Proc. Natl. Acad. Sci. USA
Vol. 77, No. 7, pp. 4160–4164, July 1980
Cell Biology

The four cytoplasmically made subunits of yeast mitochondrial cytochrome *c* oxidase are synthesized individually and not as a polyprotein

(*in vitro* and *in vivo* synthesis/immunoprecipitation by subunit-specific antisera/*N*-formyl-[^{35}S]Met-tRNA/β and γ subunits of F_1-ATPase/protein import into mitochondria)

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Communicated by James E. Darnell, April 25, 1980

ABSTRACT **Subunit-specific antisera prepared against each of the four cytoplasmically made subunits (IV, V, VI, and VII) of yeast mitochondrial cytochrome *c* oxidase (EC 1.9.3.1) were used to precipitate immunoreactive polypeptides that were synthesized either *in vitro*, in a cell-free protein-synthesizing system programmed with total yeast mRNA, or *in vivo*, in intact cells and in spheroplasts, under conditions of pulse labeling, pulse-chase labeling, and continuous labeling. Using *N*-formyl-[^{35}S]Met-tRNA as the only radioactively labeled component in the cell-free system, we demonstrate (*i*) that each of the four cytoplasmically made subunits is synthesized as a separate entity and not as part of a polyprotein as was claimed by others; (*ii*) that subunits IV, V, and VI are synthesized as precursors, larger by 1500–3000 daltons than their mature counterparts; in contrast, subunit VII is not synthesized as larger precursor. Precursor forms of subunits IV, V, and VI identical to those synthesized *in vitro* were also detected *in vivo* by pulse-labeling of spheroplasts. The observed disappearance of these larger forms after a chase is compatible with the notion that they represent short-lived precursors that are rapidly converted to their mature counterparts during or shortly after import into mitochondria. Furthermore, using *N*-formyl-[^{35}S]Met-tRNA, we provide definitive evidence that two of the cytoplasmically made subunits (β and γ) of another oligomeric inner mitochondrial membrane protein (F_1-ATPase, EC 3.6.1.3) are not synthesized as part of a polyprotein but as individual precursors.**

Cytochrome *c* oxidase (EC 1.9.3.1) is located in the inner mitochondrial membrane. As the last member protein of the mitochondrial electron transport chain it catalyzes the translocation of electrons from cytochrome *c* in the intermembrane space across the inner mitochondrial membrane to molecular oxygen in the mitochondrial matrix. In mitochondria of yeast (1) cytochrome *c* oxidase is an oligomeric protein consisting of seven nonidentical subunits. Three of the seven subunits (I–III) are encoded in the mitochondrial genome and are synthesized within mitochondria. The other four subunits (IV–VII) are encoded in the nuclear genome and are synthesized in the cytoplasm.

Poyton and colleagues (2–6) have recently reported that all four cytoplasmically made subunits from yeast (*Saccharomyces cerevisiae*) are initially synthesized as a "polyprotein" of 55,000 molecular weight, larger by ≈8000 than the combined molecular weights of the four cytoplasmically synthesized subunits. This polyprotein was isolated from a postmitochondrial supernatant of yeast cells by immunoprecipitation, being immunoreactive with subunit-specific antisera that were raised against subunit IV, subunit VI, and a mixture of subunits V and VII (2–6). On the basis of data obtained from pulse–chase experiments, subcellular fractionation, and tryptic peptide analysis, Poyton and colleagues suggested that this polyprotein is posttranslationally transported into mitochondria, where it becomes associated with the inner mitochondrial membrane and where it is then proteolytically processed in a stepwise fashion to yield subunits IV, V, VI, and VII.

In this paper we describe our studies on the biosynthesis of subunits IV, V, VI, and VII of yeast cytochrome *c* oxidase. At variance with the data of Poyton and coworkers (2–6), we report here that subunits IV, V, VI, and VII of cytochrome *c* oxidase are synthesized not as parts of a polyprotein but as individual polypeptides. Furthermore, we provide definitive evidence that two of the cytoplasmically made subunits (β and γ) of another oligomeric inner mitochondrial membrane protein (F_1-ATPase, EC 3.6.1.3), of yeast are also synthesized as individual polypeptides rather than as parts of a polyprotein.

METHODS

Purification of Cytochrome *c* Oxidase and of F_1-ATPase. Mitochondria and submitochondrial particles were prepared from pressed yeast cells (Budweiser yeast) according to the method of Tzagoloff (7). Cytochrome *c* oxidase was extracted from submitochondrial particles with potassium cholate, fractionated with ammonium sulfate (8), and purified by chromatography on octyl-Sepharose CL-4B (9). After electrophoresis in a 10–15% polyacrylamide gradient gel in the presence of $NaDodSO_4$, the characteristic seven subunits of cytochrome *c* oxidase could be clearly identified (Fig. 1). F_1-ATPase was solubilized from submitochondrial particles with chloroform and partially purified by DEAE-cellulose chromatography according to a method of Takeshige *et al.* (10). The characteristic subunits of F_1-ATPase could be readily identified in a $NaDodSO_4$/10–15% polyacrylamide gradient gel (data not shown).

Preparation of Subunit-Specific Antibodies. The cytoplasmically synthesized subunits IV, V, VI, and VII of cytochrome *c* oxidase separated on polyacrylamide slab gels and stained with Coomassie brilliant blue (Fig. 1) were excised from the gel. Because subunits IV and V were barely separated from each other, they were excised together, whereas the clearly separated subunits VI and VII were excised each in separate slices. Gel slices containing both subunits IV and V, subunit VI, and subunit VII were homogenized in water. Each of the resulting slurries was mixed with an equal volume of Freund's complete adjuvant and injected subcutaneously into rabbits, four to six times at weekly intervals. Antibody production was monitored by Ouchterlony double-diffusion analysis of the antisera. An IgG fraction specific to subunits IV and V, subunit VI, and subunit VII was isolated by affinity chromatography (11) on Sepharose 4B conjugated to $NaDodSO_4$-denatured cytochrome *c* oxidase. Subunit-specific antibodies to the β and γ subunits of F_1-ATPase were prepared in an identical fashion.

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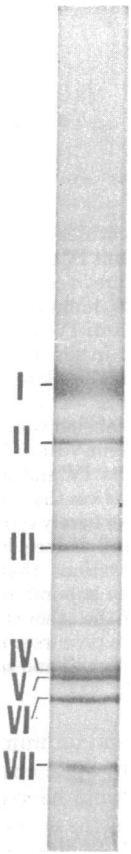

FIG. 1. Electrophoretogram of purified yeast cytochrome *c* oxidase. Purified yeast cytochrome *c* oxidase was analyzed by electrophoresis on a 10–15% polyacrylamide gradient slab gel in Na-DodSO$_4$. Shown is the Coomassie brilliant blue staining pattern. Roman numerals I to VII indicate position of subunits I–VII of cytochrome *c* oxidase.

Growth and Labeling of Cells and Spheroplasts. *S. cerevisiae* stain D273-10B (ATCC 24657) was grown to midlogarithmic phase either in rich medium (12) (for RNA extraction or for pulse and pulse–chase labeling of spheroplasts) or in sulfate-free semisynthetic medium (13) (for continuous labeling of cells). For continuous labeling of cells, growth was for at least 10 generations in sulfate-free semisynthetic medium supplemented with 20 mCi of carrier-free $^{35}SO_4^{2-}$ per liter (1 Ci = 3.7 × 10^{10} becquerels).

Pulse labeling and pulse–chase labeling of spheroplasts were carried out essentially as described (14, 15). Spheroplasts were resuspended to 200 mg wet weight per ml in 0.061% $MgCl_2 \cdot 6H_2O$/0.1% KH_2PO_4/0.05% NaCl/0.04% $CaCl_2$/1% ethanol/0.3% galactose/1.3 M sorbitol at pH 6.0 (15). Two aliquots, 1 ml each, of the spheroplast suspension were preincubated at 25°C for 60 min. Preincubation was followed by the addition of 0.5 ml of the suspending medium and 60 μl containing 600 μCi of [^{35}S]methionine, and by subsequent incubation at 26°C for 4 min. Labeling was terminated (see below) in one of the two aliquots (pulse). For pulse–chase labeling, the other aliquot was centrifuged to separate the spheroplasts from the pulse medium. The spheroplasts were then washed once with 15 ml of suspending medium containing 5 mM unlabeled methionine, resuspended in 1.5 ml of the same medium, and incubated at 26°C for 90 min. Pulse–chase labeling (as well as pulse labeling, see above) was terminated exactly as described (14, 15) by the addition of NaDodSO$_4$ and incubation at 100°C. In preparation for immunoprecipitation, the solutions were adjusted to contain 2% Triton X-100/0.4% NaDodSO$_4$/150 mM NaCl/40 mM Tris·HCl, pH 7.4/5 mM EDTA/200 units per ml of Trasylol in a total volume of 15 ml and centrifuged for 15 min at 12,000 × *g*. Aliquots (5 ml of the supernatant) were used for immunoprecipitation.

Preparation of *N*-Formyl-[^{35}S]Met-tRNA$_i^{Met}$. Calf liver tRNA was aminoacylated in the presence of [^{35}S]Met with highly purified *Escherichia coli* Met-tRNA synthetase and was formylated with highly purified *E. coli* transformylase (16). Both enzymes were a generous gift of H. Weissbach. Met-tRNA synthetase was prepared by C. Bruton.

It is known (17) that Met-tRNA synthetase of *E. coli* charges only one of the two mammalian cytoplasmic Met-accepting tRNA species, namely the initiator tRNA (tRNA$_i^{Met}$), but not the other Met-accepting tRNA (tRNA$_m^{Met}$), which donates Met to internal positions of the polypeptide. The "specific activity" of the *N*-formyl-[^{35}S]Met-tRNA containing preparation was 6.4 × 10^6 cpm per 1.0 A_{260} unit of tRNA.

Cell-Free Protein Synthesis. Translation of total yeast RNA (extracted as described in ref. 14) or of anterior bovine pituitary RNA (18) in a nuclease-treated rabbit reticulocyte lysate was as described (19). A typical 100-μl translation mixture contained 50 μl of lysate, 74 μCi of [^{35}S]methionine, and 1.0 A_{260} unit of RNA. In the translation experiment using *N*-formyl-[^{35}S]-Met-tRNA, [^{35}S]methionine was replaced by 30 μM unlabeled methionine; 8.1 × 10^6 cpm of *N*-formyl-[^{35}S]Met-tRNA were present per 100-μl translation mixture. In the translation experiment with microsomal membranes of dog pancreas (19), 0.5 A_{260} unit of the membranes was used per 100 μl of translation mixture.

Immunoprecipitation in the presence of NaDodSO$_4$ and Triton X-100, but using staphylococcal protein A-Sepharose instead of *Staphylococcus aureus* cells, was as described (20). Analysis of the immunoprecipitates by 10–15% polyacrylamide gradient slab gel electrophoresis in NaDodSO$_4$ and subsequent fluorography (21) of the fixed and dried slab gels were as described (22).

Source of Materials. Carrier-free $H_2{}^{35}SO_4$ from Amersham; [^{35}S]methionine (850–950 Ci/mmol) from New England Nuclear; octyl-Sepharose CL-4B and protein A-Sepharose from Pharmacia; Trasylol from Mobay Chemical, New York, NY; pressed yeast cells (Budweiser yeast) from Anheuser-Busch, St. Louis, MO; calf liver tRNA from Boehringer Mannheim.

RESULTS

To study the biosynthesis of the cytoplasmically made subunits of yeast cytochrome *c* oxidase, we combined an *in vitro* and an *in vivo* biosynthesis approach and used immunoprecipitation with subunit-specific IgG as a means to isolate immunoreactive polypeptides from the total products. *In vitro* biosynthesis was achieved by translating total yeast mRNA in a reticulocyte cell-free system (see lanes 1 of Fig. 2). *In vivo* biosynthesis was monitored after pulse labeling of spheroplasts (lanes 2 of Fig. 2), pulse–chase labeling of spheroplasts (lanes 3 of Fig. 2), or continuous labeling of intact cells (lanes 4 of Fig. 2). It is clear from the data shown in Fig. 2 that a polyprotein of ≈55 kilodaltons, allegedly containing all four cytoplasmically made subunits of cytochrome *c* oxidase (2–6), was detected neither after *in vitro* (lanes 1) nor after any of the *in vivo* biosynthesis experiments (lanes 2, 3, and 4). Instead, each of the four subunits appeared to be synthesized individually—i.e., as a "monoprotein." *In vitro*, subunits IV, V, and VI were synthesized as forms that were larger by ≈1500–3000 daltons than the mature forms (compare lanes 1 to lanes 4); in contrast, subunit VII was apparently not synthesized as a larger form. Forms that were identical in their molecular weight to those of the *in vitro* synthesized forms of the four subunits were detected also *in vivo*, after a 4-min pulse labeling of spheroplasts (lanes 2). In addition to the precursor forms, and consistent with the notion that *in vivo* import into mitochondria and accompanying conversion of the precursors to the mature forms proceed rapidly (14), there was appreciable synthesis of mature forms

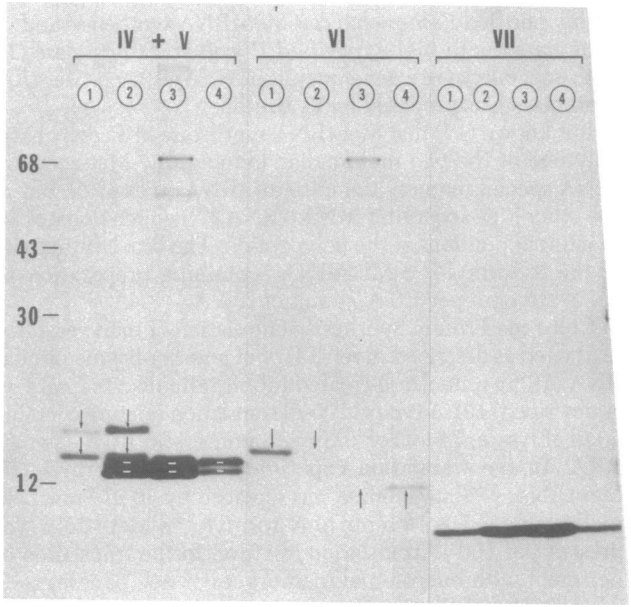

FIG. 2. Comparison of subunits IV, V, VI, and VII of cytochrome *c* oxidase synthesized *in vitro* and *in vivo*. Shown are products that were immunoprecipitated with an IgG fraction specific to subunits IV and V, to subunit VI, and to subunit VII. Lanes 1, products synthesized *in vitro* by translation of total yeast RNA in 50- to 100-μl translation mixture; lanes 2, products synthesized *in vivo* by pulse labeling of spheroplasts; lanes 3, products synthesized *in vivo* by pulse-chase labeling of spheroplasts; lanes 4, products synthesized *in vivo* by continuous labeling of cells. Numbers next to bars on the left indicate molecular weight ($\times 10^{-3}$) of marker proteins. Downward-pointing arrows indicate precursor for subunits IV, V, and VI. Upward-pointing arrows or white bars indicate mature forms of subunit VI or subunits IV and V, respectively. There is a band above the mature subunit VI in lane 3 (VI) that is most likely a contaminant in the immunoprecipitate. Mature subunit VI contains only one Met (23) and is therefore weakly labeled.

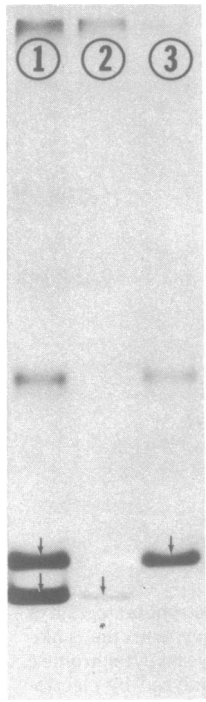

FIG. 3. Identification of precursor for subunit IV and for subunit V. Shown are *in vitro* synthesized products immunoprecipitated with an anti-IV and V IgG fraction, either in the absence of unlabeled subunits (lane 1), or in the presence of competing amounts of unlabeled subunit IV (lane 2) or subunit V (lane 3). Unlabeled subunits IV and V were eluted from gel slices that were cut between the two barely separated subunits IV and V (see Fig. 1), yielding preparations that were enriched in one subunit but not entirely free of the other subunit. Arrows indicate precursor to subunit IV (upper band) and subunit V (lower band).

already at the 4-min pulse (lanes 2, particularly evident in the case of subunits IV and V). After a pulse–chase experiment (lanes 3), labeled precursor forms were no longer detectable. The chased products appeared to be identical in molecular weight to the products synthesized in intact cells by continuous labeling (lanes 4).

Because subunit VII is a small protein (estimated molecular weight of ≈5000 (23) and because molecular weight differences of proteins in the 5000–10,000 range are poorly resolved by the type of gel used in Fig. 2, we analyzed the *in vitro* and *in vivo* synthesized forms of subunits VII in a polyacrylamide gel containing $NaDodSO_4$ *and* urea (such a gel system is able to reveal molecular weight differences of at least 1000 (24). However, even in this gel system there was no mobility difference detectable between the *in vitro* and *in vivo* synthesized form of subunit VII (data not shown), suggesting that subunit VII is not synthesized as a larger precursor.

To settle the question which of the two bands that were immunoprecipitated from the *in vitro* products by the IgG fraction against a mixture of subunits IV and V [see Fig. 2, lane 1 (IV and V) and Fig. 3, lane 1] is the precursor to subunit IV and which is the precursor to subunit V, immunoprecipitation was carried out in the presence of competing amounts of either unlabeled subunit IV (Fig. 3, lane 2) or unlabeled subunit V (Fig. 3, lane 3). It is clear from these competition experiments that the slower-moving band is pre-IV and the faster moving band is pre-V.

Although the data shown in Fig. 2 have failed to reveal the 55-kilodalton polyprotein that was detected by Poyton and coworkers (2–6), they do not rule out its existence. Our procedures for the preparation of antibodies and for immunoprecipitation are significantly different from those employed by Poyton's group, and these differences could have resulted in our failure to detect the polyprotein among the *in vivo* products. Furthermore, it is conceivable that the *in vitro* synthesized subunits do not represent primary translation products. Putative processing enzymes (that convert the polyprotein) could have leaked from mitochondria during the hypotonic lysis of rabbit reticulocytes, and thus might be present in the postmitochondrial supernatant that is used for cell-free protein synthesis. A similar argument could be made also in the case of the previously reported data (14) on the cytoplasmically synthesized subunits of another oligomeric mitochondrial protein, yeast F_1-ATPase, in which the *in vitro* synthesized pre-α subunit, pre-β subunit, and pre-γ subunit may actually represent cleavage products of a polyprotein, rather than individual primary translation products.

A definitive experiment that could settle these questions is to translate yeast mRNA in the presence of *N*-formyl-[^{35}S]-Met-tRNA as the only radiolabeled component in the cell-free system. Because the formyl methionine residue that is donated by this tRNA to the amino-terminal position of the nascent chain cannot be cleaved by the Met aminopeptidase present in the eukaryotic cell-free system (25) it serves as a marker for an initiator Met. An important prerequisite for the validity of such experiments is to demonstrate that the *N*-formyl-[^{35}S]Met-tRNA$_i^{Met}$ preparation is absolutely free of [^{35}S]Met-tRNA$_m^{Met}$ (which would insert internal Met) and, subsequently, that the newly synthesized chains contain label exclusively in the amino-terminal initiator Met and not in any internal Met.

To meet the former condition we charged unfractionated mammalian tRNA with [^{35}S]Met, using purified *E. coli* Met-tRNA synthetase. It has been established (17) that the prokaryotic enzyme is able to charge only the tRNA$_i^{Met}$ species and not the tRNA$_m^{Met}$ species of the two mammalian cytoplasmic Met-accepting tRNA species.

To ascertain that the *N*-formyl-[^{35}S]Met-tRNA preparation labeled only the initiator Met of newly synthesized chains, we took advantage of an assay in which cleavage of a distinct amino-terminal portion of the nascent chain (containing the

initiator Met) can be readily demonstrated (26, 27). Cleavage at such a distinct site occurs when mRNA for a secretory protein (in the present case, anterior bovine pituitary mRNA containing primarily prolactin mRNA) is translated in a cell-free system supplemented with microsomal membranes from canine pancreas. It has been demonstrated (18) that the amino-terminal signal sequence of nascent preprolactin (addressed to the rough endoplasmic reticulum) is cleaved by the membrane-associated signal peptidase during the translation-coupled translocation of nascent preprolactin across the microsomal membrane. As expected, therefore, in a control experiment in which the cell-free system contained [^{35}S]Met, there was synthesis of only the preprolactin form in the absence of membranes (Fig. 4, lane 1), and primarily of the mature prolactin form when membranes were present during translation (Fig. 4, lane 2). In a cell-free system containing *N*-formyl-[^{35}S]Met-tRNA there was synthesis again of only the preprolactin form in the absence of membranes (Fig. 4, lane 3), but there was no label detectable in the mature prolactin form synthesized in the presence of membranes (Fig. 4, lane 4), although the conversion of nascent preprolactin to prolactin clearly occurred (note the largely diminished preprolactin band in lane 4). Thus it is clear that cell-free synthesis in the presence of our *N*-formyl-[^{35}S]Met-tRNA preparation yielded polypeptide chains that were labeled exclusively in their initiator Met.

To determine whether the cytoplasmically synthesized subunits IV, V, VI, and VII of yeast cytochrome *c* oxidase and β and γ of yeast F_1-ATPase are synthesized as a polyprotein (see above), we translated yeast mRNA in a cell-free system containing our *N*-formyl-[^{35}S]Met-tRNA preparation (Fig. 5, lanes 1–3 and Fig. 6, lanes 1 and 2). For comparison, translation was also carried out in a cell-free system containing [^{35}S]Met (Fig. 5, lanes 4–6). Analysis of the immunoreactive products precipitated by anti-IV and V (Fig. 5, lanes 1 and 4) by anti-VI (Fig. 5, lanes 2 and 5) and by anti-VII (Fig. 5, lanes 3 and 6) as well as by anti-β (Fig. 6, lane 1) and anti-γ (Fig. 6, lane 2) clearly shows that each of the *in vitro* synthesized forms of subunits IV, V, VI, and VII of cytochrome *c* oxidase as well as of subunits β and γ of F_1-ATPase was labeled when synthesized in the presence of *N*-formyl-[^{35}S]Met-tRNA as the sole source of radioactivity in the cell-free system. These results establish unequivocally that each of the four subunits of cytochrome *c* oxidase is synthesized as a "monoprotein" and not as a polyprotein as was claimed by Poyton's group (2–6). Similarly, the previously (14) detected precursors to the cytoplasmically made subunits of F_1-ATPase are definitely primary translation products and therefore are also synthesized as monoproteins rather than as parts of a polyprotein.

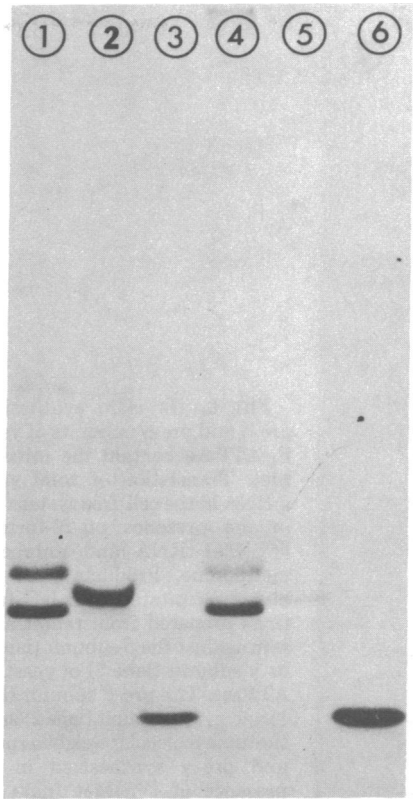

FIG. 5. Each of the *in vitro* synthesized subunits IV, V, VI, and VII of cytochrome *c* oxidase contains the initiator Met. Comparison of subunits IV, V, VI, and VII synthesized *in vitro* either in the presence of *N*-formyl-[^{35}S]Met-tRNA and unlabeled methionine (lanes 1–3) or in the presence of [^{35}S]methionine (lanes 4–6), in 300- to 500-μl or 50- to 100-μl translocation mixtures, respectively. Products were immunoprecipitated with: anti-IV and V IgG fraction (lanes 1 and 4), anti-VI IgG fraction (lanes 2 and 5), and anti-VII IgG fraction (lanes 3 and 6). The low recovery in lane 5 was due to losses during immunoprecipitation.

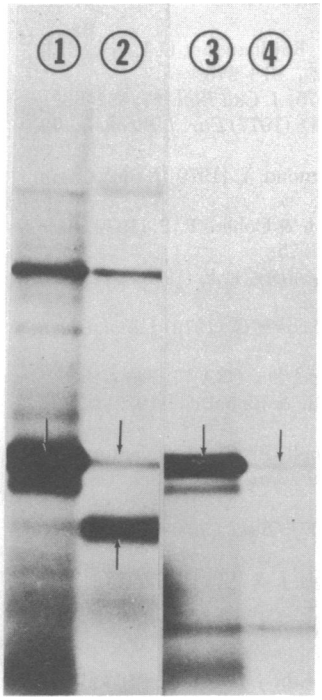

FIG. 4. *N*-formyl-[^{35}S]Met-tRNA$_f^{Met}$ preparation is free of [^{35}S]Met-tRNA$_m^{Met}$. Bovine anterior pituitary RNA was translated in the cell-free system either in the presence of [^{35}S]Met (lanes 1 and 2) or in the presence of *N*-formyl-[^{35}S]Met-tRNA and unlabeled Met (lanes 3 and 4). Microsomal membranes from dog pancreas were either absent (lanes 1 and 3) or present (lanes 2 and 4) during translation. Downward-pointing arrows indicate preprolactin and upward-pointing arrow indicates mature prolactin. Note the absence of labeled mature prolactin in lane 4.

DISCUSSION

Our results here demonstrate unequivocally that all of the four cytoplasmically made subunits (IV, V, VI, and VII) of yeast mitochondrial cytochrome *c* oxidase are synthesized individually as "monoproteins," rather than in the form of a polyprotein, as was claimed by Poyton and colleagues (2–6). The reasons for the detection of a polyprotein as the biogenetic precursor for all four cytoplasmically made subunits of cytochrome *c* oxidase are at present obscure and remain to be investigated. A similarly individual synthesis has been demonstrated here also for two cytoplasmically made subunits (β and γ) of yeast F_1-ATPase, thereby supporting previously published data on cell-free synthesis and import into mitochondria (14).

Like other cytoplasmically made polypeptides that are imported into the mitochondrial matrix (14, 28–30) or the intermembrane space (31), subunits IV, V, and VI of cytochrome

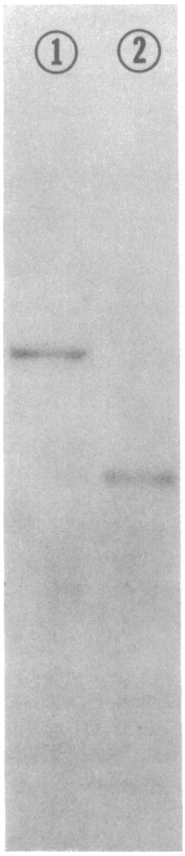

FIG. 6. *In vitro* synthesized pre-β and pre-γ subunits of yeast F_1-ATPase contain the initiator Met. Translation of total yeast mRNA in the cell-free system was in the presence of *N*-formyl-[^{35}S]Met-tRNA and unlabeled methionine. Products were immunoprecipitated with IgG fractions prepared from rabbit antisera against the β subunit (lane 1) or γ subunit (lane 2) of yeast F_1-ATPase. The pre-β subunit (lane 1) and pre-γ subunit (lane 2) are of the same molecular weight as pre-β and pre-γ synthesized in the presence of [^{35}S]Met (data not shown) and are larger than their mature counterparts (see ref. 14).

c oxidase are synthesized as larger precursors. The additional protein sequence of these larger precursors has been proposed to serve as a signal sequence for unidirectional and posttranslational translocation from the cytoplasm into mitochondria (32). This signal sequence would be cleaved either during or shortly after import into mitochondria. The existence of two distinct types of signal sequences for posttranslational translocation into mitochondria has been proposed (32): one addressed to a translocator system effecting transfer only across the outer membrane, and the other one addressed to a translocation system effecting transfer across both outer and inner mitochondrial membranes. Because the precise topological relationship of the polypeptide chains of subunits IV, V, and VI with the lipid bilayer of the inner mitochondrial membrane is unknown, it remains to be established to which of the two translocation systems the signal sequences of subunits IV, V, and VI are addressed.

Interestingly, subunit VII is not synthesized as a larger form. We propose that, like the other three cytoplasmically made subunits, subunit VII is endowed with a signal sequence addressed to one of the two mitochondrial posttranslational translocation systems, but that this signal sequence is not cleaved during or following import into mitochondria. There are now several precedents for uncleaved signal sequences. These have been detected so far among the signal sequences addressed either to the cotranslational translocation system in the rough endoplasmic reticulum (33–35) or to the cotranslational translocation system in the prokaryotic plasma membrane (36) or to the two posttranslational translocation systems in the mitochondrial membranes (37, 38).

This study was supported by Grants GM 27155 from the National Institutes of Health and NP-268 from the American Cancer Society.

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