Import of Proteins into Mitochondria

EXTRAMITOCHONDRIAL POOLS AND POST-TRANSLATIONAL IMPORT OF MITOCHONDRIAL PROTEIN PRECURSORS IN VIVO*

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Extramitochondrial pools of mitochondrial precursor polypeptides can be detected in yeast spheroplasts which had been pulse-labeled for 3 min in the absence of inhibitors or for 10 min in the presence of an uncoupler of oxidative phosphorylation. In either case, these precursors can be post-translationally converted to the mature forms during a subsequent chase. Post-translational maturation of the precursor to F1-ATPase \( \beta \)-subunit is accompanied by uptake of the polypeptide into mitochondria.

Pools of precursor polypeptides are also found in pulse-labeled intact yeast cells, the pool sizes depending on the metabolic status of the cells. For example, if the rate of protein synthesis is lowered by cycloheximide, the precursor pool size decreases. This indicates that living yeast cells can, and apparently do, import polypeptides into mitochondria post-translationally.

The translocation of at least some proteins across the endoplasmic reticulum of eukaryotic cells is obligately co-translational (1). When such proteins are synthesized in vitro, they can only be translocated if endoplasmic reticulum membranes are present during translation (1–3). In contrast, the import of proteins into mitochondria can occur post-translationally. When mRNA is translated in a reticulocyte lysate, protein synthesis is stopped and then isolated mitochondria are added. The in vitro-synthesized finished precursors can be correctly imported into mitochondria (4–8). While post-translational import has thus clearly been demonstrated in vitro, its importance in vivo has remained unclear. For example, yeast polypeptides synthesizing proteins destined for the mitochondria bind to the mitochondrial surface (9–11). Since polypeptides synthesizing proteins which are secreted across or inserted into the endoplasmic reticulum are usually found attached to this membrane (12, 13), it has been argued (9–11) that import of polypeptides into mitochondria can and does occur co-translationally. The occurrence of co-translational import in vivo also appeared to be supported by the inability to detect extramitochondrial pools of mitochondrial precursor polypeptides in yeast (14). In order to reconcile these findings with the clear evidence for post-translational import in vitro, it has recently been suggested (14) that, in vivo, both co- and post-translational import may occur side by side, the relative importance of each depending on physiological factors. The results reported here and in the accompanying papers (15, 16) show that mitochondrial precursor polypeptides can transiently accumulate outside the mitochondria and then be translocated into mitochondria, and processed, post-translationally. We also demonstrate that the transient accumulation of precursor polypeptides depends on physiological factors and can be decreased by lowering the rate of protein synthesis.

MATERIALS AND METHODS

Yeast Strains and Culture Conditions—The wild type Saccharomyces cerevisiae strain D273-10B (\( \sigma^{+} \), ATCC 25657) and the corresponding rho' mutant D273-10B-1 were grown on a low sulfate semisynthetic medium (17). Cell density was monitored with a Summerson-Klett colorimeter using the red filter. Cells were harvested in midexponential phase (130–160 Klett units) by centrifugation at room temperature (\( -21^\circ \mathrm{C} \)).

Preparation of Spheroplasts—Harvested wild type cells were suspended for 5 min at room temperature in 0.1 M Tris-\( \mathrm{SO}_{4}, \) pH 9.4, containing 10 mM dithiothreitol, centrifuged, and resuspended to 0.1 g wet weight/ml in 40 mM KP, pH 6.0, containing an energy source as detailed in the figure legends, aerated by vigorous shaking at 30 °C, and immediately labeled with \([^{35} \mathrm{S}]\)methionine (250 \( \mu \mathrm{Ci} / \mathrm{ml} \) ) to achieve high incorporation of amino acid into protein. For a subsequent chase, unlabeled l-methionine was added to 2 mM. Reactions were stopped by adding 0.2 volume of 100% (v/v) trichloroacetic acid. The effectiveness of each chase in stopping further incorporation of radioactivity into protein was checked by determining the acid-precipitable radioactivity in a 5-ml sample, collected, and thoroughly washed on a Whatman GF/A glass-fiber filter (19). Spheroplasts were collected by centrifugation and suspended in 1.3 M sorbitol, sulfate-free salts (as in the growth medium), 1% Na lactate, pH 6.0, plus 0.5% (w/v) glycerol. The suspension was shaken at 30 °C and \([^{35} \mathrm{S}]\)methionine (300 \( \mu \mathrm{Ci} / \mathrm{ml} \) ) was added immediately to achieve high labeling rates. Labeling was stopped by addition of 2 volumes of ice-cold labeling buffer and chilling on ice. At all times during subsequent fractionation, the samples were kept at 0 °C.

Spheroplasts were centrifuged and resuspended in 0.6 M mannitol, 10 mM Tris-Cl, pH 7.4. Mitochondria and submitochondrial fractions were isolated as described (20) except that the unbroken spheroplast pellet was not rehomogenized, but discarded.

Immunoprecipitation—Pulse-labeled cells were treated with trichloroacetic acid, broken, and extracted with SDS1 and the extracted proteins were subjected to immunoprecipitation as described (16, 21). Spheroplasts and fractions obtained from them were dissociated directly with 0.4 ml of 25% (w/v) SDS, diluted to 40 ml with Trition buffer (1% Triton X-100, 0.1 M NaCl, 5 mM EDTA, 50 mM Tris-Cl, pH 8.0) and similarly subjected to immunoprecipitation. Since the immunoprecipitated material was used to quantitate the relative amount of a labeled polypeptide in the various subcellular fractions, the abbreviations used are: SDS, sodium dodecyl sulfate; CCCP, carbonyl cyanide \( m \)-chlorophenylhydrazone.

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it was necessary to show that the efficiency of immunoprecipitation from each fraction was equal. This was achieved by adding an excess of antibody over antigen and was checked by the following control experiments. Unlabeled spheroplasts were fractionated by the same procedures used with radioactively labeled spheroplasts; the amount of starting material and all volumes were the same in each case. Such a fractionation was performed twice to determine its reproducibility. An estimation of FI-ATPase β-subunit by this method, performed under the conditions used here. A 3.3-fold underestimate of the β-subunit in the postmitochondrial fraction. The amount of β-protein A radioactivity in the antigen band of each fraction was determined after electrophoresis of the eluted material on SDS-polyacrylamide gels, fluorography, and scanning the films with a Joyce-Loebl densitometer. The amounts varied within a range of ±7%. Although this control does not prove that recovery by immunoprecipitation was complete, it does show that recovery was essentially identical for each labeled polypeptide and each subcellular fraction.

Quantitation of Antigens by the Immune Replica Method—SDS-denatured fractions from spheroplasts were subjected to electrophoresis on SDS-polyacrylamide gels and then electrochemically transferred to nitrocellulose sheets (23). Specific antigens were detected as described (20) except that the incubation with antibody was performed at room temperature. This method was used to quantitate the amounts of certain marker proteins in the various subcellular fractions, since under appropriate conditions the 125I-protein A, bound indirectly to the antigen, is proportional to the amount of antigen in the antigen band. This avoids two problems associated with this quantitation.

Firstly, proportionality is valid only up to a limited amount of antigen; this depends on the particular antigen. Secondly, the efficiency of transfer of a protein from the gel slab to the nitrocellulose sheet may be affected by large amounts of other polypeptides which co-electrophorese with the antigen of interest and may compete with the antigen for binding to the nitrocellulose. To assess any errors arising from such complications, the following control experiments were performed. Samples of postmitochondrial supernatant, mitochondria, mitoplasts, and intermembrane space were isolated from unlabelled spheroplasts as described above and fractionated with SDS. Different amounts of each fraction (covering a 10-fold range) were loaded on an SDS-polyacrylamide gel, electrophoresed, and transferred to nitrocellulose. Duplicate sheets were treated with antibody against cytochrome b2 or FI-ATPase β-subunit. In each case, the 125I-protein A radioactivity in the antigen band of each fraction was proportional to the amount of that fraction loaded on the gel up to the highest amount of each fraction loaded; this amount was equivalent to the amounts used in the actual fractionation experiments described below. Secondly, each of 3 lanes of a gel was loaded with an aliquot of the mitochondrial fraction corresponding in amount to that used in one of the previous experiments described. One of these was loaded with an equivalent amount of postmitochondrial supernatant and the same amount of postmitochondrial supernatant alone was also run in a separate lane. Similarly, one-fifth of this amount of postmitochondrial supernatant was added to one of the lanes containing mitochondria and also run separately. Duplicate gels were electrophoresed, transferred to nitrocellulose, and treated with antibody against cytochrome b2 or FI-ATPase β-subunit. Postmitochondrial supernatant was used in this experiment since, of the fractions prepared here, it contains by far the highest amount of protein derived from an equivalent amount of spheroplasts. Saturation of binding to the nitrocellulose by proteins is only likely to be a serious problem with this postmitochondrial fraction. The amounts of 125I-protein A bound to the antigen bands were determined. The amounts bound to cytochrome b2 in mitochondria and postmitochondrial supernatant were additive to within ±20%. Estimates of fluorography of polyacrylamide gels (25), and preparation of [35S]methionine (approximately 100 Ci/mmol) from "SO4" (26). Zymolase 5000 was obtained from Kirin Brewery Ltd., Tokyo, Japan. Staphylococcus aureus protein A was obtained from Pharmacia, Sweden, and iodinated by the chloramine-T method (27). Carrier-free "SO4" was obtained from Amer sham International Ltd., Amersham, U.K. and tosyl-L-phenylalanyl chloromethyl ketone-treated bovine trypsin was from Merck, Darmstadt, GFR. The pH values of all buffers referred to in this paper were measured at 21°C.

RESULTS

Pulse-labeled Precursors Are Extramitochondrial—Immunoprecipitates from yeast spheroplasts which had been pulse-labeled with [35S]methionine for 3 min showed that 10-30% of the label incorporated into several mitochondrial proteins was present in the corresponding precursor forms. In order to determine the subcellular location of these precursors, pulse-labeled spheroplasts were fractionated into a mitochondrial fraction and a postmitochondrial supernatant. The precursor of cytochrome b2, cytochrome c1, and the β-subunit of F1-ATPase were found exclusively in the postmitochondrial supernatant (Table I). In contrast, mature radiolabeled cytochrome b2 and F1-ATPase β-subunit (which were immunoprecipitated together with their precursors from the un fractionated homogenates) were found largely within the mitochondria where they were protected from externally added trypsin. Total cytochrome b2 and F1-ATPase β-subunit polypeptides (as determined by the immune replica technique) distributed similarly to the corresponding radiolabeled mature polypeptides; however, since quantitation of unlabeled F1-ATPase β-subunit by immunoprecipitation understimates the amounts of protein in the postmitochondrial supernatant (see "Materials and Methods"), quantitation of the radiolabeled mature proteins by immunoprecipitation (Table I) is the better method. Most likely, the presence of pulse-labeled mature proteins in the postmitochondrial supernatant reflects leakage from the mitochondria during the fractionation procedure rather than a physiologically significant extramitochondrial pool, since the protease which processes these precursors is located within the mitochondrial matrix (28). Alternatively, the protease may have leaked from damaged mitochondria and processed extramitochondrial precursor. No steps were taken to inhibit this protease during the fractionation other than to keep the samples cold (0°C). The addition of 1,10-phenanthroline or other inhibitors was avoided to prevent possible artifacts of redistribution.

Precursors Accumulated in the Presence of CCCP Are Extramitochondrial—Import and processing of mitochondrial polypeptide precursors by yeast mitochondria is inhibited by CCCP (5, 29). Spheroplasts pulse-labeled in the presence of CCCP were fractionated into mitochondria and postmitochondrial supernatant. Virtually all of the immunoprecipitated precursors of cytochrome b2, cytochrome c1, and F1-ATPase β-subunit were found in the postmitochondrial supernatant (Table II); about 20% of cytochrome c peroxidase precursor was apparently intramitochondrial (see Ref. 21), presumably because import of this precursor was not completely inhibited in this experiment. The mature cytochrome b2 and F1-ATPase β-subunit were quantified to measure the leakage of mitochondrial proteins into the postmitochondrial supernatant. These unlabelled markers fractionated (Table II) much the same as in the experiment described above, so CCCP does not appear to disrupt the intactness of the mitochondria.

This experiment confirms previous in vitro studies which showed that CCCP blocks the maturation of mitochondrial protein precursors by inhibiting their import into mitochon-
Preparations of mitochondrial proteins were located outside the mitochondria

Wild type yeast was grown on low sulphate semisynthetic medium containing 2% (w/v) galactose, converted to spheroplasts, and pulse-labeled for 3 min at 30 °C. The spheroplasts were rapidly chilled by adding 2 volumes of ice-cold buffer and fractionated (see "Materials and Methods"). Each fraction was divided into two halves, one of which was incubated with a Loebl densitometer. The amount of total mature F1-ATPase P-subunit was chilling and fractionated as described under "Materials and Methods." The bands were quantified by scanning the films with a Joyce-Loebel densitometer. Approximately 10, 30, and 15% of the counts in cytochrome b2, F1-β-subunit, and cytochrome c1, respectively, were found in the precursor.

### Table I

<table>
<thead>
<tr>
<th>Polypeptide</th>
<th>Postmitochondrial supernatant</th>
<th>Mitochondria</th>
<th>Mitoplasts</th>
<th>Intermembrane space</th>
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<td>F1-ATPase β-subunit precursor</td>
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<td>Cytochrome c1 precursor</td>
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<td>Mature F1-ATPase β-subunit</td>
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<td>91 74</td>
<td>62 52</td>
<td>&lt;2 &lt;2</td>
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* 100% is defined as the sum of the amounts recovered from mitochondria and from postmitochondrial supernatant.

### Table II

Mitochondrial protein precursors pulse-labeled in the presence of CCCP are found in the postmitochondrial supernatant

Wild type yeast was grown on semisynthetic medium containing 2% lactate and converted to spheroplasts. These were labeled in the presence of 20 μM CCCP with [35S]methionine (300 μCi/ml) for 10 min at 30 °C and fractionated as described in the legend to Fig. 1. Samples were subjected to immunoprecipitation with antibodies directed against cytochrome c peroxidase, cytochrome b2, β-subunit of F1-ATPase, and cytochrome c1. Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. The amounts of radioactive cytochrome c1, precursors of mitochondrial intermembrane space and matrix, respectively, were determined with each fraction by the immune replica technique (17).

#### Table III

Post-translationally matured F1-ATPase β-subunit is located within the mitochondria

Wild type yeast was grown on semisynthetic medium containing 2% lactate, converted to spheroplasts, and labeled with [35S]methionine (300 μCi/ml) in the presence of 20 μM CCCP at 30 °C. After 10 min, unlabeled methionine was added to 2 mM and 2-mercaptoethanol to 0.05% (v/v). After a further period of 30 min, the spheroplasts were chilled and fractionated as described under "Materials and Methods." Each fraction was divided into two halves, one of which was incubated with trypsin (20 μg/ml) for 15 min on ice. The reaction with protease was terminated by addition of tosyl-L-lysine chloromethyl ketone to a final concentration of 2 mM. Protease-treated and untreated samples were dissociated with SDS and subjected to immunoprecipitation with antisera directed against cytochrome b2, the β-subunit of F1-ATPase, and cytochrome c1. The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. The bands were quantified by scanning the films with a Joyce-Loebel densitometer. Approximately 10, 30, and 15% of the counts in cytochrome b2, F1-β-subunit, and cytochrome c1, respectively, were found in the precursor.

#### Table III

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<td>69 73</td>
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<td>Total F1-ATPase β-subunit</td>
<td>2b &lt;2</td>
<td>98 90</td>
<td>87 80</td>
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* 100% is defined as the sum of the amounts recovered from mitochondria and from postmitochondrial supernatant.

* May be underestimated by a factor of 3-4, as discussed under "Materials and Methods."
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Postmitochondrial supernatant fraction. This may be because the newly imported protein is not yet completely assembled and therefore more likely to leak from the mitochondria during isolation. However, we have found that the immune replica method underestimates the amount of F$_1$-ATPase $\beta$-subunit in the postmitochondrial supernatant by 3–4-fold (see "Materials and Methods"). This experiment shows that the radioactively extramitochondrial precursor of a mitochondrial polypeptide can be post-translationally chased to the intramitochondrial mature form. This could not be shown by chasing yeast cells that had been pulse-labeled in the absence of CCCP since even after a pulse as brief as 1.5 min, the radioactivity in the precursor of F$_1$-ATPase $\beta$-subunit is less than that in the mature polypeptide. For this reason, it is not possible to show that, during a subsequent chase, any observed decrease in the precursor is paralleled by a corresponding increase in intramitochondrial mature polypeptide.

Since post-translational import of precursors into mitochondria is observed most clearly in spheroplasts which had been treated with CCCP, one may ask whether this import reflects a physiologically significant event. For example, the rate of import and processing under these conditions should approach the rate of import and processing of precursor polypeptides during a pulse-chase in the absence of CCCP. This was tested by pulse-chase labeling a suspension of wild type yeast cells in the presence or absence of CCCP. The disappearance of radioactivity from the immunoprecipitated precursor of the F$_1$-ATPase $\beta$-subunit was monitored as a function of the time after initiating the chase (Fig. 1). The precursor pulse-labeled in the absence of CCCP had a half-life of approximately 0.5 min whereas the precursor pulse-labeled in the presence of CCCP had a half-life of approximately 1 min. Thus, cells which had been treated with CCCP and 2-mercaptoethanol import polypeptides into their mitochondria only 2-fold more slowly than untreated cells. The lag of 1–1.5 min between addition of 2-mercaptoethanol and the onset of precursor maturation presumably reflects the time required to inactivate the CCCP and to restore energy coupling to the mitochondria. The lag found here with lactate-grown cells was 10-fold shorter than that found with galactose-grown yeast (16, 21).

The experiments described so far indicate that import of mitochondrial proteins can occur post-translationally in vivo under physiological conditions. However, it has been suggested, based largely on the observation of polysomes bound to the mitochondrial surface and investigations of the properties of these polysomes (9–11), that polypeptides destined for the mitochondria can bind to the mitochondrial surface before their synthesis is complete, and that this binding may start co-translationally and cannot be removed by treatments such as 10 mM phenanthroline. The processed polypeptide may represent the mature species or may be subjected to a second processing step (16, 19).

**Fig. 1.** The kinetics of post-translational processing of F$_1$-ATPase $\beta$-subunit precursor in intact yeast cells. Wild type yeast was grown on semisynthetic medium containing 2% lactate, harvested, and suspended to 0.1 g wet weight/ml in 40 mM KPi, pH 6.0, containing 0.5% (w/v) glucose and 0.5% lactate, pH 6.0. Two 8-ml aliquots of the suspension were pipetted into two flaskss and shaken at 30°C. CCCP (20 µM) was added to one vessel, followed by $^{35}$S methionine (200 µCi/ml). At the same time, $^{35}$S methionine (600 µCi/ml) was added to the second cell suspension. After 1.5 min, a 1-ml sample of the CCCP-free suspension was removed and mixed with 0.2 ml of 100% (w/v) trichloroacetic acid containing 2 µmol of L-methionine; at the same time, L-methionine (2 mM) was added to the remainder of the CCCP-free suspension and 1-ml samples were withdrawn and mixed with 0.2 ml of 100% (w/v) trichloroacetic acid after various periods of chase. The suspension containing CCCP was pulse-labeled for 10 min; a 1-ml sample was then removed and mixed with 0.2 ml of 100% (w/v) trichloroacetic acid containing 2 µmol of L-methionine; at the same time, L-methionine (2 mM) and 2-mercaptoethanol (0.05%, v/v) were added to the remainder of the CCCP-containing suspension and 1-ml samples were removed after various periods of chase and mixed with 0.2 ml of 100% (w/v) trichloroacetic acid. Cells were broken and the F$_1$-ATPase $\beta$-subunit was immunoprecipitated as described under "Materials and Methods." The immunoprecipitates were subjected to SDS-10% polyacrylamide gel electrophoresis and fluorography. The radioactivity in the precursor bands was quantitated by scanning of the films with a Camag densitometer. The ordinate gives the amount of precursor remaining after various periods of chase after a pulse in the absence (– – – –) or presence (● – – ●) of CCCP. The percentage of precursor at the beginning of the chase is taken as 100%. At the beginning of the chase, the precursor form contained 15% (in the absence of CCCP) and 80% (in the presence of CCCP) of the radioactivity found in the precursor plus mature form of the F$_1$ $\beta$-subunit.

**Fig. 2.** Post-translational versus co-translational import of polypeptides into mitochondria. The synthesis of a polypeptide destined for the mitochondrion is initiated on cytosolic "free" ribosomes and chain elongation yields the nascent precursor (step 1). If the rate of precursor synthesis exceeds the rate of precursor import into the mitochondrion, the precursor is completed and discharged into an extramitochondrial presumably cytosolic pool (step 2). The precursor can then be post-translationally imported (step 3) and processed (step 4). If the rate of precursor synthesis is slower than that of precursor import and if the domains necessary for binding to the mitochondria are already present in the nascent precursor, it may be imported (step 5) and processed (step 6) co-translationally. The processed nascent chain can then be completed (step 7). Alternatively, co-translational import may be completed (step 8) prior to the first processing step. In each case, import can be blocked by uncouplers such as CCCP and the first processing step by chelators such as 1,10-phenanthroline. The processed polypeptide may represent the mature species or may be subjected to a second processing step (16, 19).
These may reflect an increase in co-translational import, but may also be explained simply as a consequence of the shorter time spent in the cytosol by a precursor awaiting import under conditions where fewer precursor molecules are competing for a limited number of uptake sites. These possibilities are further discussed below. In any case, the data of Fig. 3 suggest strongly that the sizes of precursor pools differ depending on the metabolic conditions. This is further supported by the finding (not shown) that the fraction of pulse-labeled sF, β-subunit present as a precursor after a 1.5-min pulse differs considerably depending on whether the experiment is performed with wild type cells grown in lactate, wild type cells grown in glucose, or a rho- mutant grown in glucose.

**Discussion**

In vitro experiments have shown that proteins can be imported into mitochondria after completion of translation (4-8, 31). However, it has also been suggested that import of proteins into mitochondria might exclusively or partly occur co-translationally. This possibility is supported by the findings that (i) cytoplasmic ribosomes bind to the yeast mitochondrial surface (9-11) and (ii) mRNA coding for some proteins destined for the mitochondria are enriched on this subpopulation of polysomes (15, 32). This study reports that import of proteins into mitochondria can occur post-translationally, not only in vitro, but also in vivo.

If import is post-translational in vivo, one would expect to find extramitochondrial pools of precursors awaiting uptake by mitochondria. Such pools were found by double isotope pulse-chase experiments in Neurospora (33) but were not detectable in yeast (14). The inability to detect such a pool cannot be taken to imply that no pool exists, but does define an upper limit for the size of the pool under the experimental conditions employed. The alternative interpretation of this finding is that the polypeptides were imported co-translationally (14) and thus did not pass through an extramitochondrial pool since the nascent chains would already bind to the mitochondrial surface. However, the accompanying paper demonstrates that only between 1 and 60% of any of 12 tested mRNAs coding for imported mitochondrial proteins was associated with mitochondria-bound polysomes, even under conditions which should maximize binding of cytoplasmic polysomes to the mitochondrial surface (15). Furthermore, it is shown here that in pulse-labeled yeast cells (i) a substantial fraction of radioactivity found in the sF, ATPase β-subunit is recovered with the precursor of this polypeptide, (ii) this precursor pool is extramitochondrial, and (iii) the size of the pool depends upon metabolic factors. This latter finding may at least partly explain the inability of Ade and Butow (14) to detect such a pool. While there exists no direct proof that mitochondria can import proteins co-translationally, we concur with the suggestion of Ade and Butow (14) that such a pathway may contribute to the overall import process. This view is outlined in Fig. 2. That at least some nascent precursors can bind to the mitochondrial surface seems very likely: the polysomes bound to the mitochondrial surface are enriched for most (but not all) mRNAs encoding polypeptides destined for mitochondria (15, 32) and the bound polysomes are dissociated from the mitochondrial surface by a combination of puromycin and a high salt concentration (9).

Unfortunately, there is no clear evidence as to the immediate fate of the nascent chains bound to the mitochondria. Although it has been shown by read-out experiments (in which these nascent chains are elongated to completion) that the completed polypeptides are found within the mitochondria (32), it is not clear whether the translocation process occurs before completion of the polypeptide chain. To demonstrate
that import of proteins into mitochondria can occur co-translationally will require the demonstration of a transmembranous nascent chain, as was shown in bacteria (34). In summary, the results presented here and elsewhere (15, 16, 21, 31, 33) indicate that import of at least some polypeptides into the mitochondria of living cells does not require concomitant protein synthesis. They also support the view (35) that a rigid distinction between co- and post-translational movement of polypeptides across membranes may no longer be warranted.

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REFERENCES