Import of Proteins into Mitochondria

ENERGY-DEPENDENT UPTAKE OF PRECURSORS BY ISOLATED MITOCHONDRIA*

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The post-translational import of cytoplasmically synthesized precursors of proteins of the mitochondrial matrix and inner membrane has been studied in vitro with isolated yeast mitochondria and reticulocyte lysates programmed with yeast mRNA. Import of precursors into isolated mitochondria is not a nonspecific side reaction: up to 70% of the in vitro synthesized precursor enters the mitochondria. Subfractionation of F1-ATPase can be processed to the mature form and rendered protease-resistant during incubation with mitochondria. Furthermore, precursors which were processed to their mature form prior to incubation with mitochondria do not become protease-resistant under similar conditions. Finally, a brief trypsin treatment of mitochondria prior to incubation with labeled F1-β-subunit precursor abolished uptake of this precursor into the mitochondria.

In vitro import occurs into the correct intramitochondrial location: this was determined by the use of chemicals such as GTP and bathophenanthroline disulfonate which inhibit the cleavage of precursors to their mature form by a hypotonic extract from yeast mitochondria or by lysed mitochondria. The processing of these precursors by intact mitochondria, however, is not affected by such inhibitors. Since these inhibitors can cross the outer, but not the inner, mitochondrial membrane, at least part of the precursor molecule is translocated across an intact inner membrane before cleavage to the mature form occurs. Subfractionation of mitochondria after in vitro import provides further evidence for the proper localization of proteins imported into the mitochondrial matrix, intermembrane space, and membrane fraction. Import of precursors into isolated yeast mitochondria thus exhibits the topological specificity of the process as it occurs in living cells.

The in vitro import of precursor polypeptides requires energy: in respiring mitochondria, import is blocked either by protonophores or by valinomycin plus potassium, but not by oligomycin; in cyanide-inhibited mitochondria supplemented with external ATP, import is blocked by carboxyatractyloside or by oligomycin. This shows that an electrochemical gradient across the inner mitochondrial membrane, and not ATP itself, is required for protein import.

Most mitochondrial proteins are coded by nuclear genes, translated on cytoplasmic ribosomes, and imported into mitochondria (1, 2). Many of these polypeptides are initially synthesized as larger precursors which can be detected by pulse-labeling whole cells or by translating isolated mRNA in a reticulocyte lysate (1–4). These in vitro-synthesized precursors can be cleaved to their corresponding mature form and rendered insensitive to externally added proteases during incubation with intact mitochondria (1–3). Translocation across mitochondrial membranes and processing to the mature size can thus occur post-translationally. This uptake mechanism has been termed "vectorial processing" (1) to set it apart from the obligately co-translational transport of secretory proteins across the endoplasmic reticulum ("vectorial translation").

Despite the initial evidence for in vitro import by yeast mitochondria, it remained uncertain whether or not this process resembles protein import into mitochondria in vivo. In order to resolve this point, the following questions had to be answered. Is in vitro uptake efficient or merely an insignificant side reaction? Does it exhibit the characteristic energy dependence which is typical of the import process in intact yeast cells (5)? Are the precursors transported to their correct intramitochondrial location? Can mitochondria take up precursors that have been processed before being incubated with the mitochondria?

This study has attempted to answer these questions. First, a convenient import assay was developed which eliminated the need for subsequent protease treatment of the isolated mitochondria. Second, the specificity of import was assessed by a newly developed procedure for fractionating yeast mitochondria into their major compartments (6). Third, import of precursors into the matrix space was probed by a method which does not require subfractionation of mitochondria. Fourth, the energy dependence of import was studied under conditions in which the only energy donor was added ATP.

The results of this work indicate that the import of precursor polypeptides into isolated mitochondria reflects the import process as it occurs in living yeast cells. A study of this in vitro system has yielded new information on the import mechanism.

MATERIALS AND METHODS

Yeast Strain and Fractionation—The wild type Saccharomyces cerevisiae strain D273-10B (a, ATCC 25567) was used throughout this study. For the preparation of mRNA and mitochondria, the cells were grown to early logarithmic phase in a semisynthetic medium containing 2% lactate and 0.1% glucose (6). The isolation of crude mRNA (3), spheroplasts (6), mitochondria (6), and mitoplasts (6) has been described.

A crude preparation of matrix protease was prepared from yeast mitochondria essentially as reported (7) and partially purified as will be described elsewhere.1 Subfractionation of yeast mitochondria was

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1 P. C. Bögni, manuscript in preparation.
done as outlined in the accompanying paper (6).

Cell-free Protein Synthesis—Cell-free protein synthesis was pro-
grammed by crude yeast mRNA in a rabbit reticulocyte lysate (6) untreated with nuclease. Protein synthesis was stopped by chilling to
0 °C and centrifugation (143,000 × g, 40 min) to remove polysomes.
Aliquots of translated lysate were frozen in liquid N, and stored at
−80 °C. L-lysine, amino acids, and all other components of the in vitro
reaction mixture were filtered through a Sephadex G-25 column which had been equilibrated with 0.15 M KCl, 20 mM Hepes
/KOH, pH 7.4. For processing precursors in the lysate prior to incubation with mitochondria, a partially purified hypotonic extract of yeast mitochondria* was incubated with the translated lysate for 20 min at room temperature. Ortho-phenanthro-
line was then added to 2 mM in order to inhibit further processing (7) and the lysate was filtered.

In Vitro Import Conditions—The in vitro import assay contained 125–150 µl of translated reticulocyte lysate (approximately 3 × 10^9
cpm of protein-bound 35S) which had been centrifuged, gel-filtered, and adjusted to 0.6 M mannitol, 20 mM Hepes/ KOH, pH 7.4. Unless otherwise indicated, the incubation mixture also contained 1 mM ATP, 1 mM MgCl2, 5 mM phosphoenolpyruvate, 4 units of pyruvate
kinase, 1 mM dithiothreitol, and approximately 200 µg of mitochondrial
protein in a final volume of 0.4 ml. If no protease treatment was to
be performed, the mixture was adjusted to pH 7.4 with KOH and
was incubated at 0 °C for 30 min. The mixture was then centrifuged at 10,000 × g for 10 min, and the supernatant was aliquoted for
quantitative immunoprecipitation.

The mixture was incubated for 30 min at 27 °C with shaking and then
chilled on ice. For protease treatment, the sample was divided in half:
one-half received trypsin (final concentration, 120 µg/ml) and the
other half 1 mM TlCK and 1 mM phenylmethylsulfonyl fluoride.
After 30 min at 0 °C, trypsin activity was arrested by the addition of
1.2 mg/ml of soybean trypsin inhibitor and 1 mM TlCK. Mitochon-
dria were then resuspended by centrifugation (10,000 × g for 10 min)
and the pellet was assayed with antiserum, shaken for
20 min at 0 °C. In experiments in which the effect of inhibitors on import was tested, the mitochondria (10
mg/ml) were pretreated with the inhibitors for 10 min on ice. The
final inhibitor concentration was added to the mitochondria and
the supernatant following proteolysis (lane 4, Fig. 1). Similar
results were obtained for the F1 α- and γ-subunits, for cyto-
chrome c oxidase subunit V (an inner membrane protein), for cyto-
chrome b2 (an intermembrane space enzyme), and for the Mtr,
29,000 kDa "porin" protein of the outer membrane (not shown).

Quantitative Immunoprecipitation—After dissociation in SDS,
each supernatant from the in vitro import assay and an aliquot of
unchallenged lysate were mixed with 200 µg of SDS-dissociated unla-
beled yeast mitochondrial protein. This ensured that all samples
subjected to immunoprecipitation contained the same amount of any
mitochondrial antigen which might compete with the labeled poly-
peptides for binding the immunoglobulins. Each sample (10–15 µl) was mixed with an equal volume (50–70 µl) of antiserum, shaken for
16 h at 4 °C, and then mixed with an equal volume (250–350 µl) of a
10% v/v suspension of glutaraldehyde fixed Staphylococcus aureus
cells (9). Washing and elution of the immunoprecipitates (9), prepara-
tion of subunit-specific antisera (6), electrophoresis in SDS-poly-
acrylamide slab gels (10), and fluorography (11) were done by pub-
lished procedures. Whenever possible, results were quantified both
day by densitometric scanning of the fluorographs and by directly count-
ing [35S]methionine in gel slices. The gel slices were first soaked in
distilled water for 30 min, incubated overnight at 60 °C in 0.8 ml of
NCS tissue solubilizer, cooled, and counted with 10 ml of a toluene-
based scintillation fluid. Fluorograms were scanned with a Bausch-
Lomb scanner and traces were quantified with a Hewlett-Packard
integrator.

Miscellaneous—Protein was assayed according to Lowry et al.
(12). Published methods were used to measure 35S-ATP exchange
(13) and respiration of mitochondria (14) except that the mannitol
concentration was kept at 0.6 M. Spheroplasts were labeled uniformly
with [35S]methionine as in Ref. 5, except that the labeling time was
30–60 min. Cytochrome b2 was assayed as 1-lactate-ferrocyanide
reductase (15). Bathophenanthrolinesulfonate and carbamoylacta-
loside were purchased from Sigma and Boehringer Mannheim,
respectively. [35S]Methionine (approximately 100 Ci/mmol) was pre-
pared from [35S]O. (16). Ethylenediamine was a generous gift from Dr.
Henry A. Lardy (University of Wisconsin).

RESULTS

A Significant Proportion of a Given in Vitro-synthesized Precursor Can Be Imported and Protected Post-translationally by Isolated Mitochondria—Carefully isolated yeast mito-
chondria were incubated with [35S]methionine-labeled in
vitro translation products. Half of the mixture was subse-
quently incubated with trypsin to digest 35S-labeled polypep-
dides accessible to the added protease. The other half served as
the control. The mitochondria were then reisolated from each aliquot and supernatants and mitochondria were ana-
lyzed for radiolabeled F1 β-subunit (a matrix polypeptide).

The results of such an experiment are shown in Fig. 1.

Mitochondria from the control aliquot contained both the precursor and the mature form of the F1 β-subunit. In contrast, mitochondria from the protease-treated aliquot contained
only the mature form, suggesting that the labeled forms associated with the control mitochondria have different loca-
tions: the precursor is apparently outside and the mature
subunit inside the mitochondrial membranes. In this experi-
ment, the supernatant contained only the precursor (lane 2),
as did the lysate prior to incubation (not shown). (The relative
amounts of mature and precursor forms recovered from the
supernatant depend upon the intactness of the yeast mito-
chondria, since the soluble matrix processing protease (7)
leaks out of partially damaged mitochondria and converts
β-subunit precursor to mature β-subunit outside the mito-
chondria.) The efficiency of the trypsin treatment in degrading any
nonprotected β-subunit polypeptides is confirmed by the fact
that no F1 β-subunit or its precursor could be recovered from
the supernatant following proteolysis (lane 4, Fig. 1). Similar
results were obtained for the F1 α- and γ-subunits, for cyto-
chrome c oxidase subunit V (an inner membrane protein), for cyto-
ochrome b2 (an intermembrane space enzyme), and for the
Mtr, 29,000 kDa "porin" protein of the outer membrane (not shown).

Four fluorographs like the one pictured in Fig. 1 were quantified (see "Materials and Methods"). The results show that
60–70% of the in vitro-synthesized F1 β-subunit can be
protected from proteolysis upon incubation with mitochondria (Table I). The ability of the mitochondria to withstand
the trypsin treatment varies, however. Since incubation in retic-
ulocyte lysate and protease treatment destroys about 30% of
the intermembrane space enzyme cytochrome b2 (not shown),
it clearly damages the mitochondrial outer membrane and
perhaps the inner membrane as well. As a consequence, the
percentages for protected radiolabeled polypeptides given
Table I may be an underestimation. Loss of [35S]methionine
resulting from cleavage of the (presumably) NH2-terminal
extension (17) would cause an additional underestimation.

GTP Inhibits the Processing of Precursors by a Hypotonic Extract, but Not by Intact Mitochondria—A matrix-located
chelator-sensitive mitochondrial protease is responsible for the
maturation of those precursors that are transported partly
through a Sephadex G-25 column which had been equili-

The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piper-
zineethane sulfonic acid; TlCK, tosyl-L-lysyl chloromethyl
ketone; SDS, sodium dodecyl sulfate.

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**Fig. 1 (left).** $F_1 \beta$-subunit is cleaved and rendered protease-resistant during incubation with yeast mitochondria. Isolated yeast mitochondria were incubated with $[^35]S$-labeled in vitro translation products; maturation and uptake of the $F_1 \beta$-subunit precursor were checked by protease treatment, immunoprecipitation, SDS-polyacrylamide gel electrophoresis, and fluorography (see "Materials and Methods"). A photograph of the fluorogram is shown. 1. precursor and mature form of $F_1 \beta$-subunit recovered in mitochondria which had not been treated with protease; 2. $F_1 \beta$-subunit precursor recovered in the untreated supernatant; 3. $F_1 \beta$-subunit recovered in mitochondria after protease treatment; 4. supernatant after protease treatment; 5. mature $F_1 \beta$-subunit immunoprecipitated from $[^35]S$-labeled spheroplasts.

**Fig. 2 (center).** Processing of $F_1 \beta$-subunit precursor by a hypotonic extract from yeast mitochondria is inhibited by GTP. Yeast mitochondria were incubated in 20 mM Hepes/KOH, pH 7.4, 0.5 mM phenylmethylsulfonyl fluoride for 30 min at 0°C. The mixture was centrifuged for 1 h at 143,000 × g and the supernatant was incubated for 45 min at 27°C with $[^35]S$-labeled in vitro translation products coded by yeast mRNA (see "Materials and Methods"). Each incubation contained 1 mM ATP, 1 mM MgCl$_2$, 5 mM phosphoenolpyruvate, 4 units of pyruvate kinase, and 20 mM Hepes/KOH, pH 7.4; GTP was added as indicated. $F_1 \beta$-subunit was immunoprecipitated and analyzed by SDS-polyacrylamide gel electrophoresis.

**Table I**

Quantitation of precursor import into isolated yeast mitochondria

Experiments similar to the one in Fig. 1 were quantified as described under "Materials and Methods." The amount of radiolabeled precursor immunoprecipitable from lysate before incubation with mitochondria is taken as 100%.

| Lysate (precursor) | -Trypsin | +Trypsin |
|-------------------|----------|----------|
|                    | Pellet   | Supernatant | Pellet | Supernatant |
| $F_1 \beta$-subunit | 81       | 7         | 63     | 0         |
| (100)              |          |           |        |           |
| (100)              | 87       | 21$^a$    | 71     | 0         |
| (100)              | 71$^b$   | 27$^c$    | 16     | 9         |
| Cytochrome $b_2$   | 50       | 42$^d$    | 47     | <1        |

$^a$ 21% is the sum of the mature forms (16%) and precursor (5%).
$^b$ 71% is the sum of the mature form (44%) and precursor (27%).
$^c$ All 27% was recovered as precursor.
$^d$ 42% is a sum of the mature form (11%) and precursor (31%).

As can be overcome by adding excess Mg$^{2+}$, the inhibition is likely to result from chelation (28).

In contrast to the effect in a hypotonic extract from mitochondria, GTP has no significant inhibitory effect on the processing of $F_1 \beta$-subunit by intact mitochondria (Fig. 3). This GTP resistance is not caused by a component present in the mitochondrial membranes, since processing of the $\beta$-subunit precursor by disrupted mitochondria is GTP-sensitive (Fig. 3). The observed processing of the $\beta$-subunit precursor by intact mitochondria in the presence of GTP must then occur in the matrix compartment into which GTP cannot readily diffuse (18). GTP can, however, readily penetrate into the intermembrane space, since the outer membrane permits passive diffusion of molecules with molecular weights up to several thousands (19). Only the processing enzyme which is protected from the inhibitory concentration of GTP by an intact inner membrane can cleave mitochondrial precursors of matrix and inner membrane polypeptides. Processing by mitochondria in the presence of external GTP can thus be used as a convenient assay for the import of precursors into the matrix. The validity of this assay is based upon the fact that the outer, and not the inner, membrane of yeast mitochondria is permeable to GTP. Although our evidence suggests that GTP acts as a chelator here, proof of this is not essential for interpretation of the results.

Bathophenanthroline disulfonate is another potent inhibi-
Protein Import by Isolated Yeast Mitochondria

The mature and the precursor forms (when present) of each protein were dissociated with SDS and then mixed with SDS-dissociated, unlabeled samples of the other two mitochondrial subfractions so as to give a "reconstituted" mixture with the same composition as an SDS-dissociated mitochondrion. The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. The mature and the precursor forms (when present) of each protein are marked by arrows and the lane headings indicate the following: St, mature standard from Coomassie blue staining of isolated enzyme or from immunoprecipitation of uniformly labeled yeast spheroplasts; Pre, precursor to the mature form as immunoprecipitated from translated reticulocyte lysate; MI, whole mitochondria after incubation with labeled precursors; IMS, intermembrane space fraction; MA, matrix fraction; ME; the insoluble membrane fraction (both the inner and outer membranes) after release of the matrix and intermembrane protein of the soluble processing protease and its charge should prevent its diffusion into the matrix space of intact mitochondria. Indeed, 0.5 mM bathophenanthrolinone disulfonate blocks processing by a hypotonic mitochondrial extract completely, but has little effect on processing by intact mitochondria (not shown).

Proteins Are Imported to Their Correct Intramitochondrial Location—When isolated yeast mitochondria are first allowed to take up in vitro-synthesized precursors and then fractionated into matrix space, intermembrane space, and a membrane fraction, labeled matrix proteins are found in the matrix fraction but not the intermembrane space fraction, whereas the labeled intermembrane space enzyme cytochrome $b_6$ is found in the intermembrane space, but not the matrix. Labeled membrane proteins are found only in the membrane fraction (Fig. 4 and Table II). The low levels of labeled matrix enzymes in the intermembrane space fraction and of intermembrane space polypeptides in the matrix fraction can be fully accounted for by cross-contamination of these fractions (Table IIA). However, cross-contamination can only partly account for the result that roughly half of the imported matrix and intermembrane space enzymes are recovered in the membrane fraction. It is unknown whether these membrane-associated proteins represent assembly intermediates or a side reaction. Some of the precursors may be trapped "in transit" across the membranes. Nonspecific adsorption may be an additional contributing factor.

Import and processing of in vitro-synthesized precursors by isolated mitoplasts sheds further light on the localization of matrix and intermembrane space polypeptides. The mitoplasts retain a large portion of their outer membrane even though >90% of an intermembrane space marker enzyme is released (6) and they are able to process the precursors of both F$_1$, $\beta$-subunit and cytochrome $b_6$ to their corresponding mature forms (Table III). When mitoplasts are reisolated through a 0.625 M sucrose cushion from the standard incubation mixture for in vitro import, mature radiolabeled F$_1$, $\beta$-subunit is recovered with the mitoplast pellet, whereas processed radiolabeled cytochrome $b_6$ is released to the suspending medium. This suggests that the processed F$_1$, $\beta$-subunit is sequestered by the mitoplasts in the matrix space, whereas the processed cytochrome $b_6$ remains outside the mitochondrial inner membrane. Whole mitochondria, radiolabeled processed cytochrome $b_6$ is recovered completely with the mitochondrial pellet (Table III), presumably because the processed polypeptide is sequestered in the intermembrane space.

The recovery of a large part of processed radiolabeled cytochrome $b_6$ with membranes after import with whole mitochondria (see Table II) might, therefore, reflect nonspecific adsorption of the processed cytochrome $b_6$ to outer or inner membranes. When the mitoplasts are reisolated through the sucrose cushion, approximately 50 mM KCl and 30 mM Hepes/KOH, pH 7.4, are present in the incubation mixture. The release of intermembrane space from whole mitochondria, on the other hand, is done with 0.1 M mannitol and 10 mM Tris-Cl, pH 7.4. The higher ionic strength present during the mitoplast reisolation may be sufficient to eliminate the adsorption of processed cytochrome $b_6$ to the membranes, as is seen in the fractionation results.

In order to check the submitochondrial distribution of non-specifically imported precursors, the mitoplast reisolation might, therefore, reflect nonspecific adsorption of the processed cytochrome $b_6$ to outer or inner membranes. When the mitoplasts are reisolated through the sucrose cushion, approximately 50 mM KCl and 30 mM Hepes/KOH, pH 7.4, are present in the incubation mixture. The release of intermembrane space from whole mitochondria, on the other hand, is done with 0.1 M mannitol and 10 mM Tris-Cl, pH 7.4. The higher ionic strength present during the mitoplast reisolation may be sufficient to eliminate the adsorption of processed cytochrome $b_6$ to the membranes, as is seen in the fractionation results.

In order to check the submitochondrial distribution of non-

Fig. 4. In vitro-synthesized precursors are imported into their correct mitochondrial location. Mitochondria were incubated for 30 min at 27 °C with [35S]methionine-labeled products of total yeast mRNA under conditions described earlier, including 3 mM GTP. The mitochondria were reisolated and washed once in 0.6 M mannitol prior to subfractionation into intermembrane space, matrix, and membranes. Each sample was quantitatively immunoprecipitated. In order to equalize the antigen concentrations of all samples, each was associated with SDS and then mixed with SDS-dissociated, unlabeled samples of the other two mitochondrial subfractions so as to give a "reconstituted" mixture with the same composition as an SDS-dissociated mitochondrion. The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. The mature and the precursor forms (when present) of each protein are marked by arrows and the lane headings indicate the following: St, mature standard from Coomassie blue staining of isolated enzyme or from immunoprecipitation of uniformly labeled yeast spheroplasts; Pre, precursor to the mature form as immunoprecipitated from translated reticulocyte lysate; MI, whole mitochondria after incubation with labeled precursors; IMS, intermembrane space fraction; MA, matrix fraction; ME, the insoluble membrane fraction (both the inner and outer membranes) after release of the matrix and intermembrane protein of the soluble processing protease and its charge should prevent its diffusion into the matrix space of intact mitochondria. Indeed, 0.5 mM bathophenanthrolinone disulfonate blocks processing by a hypotonic mitochondrial extract completely, but has little effect on processing by intact mitochondria (not shown).

Space. A, cytochrome $b_6$ (b$_6$); B, F$_1$, $\beta$-subunit (F$_1$$\beta$); C, cytochrome c oxidase subunit V (V); D, isopropyl malate synthase (IPMS); E, cytochrome c$_1$ (c$_1$), the precursor of cytochrome c$_1$ is processed by isolated mitochondria only to an intermediate form (U; cf. Ref. 20); F, glyceraldehyde-3-P dehydrogenase (GAPDH); LYS, in vitro product.
Protein Import by Isolated Yeast Mitochondria

**TABLE II**

| Location in vitro | Marker                                                        | Intermembrane space | Matrix | Membrane fraction |
|-------------------|---------------------------------------------------------------|---------------------|--------|------------------|
| Intermembrane space | Cytochrome b₉                                                | 64                  | 24     | 12               |
| Matrix            | Fumarase                                                     | 6                   | 73     | 22               |
| Inner membrane    | Cytochrome c oxidase subunit III                             | <1                  | <1     | <1               |

**A. Distribution of unlabeled marker enzymes**

**TABLE III**

| Location in vitro | Enzyme                              | % recovered with mitochondria | Intermembrane space | Matrix | Membrane fraction |
|-------------------|-------------------------------------|------------------------------|---------------------|--------|------------------|
| Intermembrane space | Cytochrome b₉                        | 62                           | 47                  | 7      | 46               |
| Matrix            | Isopropyl malate synthase            | Not determined               | 3                   | 43     | 54               |
| Inner membrane    | Cytochrome c₁                        | 34                           | 36                  | 61     |
| Cytosol           | Glyceraldehyde-3-phosphate dehydrogenase | <2                           | 5                   | 2      | 93               |

* 100% = radioactive in vitro product incubated with mitochondria.

* This polypeptide is not made as a larger precursor (not shown).

**In vitro-synthesized cytochrome b₉ is processed, but not sequestered by mitoplasts**

In *in vitro*-synthesized polypeptides were incubated with 200 μg of either mitoplasts or mitochondria under the standard conditions for *in vitro* import, including 5 mM GTP. After 30 min at 27 °C, each incubation mixture was layered over a 0.625 M sucrose cushion and centrifuged for 15 min at 36,000 × g. Pellets and supernatants were dissociated with 3% SDS at 95 °C. Samples were quantitatively immunoprecipitated for cytochrome b₉ and F₁β-subunit. The immunoprecipitates were analyzed by SDS-gel electrophoresis and fluorography, and the fluorograms were quantified by densitometric scanning. 100% refers to the total amount of immunoprecipitable [³⁵S]methionine-labeled polypeptide recovered from each incubation condition.

**B. Distribution of imported labeled in vitro products**

| Location in vitro | Enzyme                              | % recovered with mitochondria | Intermembrane space | Matrix | Membrane fraction |
|-------------------|-------------------------------------|------------------------------|---------------------|--------|------------------|
| Intermembrane space | Cytochrome b₉                        | 62                           | 47                  | 7      | 46               |
| Matrix            | Isopropyl malate synthase            | Not determined               | 3                   | 43     | 54               |
| Inner membrane    | Cytochrome c₁                        | 34                           | 36                  | 61     |
| Cytosol           | Glyceraldehyde-3-phosphate dehydrogenase | <2                           | 5                   | 2      | 93               |

of the mitochondria with a reticulocyte lysate drastically lowered the recovery of outer membrane. Still, it was possible to show that in *in vitro*-synthesized cytochrome c oxidase subunit V becomes associated only with the inner, and not with the outer, membrane (not shown). The submitochondrial distribution of imported polypeptides argues strongly that polypeptide import by isolated mitochondria mirrors the import process in *in vivo*.

**Processed Precursor Is Not Imported by Isolated Mitochondria**—The results described so far indicate that processing of the precursors to matrix and inner membrane enzymes occurs after, not before, import. This implies that import might be blocked if the precursors are artificially processed before they are incubated with isolated mitochondria. This is indeed the case (Fig. 5). A translated reticulocyte lysate was incubated with purified processing protease isolated from yeast mitochondria. About 75% of the *in vitro*-synthesized precursor of subunit V of cytochrome c oxidase was thereby converted to the mature form as determined by SDS-gel electrophoresis and counting of the precursor and mature polypeptide band. As a control, another aliquot of the labeled lysate was incubated with processing protease which had been inhibited by α-phenanthroline. Both aliquots were filtered through a Sephadex G-25 column and incubated with isolated mitochondria under the usual conditions of *in vitro* import. In this way, one lysate contained mainly processed polypeptides and the other only the precursors. After a subsequent trypsin treatment of the mitochondria, mature subunit V was protected from proteolysis in the control sample, but not in the sample in which the precursors had been processed to their mature forms prior to incubation. Since the processing activity is purified from a soluble hypotonic extract and subunit V is a hydrophobic membrane protein (21), our preparation of processing enzyme is free of unlabeled mature subunit V, which could dilute the radiolabeled subunit V and thereby complicate interpretation of the experiment.

**In Vitro Import Requires a Protease-sensitive Component**

specifically adsorbed *in vitro* translation products, we followed the distribution of the glycolytic enzyme glyceraldehyde-3-P-dehydrogenase. As shown in Table II, *in vitro*-synthesized glyceraldehyde-3-P-dehydrogenase (a cytosolic enzyme) binds only slightly to mitochondria, but whatever is bound is recovered in the membrane fraction. This distribution shows that an *in vitro* translation product nonspecifically adsorbed to mitochondria appears neither in the matrix nor the intermembrane space fractions.

Attempts to separate the inner and outer membranes after *in vitro* import were only partly successful, since incubation
on the Outer Face of the Mitochondrial Outer Membrane—

Mitochondria that had been exposed to as little as 10 μg of trypsin/ml at 0 °C for 10 min prior to the incubation with in vitro-synthesized precursors did not import precursors nor cleave them to their mature forms (Table IV). After trypsin treatment, at least 95% of the intermembrane space enzyme cytochrome b₂ was still inaccessible to externally added cytochrome c, showing that the outer membrane had remained largely intact. Since import by the treated mitochondria was strongly inhibited, a protein exposed on the cytoplasmic face of the outer membrane may be necessary for the uptake of precursors into mitochondria.

In Vitro Import, but Not Cleavage, of the Precursor Is Energy-dependent—We have previously reported that, in intact yeast cells, maturation of mitochondrial precursor polypeptides is blocked by conditions which lower the ATP level in the matrix (5). Protein import by isolated mitochondria also exhibits this characteristic energy dependence. By removing small molecules from a labeled reticulocyte lysate (see "Materials and Methods"), we could reconstitute an in vitro import system that was completely dependent on added ATP (Fig. 6). In this experiment, import of the F₁ β-subunit was assessed by the presence of processed, labeled F₁ β-subunit in the mitochondria (see preceding sections). The mitochondrial pellet and supernatant incubated without ATP show only the precursor, most of which remains in the supernatant. After incubation in the presence of ATP and an ATP-regenerating system, however, 52% of the radiolabeled F₁ β-subunit precursor is recovered as mature form with the mitochondrial pellet. In order to show that ATP is required for translocation, and not cleavage, of the precursor, the labeled lysate was incubated with hypotonically disrupted mitochondria: nearly complete conversion of the precursor to the mature form was observed even in the absence of added ATP. Thus, energy is

![Figure 5](image)

![Figure 6](image)

### Table IV

| Experiment | Polypeptide imported | Percentage of radiolabeled polypeptide imported after pretesting mitochondria with |
|------------|----------------------|--------------------------------------------------------------------------------|
| 1          | F₁ β-subunit         | 25%                                                                              |
| 2          | F₁ β-subunit         | 15%                                                                              |
| 3          | F₁ β-subunit         | 13%                                                                              |
| 1          | Cytochrome b₂        | <1%                                                                              |
| 3          | Cytochrome b₂        | 5%                                                                               |

n.a., not assayed.
required for translocation of the precursors, not for their cleavage.

Import of Precursors into the Mitochondrial Matrix Requires an Electrochemical Gradient across the Inner Membrane—ATP-driven import of the F₁ β-subunit precursor is blocked by carboxyatractylsise, a specific inhibitor of adenine nucleotide translocation across the mitochondrial inner membrane (Fig. 7). This shows that the added ATP acts from within the matrix. (In these experiments, KCN was added to block oxidative phosphorylation resulting from respiration of endogenous substrates.) This confirms the results obtained earlier with intact yeast cells (5).

ATP-dependent import of the F₁ β-subunit precursor is also inhibited by oligomycin which blocks the mitochondrial ATPase complex (Fig. 7). Thus, the added ATP must be hydrolyzed by this complex for import to occur. The most likely explanation for this would be that ATP is used to generate an electrochemical gradient across the mitochondrial inner membrane. Indeed, ATP-dependent import is abolished if any such gradient is collapsed by valinomycin plus K⁺ or by carbonyl cyanide m-chlorophenyl hydrazone (Fig. 7). In these experiments, oligomycin was added as well to block rapid hydrolysis of ATP. The essential role of an electrochemical gradient across the inner membrane is further documented by the observation that oligomycin does not block import of the F₁ β-subunit if energy is supplied by respiration instead of by added ATP (Fig. 8). This shows that the inhibitory effect of oligomycin with added ATP as energy donor does not merely reflect a general disruptive effect of oligomycin on mitochondrial integrity. Valinomycin, on the other hand, blocks import even in respiring mitochondria. Polarographic measurements clearly confirmed that the inhibitors employed here act in the expected manner: oligomycin blocks stimulation of respiration by ADP, but not that by the uncoupler carbonyl cyanide m-chlorophenyl hydrazone or by valinomycin plus K⁺ (not shown).

**DISCUSSION**

In order to understand how proteins are imported into mitochondria, one must first be able to reproduce the import process in vitro. It had already been shown that isolated mitochondria can process in vitro-made mitochondrial precursor polypeptides and that the resulting mature polypeptides are inaccessible to externally added proteases (1, 2). However, it remained to be shown that this in vitro process is an acceptable representation of mitochondrial protein import in vivo. The present work suggests that this is indeed the case.

The relatively simple in vitro system described here exhibits several features which characterize protein import by mitochondria in living cells. First, a large fraction of a given in vitro-made precursor is taken up by the mitochondria. Second, import of precursors into or across the mitochondrial inner membrane requires energy.

Study of this in vitro system has yielded new information on the import process. For example, it was possible to show that precursors processed before incubation with mitochondria are not imported. In other words, uptake occurs before, not after, processing. Independent evidence for this comes from the demonstration that the processing protease for precursors imported across the inner membrane is located in the matrix and that the extracted protease is not energy-dependent (7). It was also found that import could be abolished by treating isolated mitochondria with low amounts of trypsin,
which apparently alters but does not disrupt the outer membrane. This treatment also abolished binding of precursors to outer membrane vesicles which had been subsequently purified from these mitochondria. These results suggest that import requires protein "receptors" on the outer face of the mitochondrial outer membrane (1).

The in vitro system described here was particularly useful for identifying the energy requirement of protein import into mitochondria. In our earlier work we had found that processing of precursors in intact yeast cells required the presence of ATP in the mitochondrial matrix. Since in vivo processing still occurs in rho- mutants which lack respiration as well as a functional mitochondrial ATPase complex (22), we suggested that it was ATP itself, rather than an energized membrane, that functioned as an energy source (5). Results from an in vitro import system using Neurospora mitochondria showed that carbonyl cyanide m-chlorophenyl hydrazone blocks the conversion of the in vitro-synthesized ADP/ATP translocator to a protease-resistant form (23). However, since carbonyl cyanide m-chlorophenyl hydrazone, in the absence of oligomycin, depletes the matrix of ATP as well as collapsing the mitochondrial potential, these results were inconclusive as to the immediate energy donor. The results obtained with the in vitro system here show that the immediate energy donor is not ATP itself, but an electrochemical gradient across the mitochondrial inner membrane. In this respect, protein import into mitochondria resembles the transport of proteins into or across the cytoplasmic membrane of Escherichia coli (24, 25). Since valinomycin-treated mitochondria appear to catalyze a H+/K+ exchange (26), it is not possible to tell from the available data whether protein import requires a pH gradient, an electrical potential, or both.

These results explain our earlier observation that a buildup of precursors to mitochondrial proteins can be seen more easily in rho- yeast cells than in wild type cells (5). The fact that protein import is not completely blocked in these mutants suggests that they can generate an electrochemical gradient across the mitochondrial inner membrane by some process(es) other than respiration or ATP hydrolysis. For example, the ATP/ADP exchange via the adenine nucleotide translocator appears to be coupled to a membrane potential (27). Since any electrochemical gradient generated by the translocator would be small, import of polypeptides should require a significantly smaller electrochemical gradient than ATP synthesis requires.

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