. When the inhibition of processing by chelating agents was reversed by adding excess metal, both imported subunit 9 and precursor of $F_1\beta$ could be processed to their mature size. This processing was independent of the mitochondrial membrane potential. Obviously, the imported precursor forms are not "dead end" or denatured forms. Both precursors have apparently already reached the matrix space, the location of the processing activity. Thus processing does not appear to "drive" import and it is not processing that renders import a vectorial reaction. There was no indication that the precursors leak out of the mitochondria upon transfer into a medium free of any precursors (see Fig. 5).

The pattern of inhibition by chelating agents strongly supports the notion that processing in mitochondria is mediated by the matrix-localized, soluble protease activities detectable in mitochondrial extracts. As previously observed with yeast mitochondria (18) hydrophilic chelators that are effective inhibitors of the processing by extracts were unable to inhibit processing by intact *Neurospora* mitochondria. This is to be expected as such chelators should be unable to penetrate to the matrix. The hydrophobic chelator *o*-phenanthroline, which should be able to penetrate to the matrix, was an effective inhibitor of processing by mitochondria. This inhibition was fully reversible by metals and was specific in the sense that import of proteins continued in the treated mitochondria.

Fig. 6 summarizes our view of the import of $F_1\beta$ and subunit 9. It should be emphasized that this is merely our current working hypothesis and is not meant as a definitive statement of the mechanism of import of subunit 9 and $F_1\beta$. While most features of the model have some experimental support, other pathways could be proposed on the basis of the available data. The first step in the import of both proteins is their binding to receptors on the outer surface of the mitochondria. In the

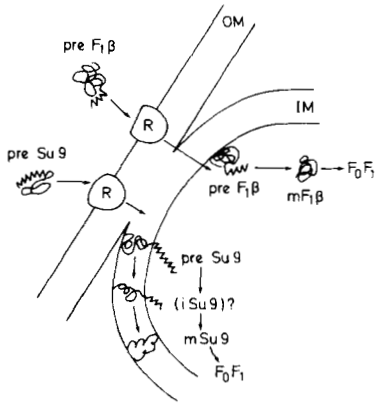

FIG. 6. **Hypothetical pathways for the transport of subunits $F_1\beta$ and of subunit 9 of ATPase into the mitochondria.** *R*, receptor, ∿∿: additional sequence; F_0F_1, oligomycin-sensitive ATPase; *OM*, outer membrane; *IM*, inner membrane; *pre*, precursor; *Su*, subunit; *m*, mature.

absence of a membrane potential, mitochondria-bound precursor can be detected for both subunit 9 and $F_1\beta$ (Fig. 1, *A* and *B*).[2] Upon re-establishment of the membrane potential, the protease-accessible bound forms of precursor subunit 9 can be transferred into the mitochondria and processed[2] suggesting it represents a true intermediate in the import process (12).

In the presence of a membrane potential across the inner membrane, precursors are rapidly transferred across the outer and inner membranes. This translocation could occur at sites of contact between the inner and outer mitochondrial membranes as indicated or could require separate translocation across each membrane. In the case of precursor of $F_1\beta$, this results in complete translocation across the inner membrane yielding an intramitochondrial form that is loosely bound to the inner membrane. In contrast, we suggest that translocation of precursor of subunit 9 results in an integrally bound membrane precursor disposed such that the cleavage site(s) faces the mitochondrial matrix. Once exposed to the matrix, the precursors are then processed to their mature size by the soluble processing enzyme(s) localized there.

The processing of precursor of $F_1\beta$ appears to proceed in a single step. The precursor to subunit 9, which possesses a very long 66 amino acid extension (28), may be processed in two steps. Treatment of mitochondria with chelators results in the intramitochondrial accumulation of both precursor of subunit 9 and of a polypeptide (intermediate subunit 9) of apparent molecular weight intermediate between precursor of subunit 9 and mature subunit 9. Intermediate subunit 9 is protease-resistant and disappears along with precursor subunit 9 upon readdition of metal to inhibited mitochondria. A polypeptide with the electrophoretic mobility of intermediate subunit 9 is also produced by the soluble processing activity from *Neurospora* mitochondria and appears to be an intermediate in the production of the mature subunit 9.[2] Definite assignment of this polypeptide as an intermediate in subunit 9 processing in mitochondria is not possible at this time however.

The path followed by $F_1\beta$ and subunit 9 subsequent to their processing is as yet completely unknown. In both cases (16) these subsequent steps must include assembly of the polypeptide in large multisubunit complexes.

Acknowledgments—We are grateful to Hannelore Strulik for excellent technical assistance. We thank Dr. R. Ansorg for supplying us with *Staphylococcus aureus* cells.

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