Proc. Natl. Acad. Sci. USA
Vol. 77, No. 7, pp. 3998–4002, July 1980
Biochemistry

Cytoplasmically made subunits of yeast mitochondrial F_1-ATPase and cytochrome *c* oxidase are synthesized as individual precursors, not as polyproteins

(*in vitro* protein synthesis/immunoprecipitation/*N*-formylmethionine-tRNA/mitochondrial biogenesis)

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Communicated by George E. Palade, April 25, 1980

ABSTRACT **At least three subunits of yeast mitochondrial F_1-ATPase (ATP phosphohydrolase, EC 3.6.1.3) and at least two subunits of cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) are synthesized outside the mitochondria and imported into the organelles as individual precursors that are between 2000 and 6000 daltons larger than the mature subunits. These precursors were shown to be primary translation products. Therefore, neither the five F_1 subunits nor the four small cytochrome *c* oxidase subunits are synthesized as a single polyprotein.**

Most of the mitochondrial proteins are encoded by nuclear genes, synthesized on extramitochondrial ribosomes, and then transferred into the mitochondria (1). This process is independent of translation (2, 3) and, in most instances, is coupled to the energy-dependent proteolytic processing of a larger precursor (4). The *bona fide* precursors of mitochondrial proteins identified so far are approximately 2000 to 6000 daltons larger than the corresponding mature proteins (3, 5–7). In addition, the identification of discrete precursors to cytoplasmically made subunits of yeast mitochondrial F_1-ATPase (ATP phosphohydrolase, EC 3.6.1.3) (3) and the cytochrome bc_1 complex (5) implied that each subunit of these oligomeric mitochondrial complexes is initially synthesized as a separate precursor. In contrast, Poyton *et al.* (8–11) have reported that the four cytoplasmically made subunits of cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) are initially synthesized as a single "polyprotein" precursor that is transported into the mitochondria and then cleaved to the mature subunits.

In this study we have identified the primary translation products of several cytoplasmically made subunits of cytochrome *c* oxidase and F_1-ATPase. Because protein synthesis is initiated with methionine bound to a specific "initiator" tRNA ($tRNA_f$), a primary translation product can be NH_2-terminally labeled by synthesizing it in a reticulocyte lysate with *N*-formyl[^{35}S]methionine-$tRNA_f$ as radioactive tracer (12). In this way, the NH_2 terminus is selectively and stably labeled with *N*-formyl[^{35}S]methionine because eukaryotic cells lack enzymes for removing NH_2-terminally blocked methionine (12). Proteins labeled in this manner must have their original NH_2 termini intact because proteolytic cleavage of an NH_2-terminal fragment should remove all radioactivity from the remainder of the polypeptide chain.

The results of these experiments confirm the original observation (3, 5) that individual cytoplasmically made subunits of oligomeric mitochondrial complexes are synthesized as discrete precursors, not as polyproteins.

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MATERIALS AND METHODS

The wild-type *Saccharomyces cerevisiae* strain D273-10B, growth and *in vivo* labeling of yeast cells, extraction of RNA, *in vitro* protein synthesis in a nuclease-pretreated reticulocyte lysate, and immunoprecipitation after dissociation with NaDodSO$_4$ have been described (3, 5). Published methods were used for pulse-labeling of yeast spheroplasts in the presence of the uncoupler carbonylcyanide *m*-chlorophenylhydrazone (4), immunoprecipitation of cytochrome *c* oxidase from cholate/KCl extracts of mitochondria (13), preparation of subunit-specific antibodies (14), one-dimensional proteolytic "fingerprinting" (15), measurement of incorporation of radioactivity into protein (3), and electrophoresis in NaDodSO$_4$/10% polyacrylamide slab gels for analyzing F_1 subunits (16) or in NaDodSO$_4$/15% polyacrylamide/8 M urea gels for cytochrome *c* oxidase subunits (17) and fluorography (18).

Yeast initiator $tRNA_f$, isolated by countercurrent distribution and chromatography on benzoylated DEAE-cellulose, was a generous gift of R. Giége (Institut de Biologie Moléculaire et Cellulaire du Centre National de la Recherche Scientifique, Strasbourg, France). This pure preparation exhibited only initiator methionine-accepting activity (19). *Escherichia coli* aminoacyl tRNA synthetases were prepared and yeast $tRNA_f$ was acylated and formylated as described (20) except that acylation and formylation were done in the absence of unlabeled methionine and other amino acids but in the presence of 100 μg of bovine serum albumin, 2 nmol of $tRNA_f$, and 1 mCi (1 Ci = 3.7×10^{10} becquerels) of L-[^{35}S]methionine (>1000 Ci/mmol; Amersham/Searle) per ml. After 30 min, >80% of the ^{35}S was usually precipitable by cold 5% trichloroacetic acid. Acylation was stopped by extraction with an equal volume of water-saturated phenol. The aqueous layer was made 1.2 M in LiCl and passed through a column of Sephadex G-50 (fine) equilibrated with 0.2 M Na acetate, pH 5.0/0.01 M Mg acetate. Radioactive fractions in the excluded peak were pooled, mixed with total yeast RNA as carrier, and precipitated with 2 vol of ethanol at −20°C.

For *in vitro* protein synthesis with *N*-formyl[^{35}S]methionine-$tRNA_f$, an aliquot (sufficient to give 50 μCi of ^{35}S per ml of final reaction mixture) of the ethanol precipitate (from above) was washed with ethanol at 0°C and dried under reduced pressure for 5 min. To the same vessel was added a nuclease-treated reticulocyte lysate (21), yeast RNA (3), and 5 mM unlabeled L-methionine to suppress any labeling reaction except that involving *N*-formyl[^{35}S]methionine-$tRNA_f$. All other steps were as described (3).

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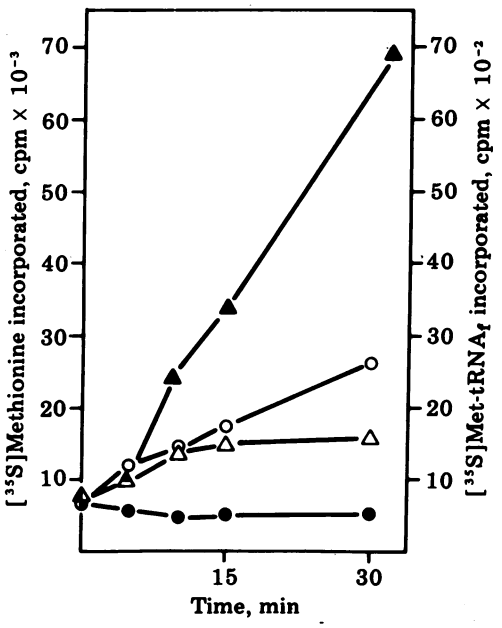

FIG. 1. Aurin tricarboxylic acid immediately inhibits labeling of polypeptides by *N*-formyl[^{35}S]methionine-tRNA$_f$ but not by [^{35}S]methionine. Equal aliquots of a reticulocyte lysate programmed with yeast RNA were labeled with the two different radioactive precursors, and incorporation of ^{35}S into protein was measured (3). ▲, [^{35}S]Methionine; △, [^{35}S]methionine plus 0.1 mM aurin tricarboxylic acid; ○, *N*-formyl[^{35}S]methionine-tRNA$_f$; ●, *N*-formyl[^{35}S]methionine-tRNA$_f$ plus 0.1 mM aurin tricarboxylic acid.

RESULTS

***In Vitro* Protein Synthesis with *N*-Formyl[^{35}S]Methionine-tRNA$_f$.** [^{35}S]Methionine was efficiently incorporated into protein *in vitro* whether added as free methionine or as *N*-formylmethionine-tRNA$_f$. As expected, labeling by free methionine, but not by *N*-formylmethionine-tRNA$_f$, was completely suppressed by 5 mM nonradioactive methionine (not shown). All labeling experiments with the charged tRNA$_f$ were therefore done in the presence of 5 mM nonradioactive methionine in order to prevent internal labeling of polypeptides via free [^{35}S]methionine that might be generated by deacylation or other side reactions. When low concentrations of aurin tricarboxylic acid were used to inhibit initiation of protein synthesis (22), incorporation of [^{35}S]methionine bound to "initiator" tRNA stopped immediately whereas incorporation of free [^{35}S]methionine only stopped after a lag of about 10 min (Fig. 1). These data, and those of others (12, 22, 23), leave little doubt that *N*-formyl[^{35}S]methionine-tRNA$_f$ in the presence of excess unlabeled methionine exclusively labels the NH_2 terminus. Additional support for this view is provided by the processing experiments described below.

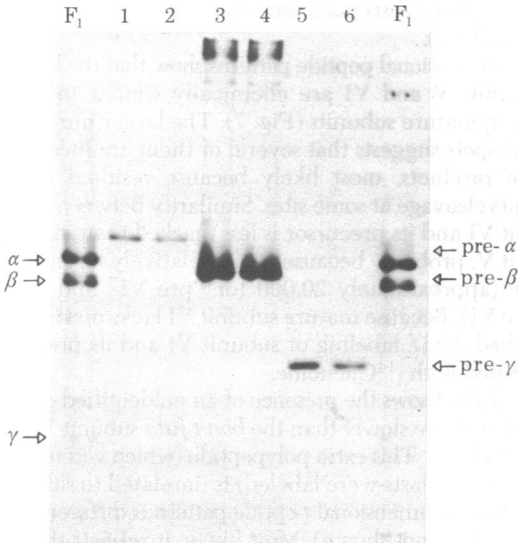

FIG. 2. Fluorogram of a 10% polyacrylamide gel slab showing that the three largest F_1-ATPase subunits are synthesized as individual precursors. Lanes: 1 and 2, α-subunit precursor (pre-α) labeled with [^{35}S]methionine and *N*-formyl[^{35}S]methionine-tRNA$_f$, respectively; 3 and 4, β-subunit precursor (pre-β) labeled as in 1 and 2; 5 and 6, γ-subunit precursor (pre-γ) labeled as in 1 and 2; F_1, mature F_1-ATPase subunits immunoprecipitated from yeast cells grown overnight in $^{35}SO_4^{2-}$.

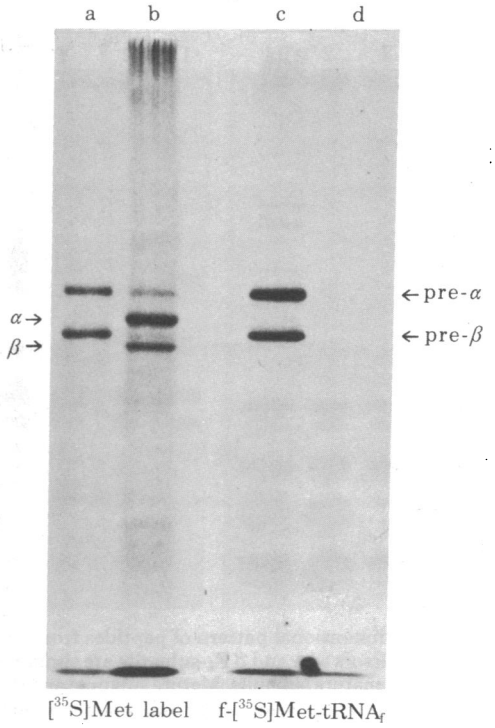

FIG. 3. Fluorogram of a 10% polyacrylamide slab gel showing that processing of NH_2-terminally labeled precursors to F_1-subunits generates unlabeled mature subunits. A reticulocyte lysate programmed with yeast RNA was labeled for 60 min with either [^{35}S]-methionine or *N*-formyl[^{35}S]methionine-tRNA$_f$. One half of each labeled lysate was then made 3% in $NaDodSO_4$, heated to 100°C for 3 min and subjected to immunoprecipitation with a mixture of anti-α and anti-β antiserum (lanes a and c). The remaining half was adjusted to 0.8 M in sorbitol and incubated for 30 min at 27°C with yeast mitochondria (final concentration, 5 mg of mitochondrial protein per ml); after addition of protease inhibitors (3), the samples were dissociated in $NaDodSO_4$ and immunoprecipitated as described above (lanes b and d). The lanes devoid of radiolabeled F_1-subunits clearly displayed F_1-subunits upon staining with Coomassie blue, showing that immunoprecipitation of the mature subunits had been effective (not shown).

The Three Largest F_1-ATPase Subunits Are Made as Separate Precursors. The three largest F_1-ATPase subunits (α, β, and γ) were synthesized in a reticulocyte lysate in the presence of either free [^{35}S]methionine or *N*-formyl[^{35}S]methionine-tRNA$_f$; they were then immunoprecipitated with subunit-specific antisera and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis followed by fluorography. Fig. 2 shows that the NH_2-terminally labeled precursors were indistinguishable from the precursors labeled in all methionine residues. Because the same precursors are also found in pulse-labeled intact cells (3), we conclude that the α, β, and γ subunits of F_1-ATPase are made as individual precursors.

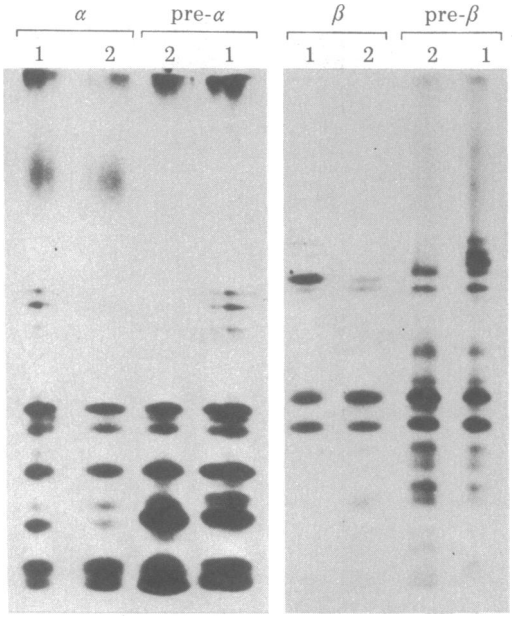

FIG. 4. One-dimensional pattern of peptides from the *in vitro*-synthesized precursors to α and β F_1-subunits are similar to those of the corresponding mature subunits. Mature subunits and their *in vitro* synthesized precursors (labeled with [^{35}S]methionine) were prepared as in Fig. 2, separated on preparative 10% polyacrylamide gels, and subjected to partial proteolysis with 0.5 μg (lanes 1) or 5 μg (lanes 2) of *Staphylococcus aureus* V8 protease as described (15). The digestion products were separated on a 15% polyacrylamide gel and visualized by fluorography.

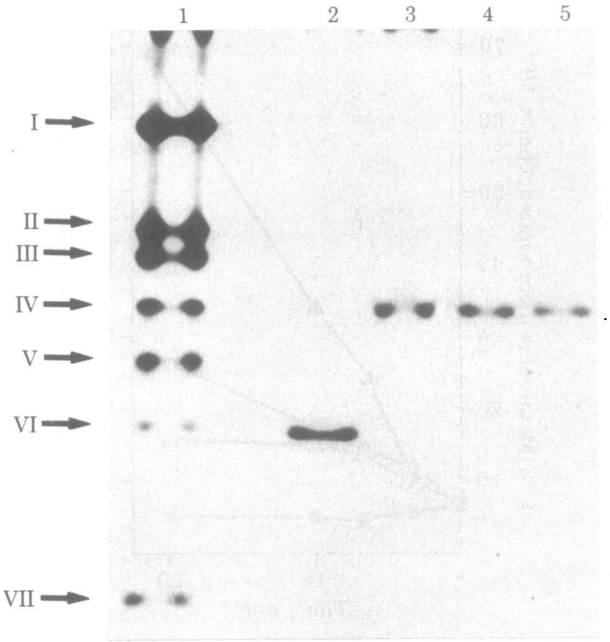

FIG. 5. Cytochrome *c* oxidase subunit VI is synthesized as an individual 20,000-dalton precursor. 1, cytochrome *c* oxidase standard. After yeast cells were grown overnight in $^{35}SO_4^{2-}$, the mitochondria were isolated and extracted with cholate/KCl (13) and the extract was immunoprecipitated with an antiserum against the holoenzyme. Lanes: 1, cytochrome *c* oxidase standard; 2, mature subunit VI immunoprecipitated from uniformly labeled spheroplasts with anti-subunit VI antiserum; 3, subunit VI precursor immunoprecipitated from spheroplasts labeled for 15 min with [^{35}S]methionine in the presence of uncoupler; 4 and 5, subunit VI precursor synthesized in a reticulocyte lysate in the presence of [^{35}S]methionine and *N*-formyl[^{35}S]methionine-$tRNA_f$, respectively. The Roman numerals indicate the seven cytochrome *c* oxidase subunits.

Processing of Precursors by Mitochondria Removes NH_2-Terminal Label. When F_1-subunit precursors were made *in vitro* in the presence of free [^{35}S]methionine and then incubated with yeast mitochondria, they were processed to their mature size (Fig. 3; see also ref. 3). In contrast, precursors labeled by *N*-formyl[^{35}S]methionine-$tRNA_f$ lost all radioactivity when they were processed to mature subunits by isolated mitochondria. These observations further demonstrate the selectivity of labeling by the $tRNA_f$ method and suggest that the α and β F_1-subunit precursors carry NH_2-terminal "extensions" which are proteolytically removed by mitochondria.

Precursors to the α and β Subunits of F_1-ATPase Yield Proteolytic Peptide Patterns Similar to Those of the Mature Subunits. The one-dimensional patterns of proteolytic products depicted in Fig. 4 show that the precursors resemble the corresponding mature subunits not only with respect to antigenic specificity but also with respect to amino acid sequence.

Subunits V and VI of Cytochrome *c* Oxidase Are Made as Individual Primary Translation Products. Depleting the mitochondrial matrix of ATP *in vivo* prevents the processing of several precursors to mitochondrial proteins to their mature forms (4). When yeast spheroplasts are labeled with [^{35}S]methionine under these conditions they accumulate higher molecular weight forms of the cytoplasmically made subunits V and VI of cytochrome *c* oxidase (24). This is confirmed in Fig. 5: only the mature subunit VI was immunoprecipitated with anti-VI from cells that had been grown overnight in $^{35}SO_4^{2-}$ (lane 2) whereas spheroplasts pulse-labeled in the presence of uncoupler yielded only a larger form of this subunit (lane 3). In order to prove that this larger form is a precursor, we checked whether it is also synthesized *in vitro* with either free [^{35}S]-methionine or *N*-formyl[^{35}S]methionine-$tRNA_f$ as tracer. Indeed, the large form was the only labeled *in vitro* product regardless of whether synthesis occurred in the presence of free [^{35}S]methionine (lane 4) or *N*-formyl[^{35}S]methionine-$tRNA_f$ (lane 5). Similar data were obtained for cytochrome *c* oxidase subunit V (Fig. 6). In contrast to many other mitochondrial precursors (3, 5, 6) the precursor to subunit V could not be detected in spheroplasts pulse-labeled in the absence of uncoupler CCCP (lane 3).

Two-dimensional peptide patterns show that the larger forms of subunits V and VI are chemically similar to the corresponding mature subunits (Fig. 7). The larger number of ^{35}S-labeled spots suggests that several of them are incomplete digestion products, most likely because residual $NaDodSO_4$ prevents cleavage at some sites. Similarity between the mature subunit VI and its precursor is less marked than in the case of subunit V, probably because of the relatively large difference in size (approximately 20,000 for "pre VI" and 12,500 for mature VI). Because mature subunit VI lacks methionine (unpublished data), labeling of subunit VI and its precursor was performed with [^{14}C]leucine.

Fig. 6 also shows the presence of an unidentified polypeptide migrating more slowly than the *bona fide* subunit V precursor (lanes 5 and 6). This extra polypeptide (which was not observed when spheroplasts were labeled) is unrelated to subunit V because its two-dimensional peptide pattern is different from that of subunit V (not shown). Most likely, it reflects the presence of a contaminating antibody in our anti-V antiserum.

The Larger Form of Cytochrome *c* Oxidase Subunit V Is a Precursor to the Mature Subunit. In order to detect *in vivo* accumulation of a cytochrome *c* oxidase precursor in the absence of inhibitors, we pulsed labeled wild-type spheroplasts. No larger form of subunit V could be detected after pulse labeling at 28°C (Fig. 6, lane 3), but it was found after pulse la-

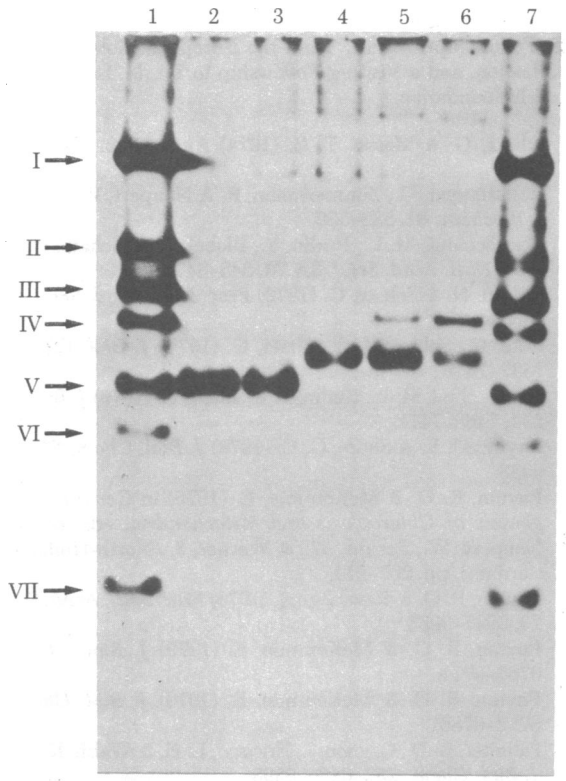

FIG. 6. Cytochrome *c* oxidase subunit V is synthesized as an individual 15,000-dalton precursor. Experimental conditions were essentially as in Fig. 5 except that the subunit-specific antiserum used was directed against subunit V. Lanes: 1 and 7, cytochrome *c* oxidase standard; 2, mature subunit V immunoprecipitated from uniformly labeled spheroplasts; 3, mature subunit V from spheroplasts labeled for 15 min with [^{35}S]methionine; 4, subunit V precursor from spheroplasts labeled for 15 min with [^{35}S]methionine in the presence of uncoupler; 5 and 6, subunit V precursor synthesized in a reticulocyte lysate in the presence of [^{35}S]methionine and *N*-formyl[^{35}S]methionine-tRNA$_f$, respectively.

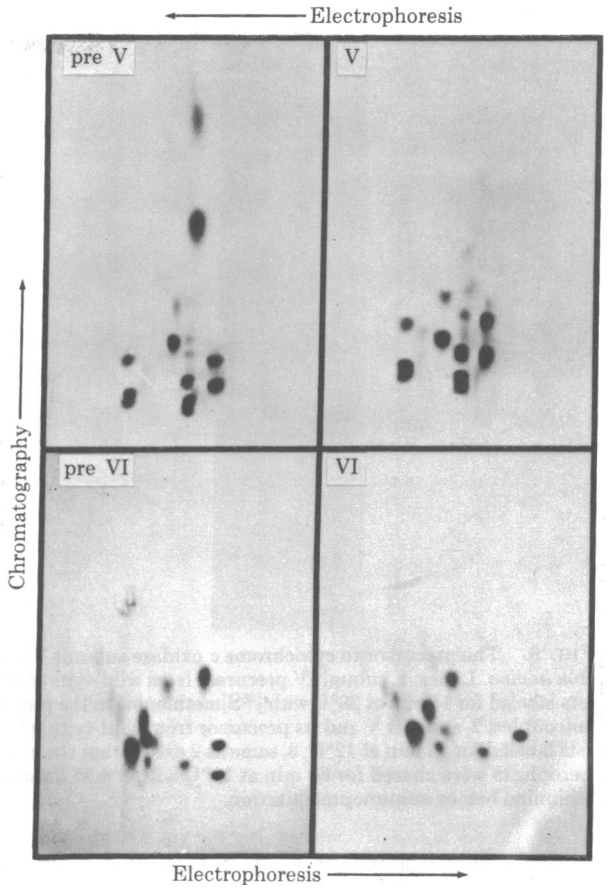

FIG. 7. The larger forms of cytochrome *c* oxidase subunits V and VI have two-dimensional tryptic peptide patterns similar to those of the corresponding mature subunits. A radioautogram of the dried cellulose plates is shown. Polyacrylamide gel slices of mature cytochrome *c* oxidase subunits V or VI or their precursors were extracted for 1 day at room temperature with 25% isopropanol/10% acetic acid, for 3 days with 25% isopropanol, and finally for 12 hr with 10% methanol. They were then digested for 20 hr at 37°C with TPCK-treated trypsin (Worthington), 30 μg/ml, in 0.2% NH_4HCO_3 buffer at pH 8. The supernatant was removed and the slices were soaked in water for 4 hr at 37°C. The supernatant from the soaked slices was combined with the first one, dried under reduced pressure, dissolved in water, and centrifuged to remove insoluble matter. The clear supernatant was dried as above, dissolved in 5 μl of 10% isopropanol, and spotted on thin-layer cellulose sheets (Polygram cel 300; 20 × 20 cm). Electrophoresis in the first dimension was carried out at 2°C and 1000 V for 30 min in pyridine/acetic acid/water, 2.5:2.5:95 (vol/vol), at pH 4.7. Chromatography in the second dimension was carried out in pyridine/*n*-butanol/acetic acid/water, 12:15:3:12 (vol/vol). Radiolabeled peptides were detected by exposing the dried chromatograms to Kodak SB-5 medical x-ray film. The labeled amino acid was [^{35}S]methionine for cytochrome *c* oxidase subunit V and [^{14}C]leucine for subunit VI.

beling at 12°C (Fig. 8, lane 2). Upon a chase with unlabeled methionine, this larger polypeptide disappeared (lane 3).

When the larger form of subunit V was synthesized in a reticulocyte lysate in the presence of free [^{35}S]methionine and then incubated with mitochondria under conditions essentially as described earlier (3), about three-fourths of it was converted to mature subunit V which then cosedimented with the mitochondria. Of the mitochondria-associated radiolabeled subunit V, 40–60% was resistant to externally added proteases. This *in vitro* import, together with the pulse–chase experiment mentioned above, supports the view that the larger form is a precursor to subunit V of cytochrome *c* oxidase.

DISCUSSION

We have demonstrated that at least three subunits of F_1-ATPase and at least two subunits of cytochrome *c* oxidase are initially made outside the mitochondria as separate precursors. In addition, we have recently detected a separate precursor to yeast cytochrome *c* oxidase subunit IV which is a primary translation product and only 3000 daltons larger than the mature subunit (not shown). The cytoplasmically made subunits of each of these two oligomeric enzymes thus are not synthesized as a common "polyprotein" precursor. Why, then, has a 55,000-dalton polyprotein precursor for the four small cytochrome *c* oxidase subunits been reported (8–11)? In trying to duplicate that work, we noted that proteins in the 50,000- to 60,000-dalton range can indeed be immunoprecipitated from yeast mitochondria or from a 100,000 × *g* supernatant with some antisera that react with one or more small cytochrome *c* oxidase subunits. However, the amount of antiserum required to immunoprecipitate these large proteins was far in excess of that needed to ensure maximal immunoprecipitation of the mature cytochrome *c* oxidase subunits. Furthermore, different large proteins were precipitated with different batches of cytochrome *c* oxidase antisera (not shown). We suggest, therefore, that the "55,000-dalton polyprotein precursor" (8–11) reflects the presence of a small population of contaminating antibodies directed against a highly antigenic unidentified 55,000-dalton polypeptide. It has been reported that such a polypeptide can specifically stimulate the synthesis of cytochrome *c* oxidase

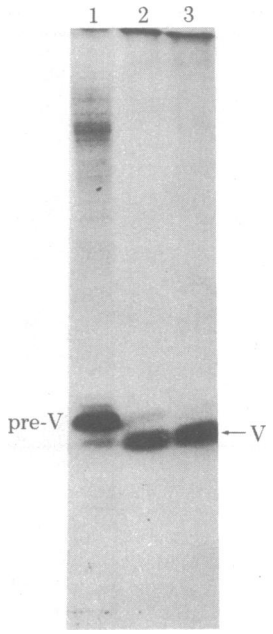

FIG. 8. The precursor to cytochrome *c* oxidase subunit V is unstable *in vivo*. Lanes: 1, subunit V precursor from wild-type spheroplasts labeled for 15 min at 28°C with [^{35}S]methionine in the presence of uncoupler; 2, subunit V and its precursor from wild-type spheroplasts labeled for 12 min at 12°C; 3, same as 2 except that the labeled spheroplasts were chased for 60 min at 12°C with 5 mM unlabeled methionine before immunoprecipitation.

subunits I–III by isolated yeast mitochondria (8, 9), but recent experiments in our laboratory do not confirm this. Although Poyton and McKemmie (10) claimed that tryptic digests of the 55,000-dalton protein revealed all the peptides typical of the four smallest subunits of cytochrome oxidase, the peptides were only separated by one-dimensional isoelectric focusing. We believe that the two-dimensional analysis used in the present study offers superior resolution and is less subject to ambiguities introduced by slicing gels with many labeled peaks.

In summary, we find no evidence for the involvement of a polyprotein precursor in mitochondrial biogenesis. On the contrary, we present evidence that individual subunits of F_1-ATPase and cytochrome *c* oxidase are synthesized and transported into the organelle as discrete precursors.

The authors are indebted to Drs. Robert Martin and Guy Dirheimer of the Institut de Biologie Moléculaire et Cellulaire du Centre National de la Recherche Scientifique in Strasbourg, France, for their advice and assistance in this project. We also thank Dr. T. D. Fox for his careful reading of the manuscript. This study was supported by Grants 3.172.77 and 3.212.77 from the Swiss National Science Foundation, a Long-Term Fellowship to A.S.L. from the European Molecular Biology Organization, and a Visiting Fellowship to T.L.M. from the Roche Research Foundation.

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