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Biochemistry

Mitochondrial membrane biogenesis: Identification of a precursor to yeast cytochrome *c* oxidase subunit II, an integral polypeptide*

(*in vitro* mitochondrial protein synthesis/pre-proteins/mitochondrial gene products/posttranslational proteolysis)

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ABSTRACT **Many of the polypeptides made on endogenous ribosomes inside of yeast mitochondria are hydrophobic "integral polypeptides" which are subunits of at least three oligomeric enzyme complexes (cytochrome *c* oxidase, rutamycin-sensitive ATPase, and coenzyme QH_2-cytochrome *c* reductase) of the inner mitochondrial membrane. In order to elucidate the pathway(s) followed by these polypeptides into the inner membrane we have used an *in vitro* mitochondrial translation system from yeast. By inhibiting this system with aurintricarboxylic acid, we have been able to demonstrate and accumulate a transient precursor to subunit II of cytochrome *c* oxidase. This precursor, designated II′, is approximately 1,500 daltons larger than mature subunit II and most likely is a form of subunit II with an NH_2-terminal extension. Although this precursor appears to be processed cotranslationally under normal conditions, it does associate in unprocessed form with mitochondrial membranes when allowed to accumulate in the presence of aurintricarboxylic acid, and it can be processed postranslationally upon removal of the drug. None of the other mitochondrial translation products made in this system exhibits larger precursors. These results indicate that at least one mitochondrial translation product has a transient "leader sequence" and is inserted into the inner mitochondrial membrane and processed cotranslationally, but they suggest that other pathways may be followed by the other translation products.**

The incorporation of integral membrane polypeptides into biological membranes is a complex process which is still only poorly understood. Some integral polypeptides appear to be made on membrane-bound polyribosomes and incorporated into the membrane cotranslationally (1–3); other integral polypeptides appear to be made on free ribosomes and incorporated into the membrane posttranslationally (4–7). As yet, the molecular determinants that favor one mode of insertion or the other and the role of transient "signal sequences" in the initial targeting of integral polypeptides to the membrane for insertion either cotranslationally or posttranslationally remain to be elucidated.

To study this process we have chosen to examine the pathways followed by the integral polypeptides that are present in the inner mitochondrial membrane of *Saccharomyces cerevisiae* and, in particular, those that are translated on the endogenous mitochondrial ribosomes bound to the inner membrane (8, 9). These endogenous mitochondrial translation products are most likely coded for by the mitochondrial genome (10) and are unique subunit polypeptides of cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1), rutamycin-sensitive ATPase (ATP phosphohydrolase, EC 3.6.1.3), and coenzyme QH_2-cytochrome *c* reductase (11). Because these mitochondrially made polypeptides represent a spectrum of hydrophobicities (12–14), vary in their topological distribution relative to the two sides of the inner membrane (15–18), and are products of a genome that is subject to easy genetic manipulation (10), they seem to be ideal for studies aimed at determining if dissimilar integral polypeptides that are inserted into the same membrane follow similar pathways. In addition, because these polypeptides are uniformly more apolar than any of the integral polypeptides whose biosynthesis has been studied thus far, they may reveal new, as yet unexpected, mechanisms for the *in vivo* insertion of proteins into membranes.

MATERIALS AND METHODS

Growth and Labeling of Cells. The wild-type *S. cerevisiae* strain D273-10B (α; ATCC 24657) was grown at 30°C as described (6) on a semisynthetic growth medium (19). Cells were harvested during the early to middle exponential growth phase at cell densities that did not exceed 5×10^7 cells per ml.

Preparation of Mitochondria for *In Vitro* Translation. Mitochondria were prepared from spheroplasted cells as described (19) except that prior to lysis the spheroplasts were allowed to recover metabolically at 28°C for 30 min in recovery medium [containing per liter: 3 g of yeast extract (Difco), 10 g of galactose, 0.8 g of $(NH_4)_2SO_4$, 0.7 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of NaCl, 1.0 g of KH_2PO_4, 0.4 g of anhydrous $CaCl_2$, 5 mg of $FeCl_3 \cdot 6H_2O$, and 182 g of sorbitol]. Spheroplasts were suspended at a concentration of 1 g (wet weight) per 100 ml of recovery medium and aerated by shaking (100 rpm).

***In Vitro* Mitochondrial Translation.** Isolated mitochondria (0.5 mg of protein per ml) were incubated in a modified "protein synthesizing mixture" (19) adjusted to contain 0.5 mM GTP, 50 μg of cycloheximide per ml, 1 μM L-methionine, and all other amino acids at 100 μM each. The mixture was aerated at 30°C with vigorous shaking (200 rpm), and labeling was initiated by the addition of L-[^{35}S]methionine (800–1100 Ci/mmol; 1 Ci = 3.7×10^{10} bequerels) to a final concentration of 0.03 mCi/ml. Aliquots were withdrawn for analysis by sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel electrophoresis and immunoreplicate electrophoresis as described in individual figure legends. The kinetics of incorporation of label into mitochondrial protein were followed by spotting 10-μl aliquots onto a Whatman 3MM filter paper grid saturated with 10% (wt/vol) trichloroacetic acid/10 mM L-methionine. After samples from all times points were taken, the filter paper was air dried and cut into individual squares containing each time point. Each square was incubated in 5% trichloroacetic acid/5 mM L-methionine for 5 min at 90°C, washed twice with cold (4°C) 5% trichloroacetic acid/5 mM L-methionine and once with ethanol/ether, 3:1 (vol/vol), and dried under a heat lamp.

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Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; ATA, aurintricarboxylic acid.

* This report is no. 3 in the series "Assembly of cytochrome *c* oxidase from *Saccaromyces cerevisiae*." The preceding paper in this series is ref. 7.

All squares were then assayed for radioactivity in a toluene-based scintillation fluid as described (6).

For labeling in the presence of inhibitors, the inhibitors were added at the times indicated in individual figure legends. Pactamycin and aurintricarboxylic acid (ATA) were made up as concentrated stock solutions in distilled water and added to give final concentrations of 1 and 500 μM, respectively.

Electrophoretic and Immunological Techniques. Labeled mitochondrial membranes (5–10 mg of protein per ml) were solubilized (20) in 10 mM $NaPO_4$, pH 7.0/1% 2-mercaptoethanol/1 mM Na_2EDTA/2% (wt/vol) $NaDodSO_4$ and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis on 15-cm-long 16% acrylamide gel slabs prepared, run, stained, and autoradiographed as described (6, 20). In all cases, electrophoresis was continued for 3–4 hr after the bromophenol blue dye front had run off the bottom of the resolving gel. Densitometric integration of autoradiograms was performed with an EC 910 transmission densitometer. Apparent molecular weights were determined on 12%, 15%, and 16% acrylamide gels with bovine serum albumin, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, ribonuclease A, and cytochrome *c* as standards.

Immunoreplication using anti-subunit II serum was performed as described (6); this antiserum was prepared as described by Poyton and Schatz (21).

Peptide Mapping. Radiolabeled membrane fractions containing the polypeptides to be used for the generation of tryptic and chymotryptic peptides were prepared and subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis as described above. The polypeptides of interest were localized on autoradiograms of the dried gels and were prepared for *in situ* hydrolysis by a modification (7) of the procedure of Elder *et al.* (22). *In situ* digestion of polypeptides in gel slices was carried out in 1 ml of 500 mM NH_4HCO_3 (pH 8.0) at 37°C for 16 hr. Aliquots of TPCK-trypsin (50 μg per aliquot; Worthington, 273 units/mg) or α-chymotrypsin (250 μg per aliquot, Worthington, 54 units/mg) were added at 4-hr intervals. Once digested, the 1-ml eluants were twice lyophilized (7), resuspended in chromatography buffer [butanol/pyridine/acetic acid/water, 32.5:25:5:20 (vol/vol)], and clarified if necessary by centrifugation for 2 min in an Eppendorf 3200 centrifuge. Small aliquots (10 μl) of each digest were analyzed on cellulose thin-layer plates (20 × 20 cm; precoated CEL MN 300, 0.1 mm; Brinkmann) developed for ascending chromatography with chromatography buffer (above) made 7% (wt/vol) in 2,5-diphenyloxazole. Chromatographed plates were air dried and subjected to fluorography at −70°C on Kodak XR-5 film.

Miscellaneous. Published procedures were followed for the measurement of protein and the recrystallization of $NaDodSO_4$ (6). All radioisotopes used in this study were purchased from Amersham/Searle. ATA was obtained from Sigma as the ammonium salt, and pactamycin was a kind gift from G. B. Whitfield (Upjohn Co., Kalamazoo, MI).

RESULTS

Translation Products of an *In Vitro* Mitochondrial Read-Out System. Previous studies aimed at identifying precursors to either secreted or membrane proteins have relied heavily on mRNA programmed in cell-free systems (1–4, 23–26). Unfortunately, because mRNA preparations from yeast mitochondria have not yet been successfully fully translated in such systems (10, 27, 28), this approach is not feasible for determining if there are precursors for yeast mitochondrial translation products. Therefore, we have adopted an alternative approach. This approach grew out of the observation that ATA, a drug which, at low concentrations, inhibits polypeptide chain initiation (29, 30) and interferes with the binding of ribosomes to membranes (31), induces the appearance of precursors to some secretory proteins when present in rough microsomal read-out systems (23).

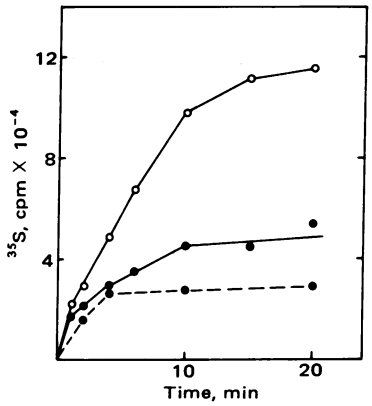

FIG. 1. Polypeptide synthesis by isolated yeast mitochondria incubated in the presence of 1 μM pactamycin (●---●) or 500 μM ATA (●—●). Aliquots containing 10 μg of mitochondrial protein were taken at the times indicated. O—O, Control.

In order to examine the feasibility of this approach for identifying precursors to mitochondrial translation products, we first assessed the ability of ATA to act on the yeast mitochondrial translation system. It was apparent (Fig. 1) that ATA is an effective inhibitor of endogenous protein synthesis in isolated yeast mitochondria. Like pactamycin, another inhibitor of polypeptide chain initiation (32), ATA led to a rapid cessation of protein synthesis with kinetics suggestive of the incorporation of label only into polypeptides being "read-out" from polysomes present at the time the drug was added. These observations, together with the findings that the concentration of drug used here effectively eliminates the incorporation of [^{35}S]methionine into formylmethionylpuromycin (unpublished observations) and that the presence of ATA does not markedly alter the size distribution of mitochondrial translation products (Fig. 2), clearly indicate that ATA is an effective inhibitor of polypeptide chain initiation, but not elongation, in isolated yeast mitochondria.

Although the size and relative amounts of most of those polypeptides made in the presence of ATA were the same as those made in its absence there was one interesting exception (Fig. 2). In mitochondria labeled in the presence of ATA, the polypeptide band corresponding to subunit II of cytochrome

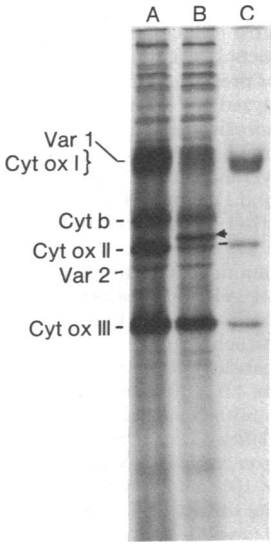

FIG. 2. Autoradiogram showing $NaDodSO_4$/polyacrylamide gel electrophoretic patterns of [^{35}S]-methionine-labeled polypeptides synthesized by isolated yeast mitochondria in the absence (lane A) or presence (lane B) of 500 μM ATA. Cytochrome *c* oxidase (Cy tox) subunits I, II, and III that had been labeled *in vitro* in the absence of ATA and immunoprecipitated from cholate/KCl extracts of mitochondrial membranes (19) were used as markers (lane C). Bands corresponding to subunits I, II, and III of cytochrome *c* oxidase, cytochrome *b*, and the Var 1 and Var 2 polypeptides (39) are as indicated. The arrow denotes polypeptide II′.

c oxidase was decreased in amount and replaced by a new polypeptide, designated II′. This polypeptide exhibited an apparent molecular weight 1500 ± 320 larger than that of mature subunit II and did not migrate anomalously in Na-DodSO$_4$ gels of different acrylamide concentrations.

Identification of II′ as a Precursor to Subunit II of Cytochrome *c* Oxidase. To examine the relationship between II′ and subunit II of cytochrome *c* oxidase and to test the hypothesis that II′ is a biosynthetic precursor to subunit II, we first tested the crossreactivity of II′ to antisera raised against subunit II. This was done by subjecting mitochondrial translation products labeled in the presence of ATA to immunoreplicate electrophoresis (33) with anti-subunit II antiserum in the agarose replicate gel. Both subunit II and II′ were strongly crossreactive to anti-subunit II antisera (Fig. 3).

To test further the degree of similarity between subunit II and II′ we compared their tryptic and chymotryptic peptides. The chymotryptic peptide patterns of subunit II and II′ were essentially identical (Fig. 4). In addition, the tryptic peptide patterns showed a high degree of similarity; four of the five peptide bands present in subunit II also were present in II′. It is interesting that II′ had two tryptic peptide bands (b and c) not present in subunit II, and subunit II had one peptide band (a) not present in II′.

A plausible explanation for these peptide differences is given in Fig. 5. According to this interpretation, II′ is a form of subunit II with an extended terminus that has a trypsin-sensitive site. Thus, peptide a in subunit II would include the mature terminus whereas one of those new peptides (b or c) found in II′, but not in subunit II, would include the new terminus of II′ and the other would include the region corresponding to the mature terminus of subunit II. If this interpretation is correct, there are no new methionine-containing chymotryptic peptides derived from the extended terminus of II′ and the chymotryptic peptide that contains the extended terminus of II′ (b and c) has the same R_F value as the peptide (a) that contains the mature terminus of subunit II. Despite the similar R_F values, we would predict nevertheless that the peptide band containing the terminus of II′ should be darker than its counterpart containing the mature terminus from subunit II because this region from II′ must have at least one additional methionine residue to be seen as a new band in the autoradiogram of the tryptic peptide patterns. This requirement is met by band d. By densitometric integration and normalization of counts applied to lanes C and D in Fig. 4, we found that peptide d from II′ had twice as much radioactivity as peptide d from subunit II. When considered together, these results strongly suggest that subunit II and II′ have a high degree of sequence similarity and that II′ has an extended NH_2 or COOH terminus.

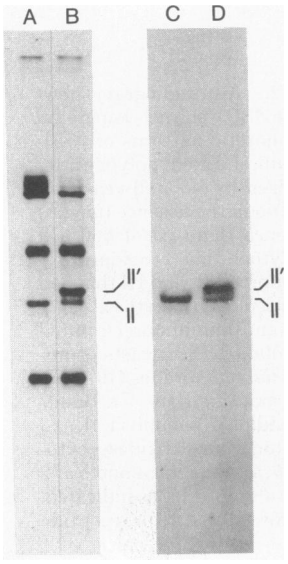

FIG. 3. Crossreactivity of polypeptide II′ to anti-subunit II antiserum. *In vitro* mitochondrial translation products labeled in the absence (lane A) or presence (lane B) of ATA were analyzed by immunoreplicate electrophoresis. The NaDodSO$_4$/polyacrylamide gel tracks (lanes A and B) were overlaid with a 1% agarose gel (lanes C and D) containing anti-subunit II antiserum prepared and characterized as described (21). Both polyacrylamide and agarose immunoreplicate gels were stained, dried, and autoradiographed. Lanes: A, *in vitro* mitochondrial translation products labeled with [^{35}S]methionine for 20 min, 4.0 × 10^4 cpm applied; B, *in vitro* mitochondrial translation products labeled with [^{35}S]methionine in the presence of 500 μM ATA for 20 min, 4.0 × 10^4 cpm applied; C, immunoreplicate of lane A; D, immunoreplicate of lane B.

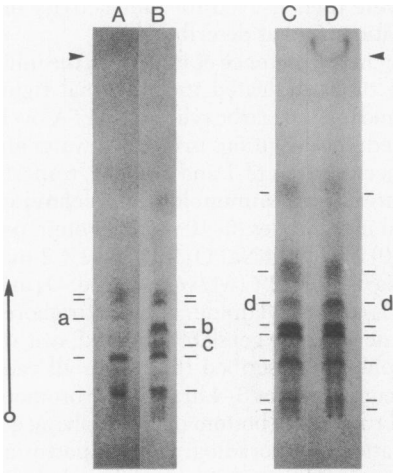

FIG. 4. Tryptic and chymotryptic maps of [^{35}S]methionine-labeled subunit II and II′. II′ was prepared from mitochondria labeled for 20 min in the presence of 500 μM ATA; subunit II was from mitochondria labeled for 20 min in its absence. Hydrolysis with trypsin and chymotrypsin was carried to completion. The tryptic and chymotryptic hydrolysates were analyzed on cellulose thin-layer chromatography plates developed with chromatography buffer containing 7% 2,5-diphenyloxazole and exposed to x-ray film at −70°C for fluorography. Lanes: A, tryptic peptide map for subunit II; B, tryptic peptide map for II′; C, chymotryptic peptide map for subunit II; D, chymotryptic peptide map for II′. The lines on each side of the fluorograms mark the positions of the peptide bands; the arrow heads denote the solvent front. Approximately 2 × 10^3 cpm was applied to each lane.

In view of the finding that II, like the precursor polypeptide of the light chain of immunoglobulin (23), is accumulated in a "read-out" translation system inhibited with ATA, we next examined the possibility that II′, likewise, has a transient NH_2-terminal extension. To do this we examined the kinetics of synthesis of II′ and subunit II. If, as suggested for the precursor of light chain polypeptide (23), the appearance and accumulation of II′ in the presence of ATA are the result of the inhibition of the binding of mitochondrial ribosomes to the inner membrane (or of the inhibition of a processing protease) and if II′ has an NH_2-terminal extension that is still intact on nascent polypeptides located on ribosomes near the 5′ end of the polysome bound mRNA, then II′ should accumulate at the expense of subunit II in a time-course experiment run in a mitochondrial "read-out" translation system inhibited with ATA.

This indeed was the case. Only mature subunit II was synthesized at early time points, and the amount of II′ synthesized relative to subunit II increased progressively with time (Fig. 6). These results parallel those derived from similar studies with

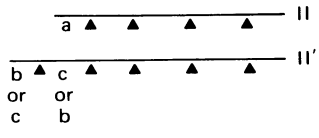

FIG. 5. Interpretation of the tryptic peptide results shown in Fig. 4. The positions of the trypsin-sensitive sites (▲) have been chosen arbitrarily. As indicated, at present it is impossible to decide if peptide band b or c includes the new terminus in II′.

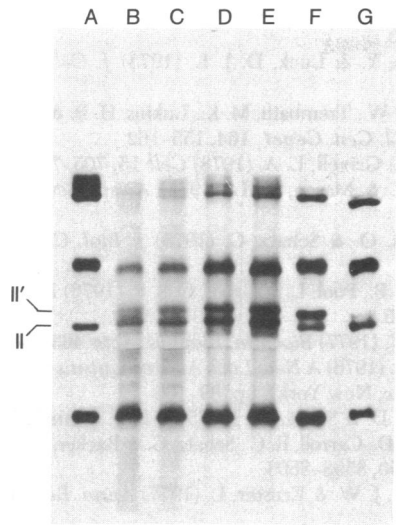

FIG. 6. Appearance of II′ during the course of *in vitro* mitochondrial read-out in the presence of [^{35}S]methionine and 500 μM ATA. Each lane received 2.0 × 10^4 cpm. Lanes: A, *in vitro* translation products labeled for 25 min in the absence of ATA; B–E, *in vitro* translation products labeled in the presence of ATA added at time 0 and terminated at 1, 6, 9 or 25 min, respectively; F, *in vitro* translation products labeled in the presence of ATA added 5 min before the isotope (in order to label II′ preferentially) and terminated 25 min after the addition of the isotope; G, *in vitro* translation products labeled as in lane F and chased for 25 min after the labeled and once-washed mitochondria were resuspended in a protein-synthesizing mixture devoid of ATA and [^{35}S]methionine. Labeling of translation products shown in lanes B–F was terminated by the addition of an equal volume of 0.2 M L-methionine to the protein-synthesizing mixture and incubation for 10 min at 28° C.

the precursor of light chain polypeptide and, by analogy, can be interpreted to mean that II′ is a precursor of subunit II with a transient NH_2-terminal extension. This extension would have already been removed from those polypeptides labeled at early times because these polypeptides, read from ribosomes near the 3′ end of the mRNA, most likely would have already associated with a processing protease of the inner mitochondrial membrane prior to the addition of ATA. It should be emphasized that this interpretation of the kinetic data implies that proteolytic processing of II′ is normally cotranslational, a conclusion that is further strengthened by the findings that: (*i*) II′ can not be observed during short pulse-labeling experiments *in vivo* or *in vitro* with isolated mitochondria (unpublished observations); and (*ii*) mature subunit II read-out in the presence of ATA is not labeled in tryptic peptide a (data not shown), the peptide

Table 1. Distribution of *in vitro* synthesized mitochondrial translation products in mitochondrial subfractions

	Protein		Trichloroacetic acid-precipitable radioactivity	
Subfraction	Control	With ATA	Control	With ATA
Whole mitochondria	100	100	100	100
Total soluble	29	32	4.5	6.5
Inner and outer membranes	71	68	95.5	94.0

Total soluble and membrane fractions were prepared by sonication (7) of mitochondria labeled *in vitro* for 20 min with [^{35}S]methionine in the absence (control) or presence of 500 μM ATA. The inner and outer membrane fraction corresponds to the submitochondrial particle membrane fraction (7).

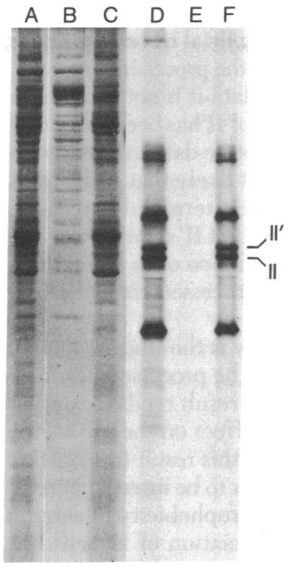

FIG. 7. Accumulation of II′ in the mitochondrial membrane fraction. Mitochondrial translation products were labeled in the presence of ATA and analyzed as in Fig. 3 (lane B). Once labeled, mitochondria were isolated by centrifugation and fractionated into membrane and soluble fractions by sonication (7). As with the *in vitro* translation products labeled in the absence of ATA (34), most (95%) of the trichloroacetic acid-precipitable radioactivity remained with the membrane fractions. Lanes: A–C, Coomassie blue-stained gel of unfractionated mitochondria (lane A, 100 μg of protein applied), the "soluble" mitochondrial fraction (lane B, 50 μg), and the submitochondrial particle fraction (lane C, 70 μg); D–F, autoradiograms of lanes A–C, respectively.

that most likely contains the mature terminus that is extended in II′.

II′ Can Be Processed Posttranslationally. In order to learn more about the processing pathway following the II′, we first allowed II′ to be accumulated in the presence of ATA and then examined its distribution in submitochondrial fractions. Although the soluble fraction accounted for 30% of the total protein, most of the radiolabeled proteins synthesized in the presence or absence of ATA were membrane-associated (Table 1). It is clear from Fig. 7 that II′ is as abundant in the mitochondrial membrane fraction as in unfractionated mitochondria (lanes D and F). It is also clear that, although a number of polypeptides are released into the soluble mitochondrial fraction (Fig. 7, lane B), II′ is not among them (Fig. 7, lane E). Thus, we conclude that most, if not all, of II′ is associated with mitochondrial membranes. Although the type of association II′ makes with the membranes is currently not known, it is apparent from our isolation procedures that II′ cannot be removed from the membrane with high- or low-salt washes. In addition, it is apparent that the II′ that is accumulated in the membrane is processed to mature II upon removal of ATA (Fig. 6, lanes F and G). These results suggest that II′ is most likely associated with the membrane as an integral polypeptide and is positioned in the membrane in such a way as to be accessible to its processing protease. These results also make clear that II′ can, under certain conditions, be processed posttranslationally.

DISCUSSION

It has been demonstrated previously that most, if not all, of the polysomes found inside of mitochondria are tightly bound to the inner mitochondrial membrane (8, 9) and that their major polypeptide products are hydrophobic "integral membrane proteins." In this study we demonstrate that at least one of these proteins, subunit II of yeast cytochrome *c* oxidase, is formed from a larger precursor, designated II′. In order to do this we have used an *in vitro* mitochondrial translation system inhibited with ATA. Although this drug is an effective inhibitor of polypeptide chain initiation in isolated yeast mitochondria, it is unlikely that the accumulation of II′ in this system is the result of an effect of ATA on polypeptide chain initiation because pactamycin, another inhibitor of polypeptide chain initiation in this system, did not elicit the formation of II′ (unpublished observations). Indeed, it appears likely that the accumulation

of II′ results from a side effect of ATA, such as its interference with the functional binding of mitochondrial ribosomes to the inner membrane (31) or its inhibition of the processing protease. Although in the absence of sequence data it is not possible to decide unequivocally which terminus of II has been extended in II′, the chymotryptic and tryptic peptide data together with the kinetic data presented here make it likely that II′ is a precursor form of II with a transient NH_2-terminal extension. When considered with the observation that II′ is not detectable during short-pulse experiments done *in vivo* or *in vitro*, these data also suggest that II′ is normally processed cotranslationally.

One surprising result from this study is the finding that II′ associates with the membrane and can be processed posttranslationally upon removal of ATA. This result tends to support the conclusion that ATA has a direct effect on the processing protease. More importantly, however, this result implies that II′ does not have to be processed in order to be inserted into the membrane. In view of the overall hydrophobicity of subunit II (12), it is conceivable that the association of II′ with the membrane is mediated not by a transient NH_2-terminal extension as suggested by the signal hypothesis but rather by the apolar regions of the mature subunit II. This mode of insertion of integral proteins into membranes was suggested some time ago by Bretscher (35).

Finally, it is interesting that subunit II of cytochrome *c* oxidase is the only mitochondrial translation product that exhibits a precursor form in the *in vitro* read-out system used here. At the moment there appear to be at least two plausible explanations for this result. On the one hand, it is possible that the other translation products also have precursors but that they are not accumulated in the presence of ATA. This might be expected if, for instance, ATA inhibits the processing protease for subunit II but not the precursors for other translation products. On the other hand, the other translation products may not have transient precursors. This possibility is supported, in part, by the observation that at least some mature mitochondrial translation products retain formate and hence must have formylmethionine, their initiating amino acid, at their NH_2 terminus (36–38). Further experimentation is required to decide which of these two possibilities is correct.

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