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Biogenesis of Mitochondrial Ubiquinol:Cytochrome *c* Reductase (Cytochrome *bc*$_1$ Complex)

PRECURSOR PROTEINS AND THEIR TRANSFER INTO MITOCHONDRIA*

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The precursor proteins to the subunits of ubiquinol:cytochrome *c* reductase (cytochrome *bc*$_1$ complex) of *Neurospora crassa* were synthesized in a reticulocyte lysate. These precursors were immunoprecipitated with antibodies prepared against the individual subunits and compared to the mature subunits immunoprecipitated or isolated from mitochondria. Most subunits were synthesized as precursors with larger apparent molecular weights (subunit I, 51,500 *versus* 50,000; subunit II, 47,500 *versus* 45,000; subunit IV (cytochrome *c*$_1$), 38,000 *versus* 31,000; subunit V (Fe-S protein), 28,000 *versus* 25,000; subunit VII, 12,000 *versus* 11,500; subunit VIII, 11,600 *versus* 11,200). Subunit VI (14,000) was synthesized with the same apparent molecular weight.

The post-translational transfer of subunits I, IV, V, and VII was studied in an *in vitro* system employing reticulocyte lysate and isolated mitochondria. The transfer and proteolytic processing of these precursors was found to be dependent on the mitochondrial membrane potential.

In the transfer of cytochrome *c*$_1$, the proteolytic processing appears to take place in two separate steps via an intermediate both *in vivo* and *in vitro*. *In vivo*, the intermediate form accumulated when cells were kept at 8 °C and was chased into mature cytochrome *c*$_1$ at 25 °C. Both processing steps were energy-dependent.

The cytochrome *bc*$_1$ complex (ubiquinol-cytochrome *c* reductase, EC 1.10.2.2) is an enzyme complex of the inner mitochondrial membrane which catalyzes the transfer of electrons from reduced ubiquinone to ferricytochrome *c*. It is thought to contain one of the sites of proton translocation in the electron transport chain (1, 2). The cytochrome *bc*$_1$ complexes from a number of species have been isolated and characterized (3-8). The enzyme from *Neurospora crassa* (7-9) was found to be a dimer with a molecular weight of 550,000. The monomeric unit contains cytochrome *b*, cytochrome *c*$_1$, an iron-sulfur protein, and probably six subunits without known prosthetic groups. Current models for the topological arrangement of the subunits suggest that cytochrome *c*$_1$ and the Fe-S protein are in the cytoplasmic side of the inner mitochondrial membrane, facing the intermembrane space, whereas subunits I and II (the so-called "core proteins") extend into the mitochondrial matrix (10-15).

In *Neurospora* and in all other organisms studied so far, the cytochrome *b* is synthesized on mitochondrial ribosomes, whereas all the other subunits of the cytochrome *bc*$_1$ complex are synthesized on cytoplasmic ribosomes (16-19).

We have studied the biogenesis of the cytochrome *bc*$_1$ complex in *Neurospora* mitochondria. Precursors to the cytoplasmically translated subunits were synthesized in a heterologous cell-free system and immunoprecipitated using antibodies prepared against individual subunits. These were compared to the mature subunits isolated or immunoprecipitated from mitochondria. Most, but not all, of the cytochrome *bc*$_1$ complex proteins in *Neurospora* mitochondria appear to be synthesized as larger molecules with additional sequences which are cleaved during or after transfer into the mitochondria.

Four of the precursor proteins were transferred into isolated *Neurospora* mitochondria in an *in vitro* system. This transfer was post-translational, like that of the other mitochondrial proteins whose import has been studied (20-27), and in contrast to the co-translational transfer that takes place in the endoplasmic reticulum (28, 29). We investigated the energy dependence of precursor processing and import into mitochondria of these four cytochrome *bc*$_1$ complex proteins. In each case, a membrane potential appears to be required for transfer.

The transfer of cytochrome *c*$_1$ is unique in that an intermediate form is observed during the conversion from precursor to mature protein both *in vitro* and *in vivo*.

EXPERIMENTAL PROCEDURES

Growth of Neurospora Cells—*Neurospora crassa* wild type 74A was cultivated in Vogel's minimal medium (30), supplemented with 2% sucrose, at 25 °C for 15-17 h under vigorous aeration and illumination with fluorescent lights. When indicated, cells were labeled with ^{35}S by growing them with a reduced concentration of sulfate (0.08 mM) in the presence of 0.5 mCi/liter of sodium [^{35}S]sulfate (10-1000 Ci/mol, New England Nuclear), or cells were labeled with ^{3}H by adding 1 mCi/liter of [^{3}H]leucine (40-60 Ci/mmol, New England Nuclear) to the culture 1 h before harvesting. Cells were harvested by filtration.

Isolation of Mitochondria—Mitochondria were prepared from ^{35}S-labeled cells by grinding with sand. One g of cells (wet weight) was mixed with 1.5 g of sterile quartz sand (Riedel de Haen, Hannover) and 2 ml of SET medium (0.44 M sucrose, 0.002 M EDTA, 0.03 M Tris-HCl, pH 7.2) and ground in a mortar for 5 min at 4 °C. The resulting slurry was centrifuged twice for 5 min at 3,500 × *g* and the pellets were discarded. The supernatant was centrifuged for 12 min at 17,400 × *g*. The mitochondrial pellet was resuspended in SET medium and recentrifuged for 12 min at 17,400 × *g*.

Unlabeled mitochondria for the *in vitro* transfer experiments were

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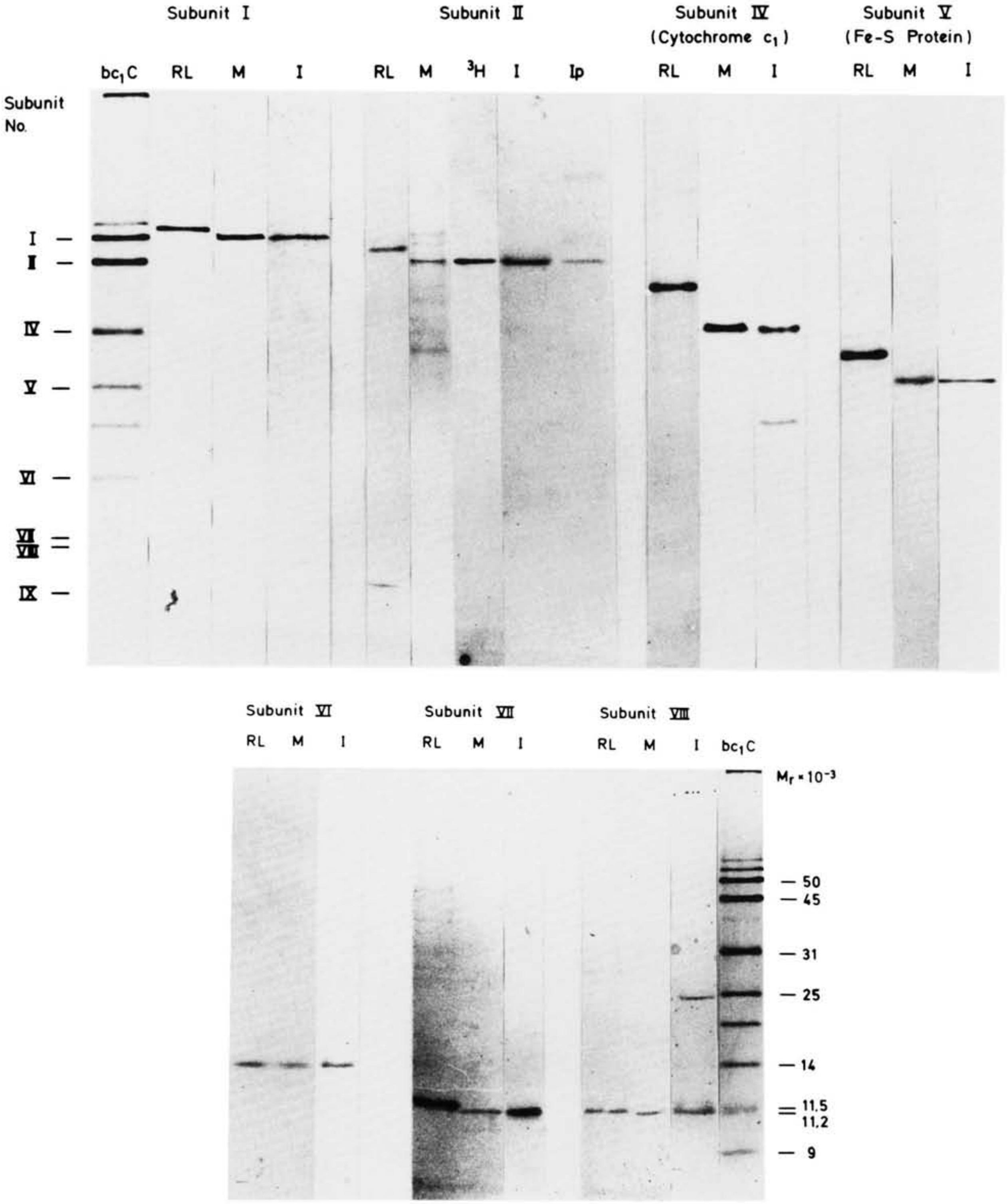

FIG. 1. **Autoradiographed SDS gels showing cytochrome *bc*$_1$ complex subunits immunoprecipitated from reticulocyte lysates incubated with *Neurospora* RNA in the presence of [^{35}S]methionine (*RL*) or from mitochondria isolated from *Neurospora* grown on [^{35}S]sulfate (*M*).** The isolated subunits are shown stained with Coomassie blue (*I*). *Lanes Ip* and ^{3}*H* represent stained and flourographed [^{3}H]leucine-labeled immunoprecipitates from mitochondria, respectively.

prepared from *Neurospora* spheroplasts as described previously (31).

Protein Synthesis in Reticulocyte Lysates—Reticulocyte lysates were prepared according to Hunt and Jackson (32) from rabbits which had been made anemic by injection of phenylhydrazine as described by Allen and Schweet (33). Cell-free protein synthesis was carried out according to Pelham and Jackson (34). One ml of incubation mixture contained 1 mg of *Neurospora* RNA (35) and 0.5 mCi of [^{35}S]methionine (New England Nuclear, specific activity 500 Ci/mmol). After the samples had been incubated for 30 min at 30 °C, a postribosomal supernatant was prepared by centrifuging for 1 h at 166,000 × *g*.

Transfer in Vitro of Cytochrome bc$_1$ *Complex Subunits*—Following incubation of a reticulocyte lysate with *Neurospora* RNA and [^{35}S]methionine and preparation of the postribosomal supernatant, methionine and sucrose were added to the supernatant to final concentrations of 50 μM and 0.2 M, respectively. Mitochondria isolated from spheroplasts were gently resuspended in this mixture (0.5–1 mg of mitochondrial protein/ml) and the suspension was incubated at 25 °C for 20–60 min with gentle shaking. The reaction was terminated by cooling to 0 °C and separating mitochondria from supernatant by centrifuging for 12 min at 17,400 × *g*. The mitochondrial pellet was

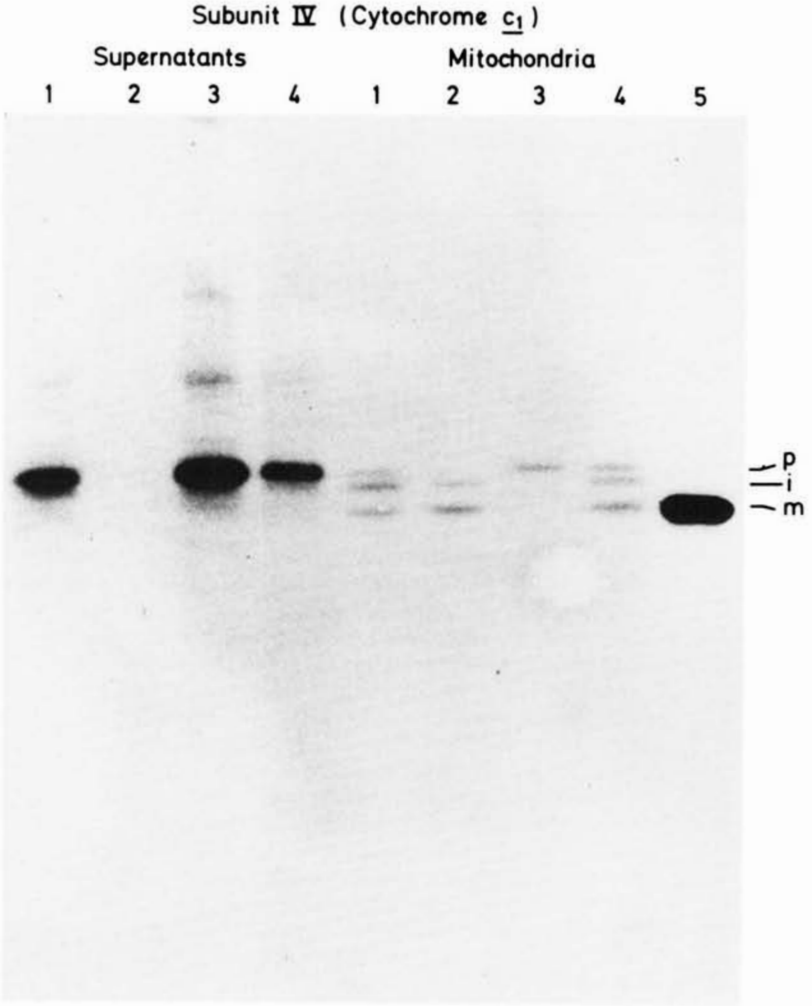

FIG. 2. **Transfer *in vitro* of cytochrome c_1.** Precursor was synthesized in a reticulocyte lysate, which was then incubated with *Neurospora* mitochondria for 1 h. The mitochondria were reisolated by centrifugation and lysed. Cytochrome c_1 was immunoprecipitated from both the supernatant and the lysed mitochondria, followed by SDS-gel electrophoresis and fluorography. *1*, control transfer; *2*, transfer followed by treatment with proteinase K; *3*, transfer in the presence of 1.5 μM valinomycin; *4*, transfer in the presence of 2 μM apocytochrome c; *5*, cytochrome c_1 from *Neurospora* grown on $[^{35}S]$sulfate.

washed once by resuspending in SET medium and recentrifuging. When indicated, proteinase K treatment was performed by adding 50 μg/ml of proteinase K (Boehringer Mannheim) to the mitochondrial pellets suspended in SET medium or to the supernatant. Samples were incubated for 30 min at 4 °C and then proteolysis was stopped by adding 0.5 μmol/ml of phenylmethylsulfonyl fluoride.

Transfer in Vivo of Cytochrome c_1—Neurospora hyphae were grown for 15 h at 25 °C as described above. The cell suspension (50–100 ml) was then cooled to 8 °C and 5 μCi/ml of $[^3H]$leucine (40–60 Ci/mmol, New England Nuclear) was added. After 6–12 min at 8 °C, unlabeled leucine and cycloheximide were added to final concentrations of 0.1 mg/ml and 0.05 mg/ml, respectively. The cells were then incubated at 25 °C for another 10–15 min. During both the pulse and the chase periods, aliquots were removed and rapidly filtered at 0 °C. The hyphae were homogenized in 1 ml of boiling 3% SDS,[1] 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, 1 mM EDTA, pH 7.5 (36) using an Ultra-Turrax (IKA, Staufen, FRG). After boiling for 5 min, the samples were cooled to 4 °C, diluted with 10 volumes of Triton buffer (1% Triton, 0.3 M NaCl, 5 mM EDTA, 10 mM Tris, pH 7.5) containing 0.1 mM phenylmethylsulfonyl fluoride and 0.1 mM *o*-phenanthroline, and centrifuged at 39,000 × *g* for 15 min. Cytochrome c_1 was immunoprecipitated from the supernatant.

Preparation of Antibodies to Cytochrome bc_1 Complex Subunits—Ubiquinol:cytochrome *c* reductase was isolated as described elsewhere (7). The polypeptides of the enzyme were separated by preparative SDS-polyacrylamide gel electrophoresis. Approximately 6 mg of protein dissolved in 2 ml of 2 mM dithioerythritol, 5 mM Tris/acetate, pH 7.0, 0.5% SDS was applied to a vertical 18% acrylamide gel (2 × 300 × 250 mm). The running buffer was 0.1% SDS, 50 mM Tris/acetate, 0.4 M glycine, pH 8.3. After electrophoresis for 16 h at 50 mA, the protein bands were stained for 2 h using 0.1% Coomassie blue in a solution containing 5 volumes of water, 1 volume of acetic acid. After destaining the gel for 24 h, the polypeptide bands were cut out of the gel and the proteins were recovered by electroelution of the gel slices.

Antibodies were raised in rabbits which were bled prior to immunization to obtain preimmune sera. Aliquots of 0.5 mg of protein in 1 ml of phosphate-buffered saline, mixed with 1 ml of incomplete Freund's adjuvant, were injected intradermally in several sites along the backs of the rabbits on the 1st, 4th, and 7th week. The rabbits were bled on the 5th week and weekly thereafter.

IgG was isolated as follows. Ten ml of antisera was passed through a protein A-Sepharose column (1 × 3 cm) (Pharmacia, Uppsala, Sweden). The column was washed with 30 ml of phosphate-buffered saline and the IgG was eluted with 5 ml of 1 M acetic acid. The eluent was dialyzed for 24 h against phosphate-buffered saline, lyophilized, and stored at 4 °C.

Immunoprecipitation—For immunoprecipitation of cytochrome bc_1 complex subunits from mitochondria, mitochondrial pellets were lysed in 50 μl of 2% SDS, 5% β-mercaptoethanol, 0.06 M Tris, pH 6.8, 5 mM phenylmethylsulfonyl fluoride, 5 mM *o*-phenanthroline for 3 min at 95 °C. Then, 1 ml of Triton buffer was added and the solution was clarified by centrifugation for 15 min at 35,000 × *g*. Twenty to fifty μl of immunoglobulin solution (1 mg/ml) was added and the mixture was shaken for 15 min at 4 °C. Sepharose-bound protein A (5 mg dry weight preswollen in 50 μl of Triton buffer) was then added and the mixture was shaken for another 15 min at 4 °C. The Sepharose beads were collected by centrifugation and washed three times with 1 ml of Triton buffer and twice with 1 ml of 10 mM Tris, pH 7.5.

Immunoprecipitates from reticulocyte lysates were obtained by adjusting the postribosomal supernatants or the *in vitro* transfer supernatants to 0.3 M NaCl and 1% Triton, followed by addition of antibodies to the cytochrome bc_1 complex subunits and Sepharose-bound protein A, as described above.

Immunoprecipitates from *in vivo* experiments were obtained by adding 50 μl of immunoglobulin solution to the 39,000 × *g* supernatant

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[1] The abbreviations used are: SDS, sodium dodecyl sulfate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

and incubating overnight at 4 °C. Then, 250 µl of a 10% suspension of *Staphylococcus aureus* cells (37) was added and the mixture was shaken at room temperature for 30 min. Cells were collected by centrifugation and washed as described for protein A-Sepharose beads above.

The protein A-Sepharose or *Staphylococcus aureus* pellets were suspended in 40 µl of 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 60 mM Tris-HCl, pH 6.8, and heated for 2 min at 95 °C. After centrifuging again, the supernatants were applied to SDS-polyacrylamide gels.

Gel Electrophoresis—Vertical slab gel electrophoresis was carried out according to Laemmli (38). Gels were stained with Coomassie blue R-250, destained in 10% acetic acid, 40% methanol, photographed, washed in 40% methanol, and treated with 1 M sodium salicylate in 40% methanol, 2% glycerol for 2 h, a procedure adapted from that of Chamberlin (39). The gels were dried and fluorographed on Agfa-Gevaert Curix RP1 x-ray film.

RESULTS

Synthesis of Precursors to Neurospora Cytochrome bc_1 Complex Subunits in Rabbit Reticulocyte Lysates—*Neurospora* RNA stimulated the incorporation of [^{35}S]methionine into proteins in rabbit reticulocyte lysates by a factor of about 10. Using antibodies directed against each of the cytochrome bc_1 complex subunits, immunoprecipitates were obtained from postribosomal supernatants of the lysates for seven of the nine subunits. Each of these immunoprecipitates migrated as a single band upon gel electrophoresis with an apparent molecular weight that was, in most cases, greater than that of the isolated subunit of the mature protein precipitated from radiolabeled mitochondria (Fig. 1). Subunits I, II, IV (cytochrome c_1), V (Fe/S protein), VII, and VIII with apparent molecular weights of 50,000, 45,000, 31,000, 25,000, 11,500, and 11,200, respectively, were synthesized as larger molecular weight precursors with molecular weights of 51,500, 47,500, 38,000, 28,000, 12,000, and 11,600, respectively. Subunit VI was synthesized with the same apparent molecular weight (14,000) as the authentic protein (Fig. 1).

A translation with *N*-formyl-[^{35}S]Met-tRNA, which would have confirmed the absence of an additional NH$_2$-terminal sequence, was not done because not enough label can be expected in the very small quantity of this protein that we could immunoprecipitate.

In the case of subunit II, relatively little radioactivity was incorporated into the mature protein when cells were grown on [^{35}S]sulfate. Much better labeling was obtained with [^{3}H] leucine; the immunoprecipitated protein could also be visualized by staining the gel (Fig. 1). This indicates that the mature protein may have relatively few methionine and cysteine residues.

Subunit III (cytochrome *b*) is known to be coded by mitochondrial DNA and synthesized on mitochondrial ribosomes (10) and was not synthesized in the reticulocyte lysates, as expected. Antibodies which precipitated isolated subunit IX (M_r = 9,000) did not precipitate any protein from the reticulocyte lysates nor were they able to precipitate the mature subunit from mitochondria labeled with [^{35}S]sulfate or [^{3}H] leucine *in vivo*.

Transfer of Cytochrome bc_1 Complex Subunits from Postribosomal Supernatants of Reticulocyte Lysates into Isolated Mitochondria—Mitochondria isolated from *Neurospora* spheroplasts were suspended in postribosomal supernatants from reticulocyte lysates which had previously been incubated with [^{35}S]methionine and *Neurospora* RNA. Transfer *in vitro* of the precursors of subunits I, IV, V, and VII into the mitochondria was investigated (Figs. 2–4). The mitochondrial fraction contained both bound precursors, which were susceptible to digestion with proteinase K, as well as subunits processed to the mature size which had been transferred into a protease-resistant position. This is shown for cytochrome c_1 and subunit VII in Figs. 2 and 3. Similar results were obtained with subunit V, but not with subunit I, where the bound precursor could not be completely digested by proteinase K. In the case of subunit IV (cytochrome c_1), precursor (M_r = 38,000) was found bound to the mitochondria and two species

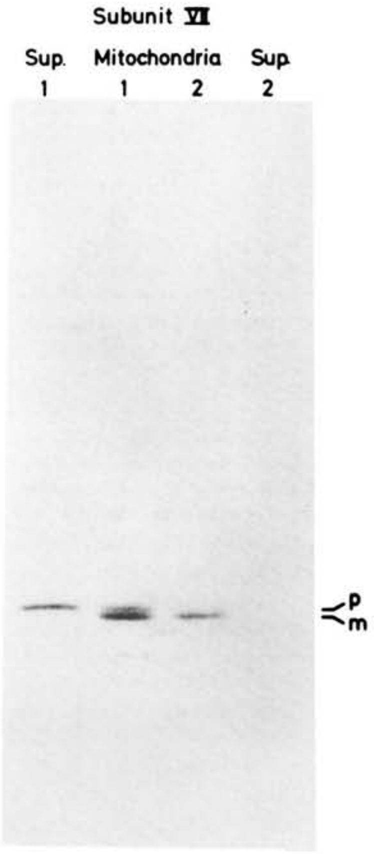

FIG. 3. **Transfer *in vitro* of subunit VII.** Conditions were the same as in Fig. 2. *1*, control transfer; *2*, transfer followed by treatment with proteinase K.

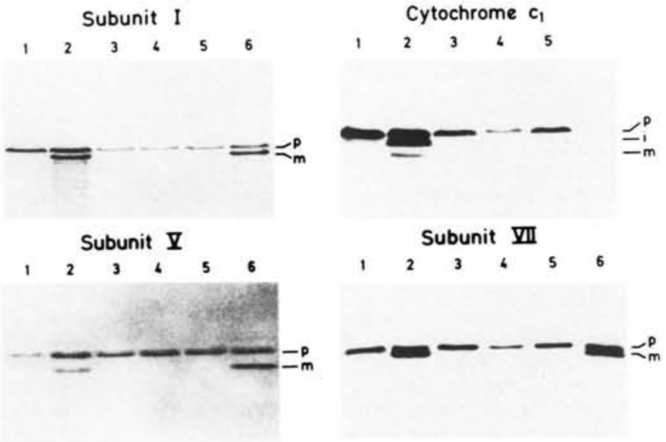

FIG. 4. **Transfer *in vitro* of cytochrome bc_1 complex subunits.** Mitochondria were incubated in reticulocyte lysate for 30 min in the case of subunits I and V, for 1 h in the case of cytochrome c_1 and subunit VII. Proteins were immunoprecipitated successively from the mitochondrial lysates using antibodies previously bound to protein A-agarose beads for 30 min at 4 °C and washed twice with Triton buffer. *1*, precursor synthesized in reticulocyte lysate; *2–6*, mitochondria following transfer in the presence of the following: *2*, control; *3*, 12.5 µM CCCP; *4*, 12.5 µM CCCP, 10 µM oligomycin, 1 mM ATP; *5*, 2 µM valinomycin, 10 µM oligomycin, 1 mM ATP; *6*, 2.5 µM carboxyatractyloside, 10 µM oligomycin.

were found transferred into proteinase K-resistant locations in the mitochondria: the mature subunit (M_r = 31,000) and an intermediate form with an apparent molecular weight of 35,000 (Fig. 2). This suggests that cytochrome c_1 may be processed in two distinct steps during its transfer into the mitochondria.

Energy Requirements for the Transfer into Mitochondria—The energy dependence of the transfer of subunit I, subunit IV (cytochrome c_1), subunit V (Fe-S protein), and subunit VII was investigated in the *in vitro* system. Both the uncoupler CCCP and the ionophore valinomycin inhibited the transfer of the four proteins studied (Fig. 4). The precursors still bound to the mitochondria under these conditions, but they were not processed to their mature forms or translocated to protease-resistant locations. The latter was verified by digesting the bound precursors with proteinase K (not shown).

CCCP acts as a protonophore and breaks down the membrane potential; valinomycin in the presence of a high concentration of K^+, such as that present in the reconstituted medium (approximately 90 mM), also causes a breakdown of the membrane potential. The inhibition of transfer by these two compounds could, however, also be caused by a secondary phenomenon. One major consequence of the dissipation of the membrane potential could be a decrease in the level of intramitochondrial ATP, due to the oligomycin-sensitive ATPase pumping out protons to compensate for the influx of H^+ or K^+ (40, 41). This ATPase activity can be inhibited by oligomycin (42).

In order to determine whether it is the mitochondrial membrane potential which is directly required for the transfer of the cytochrome bc_1 complex proteins or whether it is the intramitochondrial ATP, conditions were used under which the mitochondrial ATP level is high, whereas the membrane potential is broken down. When mitochondria are incubated in a combination of CCCP, oligomycin, and ATP, the absence of a membrane potential allows the ADP/ATP carrier to transport the external ATP into the matrix space and oligomycin inhibits the hydrolysis of this ATP, causing the matrix ATP level to rise (43). Under these conditions, transfer of cytochrome bc_1 complex proteins was inhibited (Fig. 4). Furthermore, when the membrane potential was kept intact but the mitochondria were depleted of ATP, transfer still occurred. This was accomplished by adding carboxyatractyloside to block the ADP/ATP carrier and prevent the import of ATP, as well as oligomycin to prevent the synthesis of ATP in the mitochondria (Fig. 4). These results show that it is the

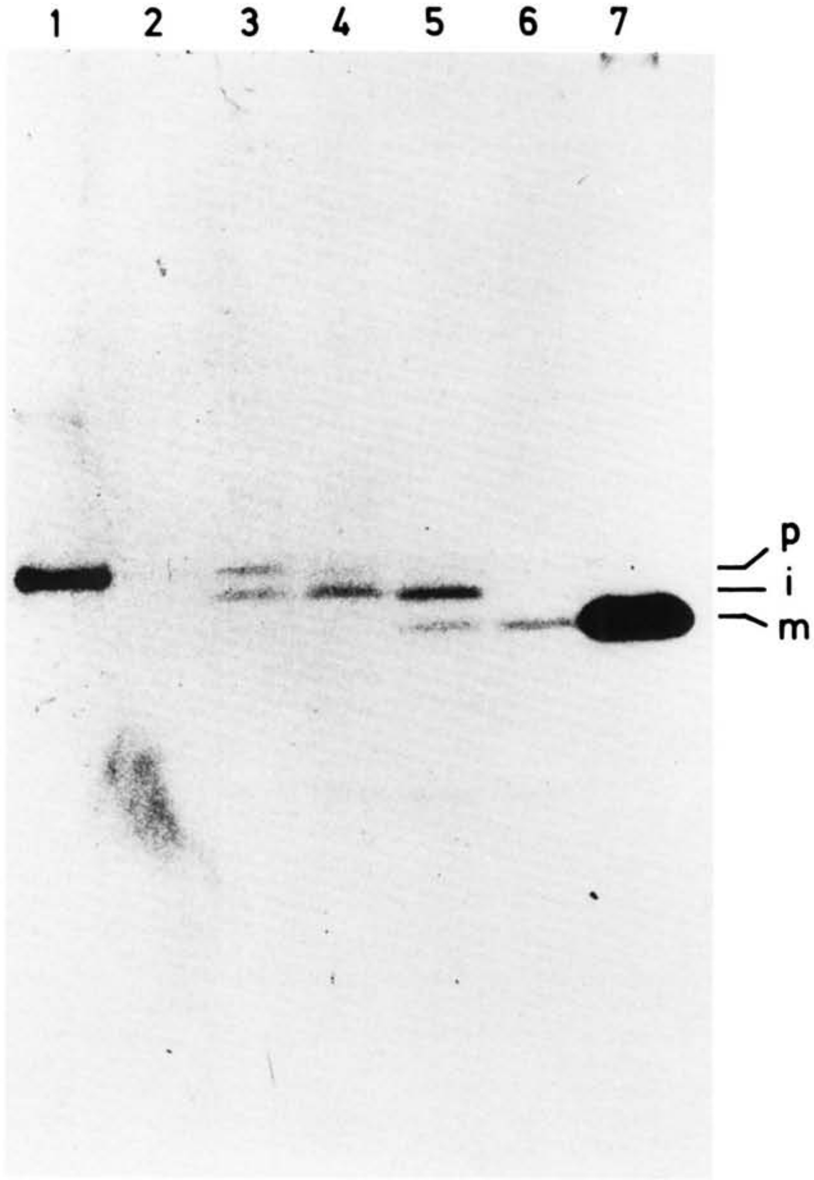

FIG. 5. **Transfer of cytochrome c_1 *in vivo.*** *Neurospora* cells were pulsed with [^{3}H]leucine at 8 °C for 12 min. Then, cycloheximide and unlabeled leucine were added and the temperature was raised to 25 °C. Aliquots were removed at various time points and harvested, lysed, and immunoprecipitated. *1*, ^{35}S-labeled cytochrome c_1 presursor from a reticulocyte lysate; *2*, [^{3}H]leucine pulse 3 min at 8 °C; *3*, 6 min at 8 °C; *4*, 12 min at 8 °C; *5*, chase for 3 min at 25 °C; *6*, chase after 12 min at 25 °C; *7*, mature cytochrome c_1 from cells grown on [^{35}S]sulfate.

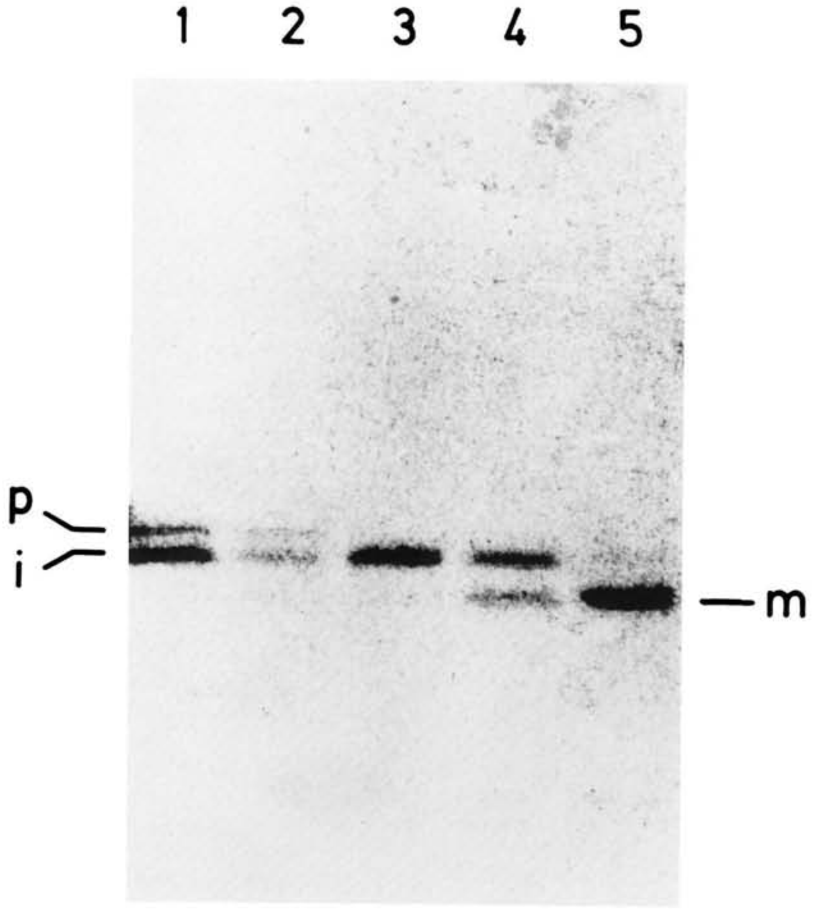

Fig. 6. **Energy dependence of cytochrome c_1 transfer *in vivo*.** *Neurospora* were labeled as follows, then immediately cooled to 0 °C, harvested, and extracted with hot SDS medium. *1*, pulse for 3 min at 8 °C with [^{3}H]leucine, then addition of cycloheximide and a leucine chase; *2*, pulse for 3 min at 8 °C, then cycloheximide and chase were added together with 12.5 μM CCCP and the sample was kept at 8 °C for 7 min; *3*, as in *2* but without addition of CCCP; *4*, pulse for 10 min at 8 °C, then addition of cycloheximide and CCCP, and further incubation for 5 min at 25 °C; *5*, as in *4* but without addition of CCCP.

mitochondrial membrane potential and not the intramitochondrial ATP that provides the energy for the import and/or processing of the precursor proteins.

Competition Experiments with Apocytochrome c—There is evidence to suggest that the transfer of cytochrome *c* into *Neurospora* mitochondria involves specific receptor sites (44, 45). The precursor, apocytochrome *c*, was shown to bind to specific sites on the mitochondria before its transfer and conversion to holocytochrome *c* by attachment of the heme group. Unlabeled apocytochrome *c* was able to compete with radioactive apocytochrome *c* for binding to these sites and addition of excess unlabeled apocytochrome *c* resulted in reduced amounts of radioactive holocytochrome *c* appearing in the mitochondria during transfer *in vitro* (44). In order to investigate whether cytochrome c_1 might be imported by the same pathway, we studied the effect of excess unlabeled apocytochrome *c* on the transfer of cytochrome c_1 *in vitro*. Fig. 2 shows that 1 μM apocytochrome *c* (lane 4) did not inhibit the transfer of cytochrome c_1 when compared to the control (lane 1). This concentration of apocytochrome *c*, however, significantly reduces the amount of radioactive holocytochrome *c* found in the mitochondria after transfer *in vitro* (44). This was confirmed by immunoprecipitating holocytochrome *c* from the samples used for Fig. 2, *lanes 1* and *4*. Cytochrome c_1 must therefore be imported by a different pathway from that of cytochrome *c*.

Transfer in Vivo of Cytochrome c_1—In order to show that the two-step processing observed for cytochrome c_1 was not an *in vitro* artifact, the transfer of cytochrome c_1 was also studied by pulse-labeling with [^{3}H]leucine *in vivo*. Since the transfer *in vivo* at 25 °C was too rapid, the cells were cooled to 8 °C before the [^{3}H]leucine was added. As can be seen in Fig. 5, both labeled precursor and labeled intermediate form appeared while the cells were at 8 °C, but conversion to the mature form did not take place. After 12 min, unlabeled leucine and cycloheximide were added to prevent any further incorporation of label and the temperature was raised to 25 °C. This caused the accumulated intermediate form to be quantitatively converted to the mature protein (Fig. 5). A second experiment was carried out where the intermediate form was allowed to accumulate during a [^{3}H]leucine pulse at 8 °C and then CCCP was added to the cell suspension along with the unlabeled leucine and cycloheximide. When the temperature was raised to 25 °C, no conversion to the mature cytochrome *c* was observed (Fig. 6). Under these conditions, the transfer of cytochrome *c*, which is not energy-dependent (49), was not inhibited by CCCP. This shows that the second proteolytic processing step in the transfer of cytochrome c_1 is also energy-dependent.

Transfer of Neurospora Precursor Proteins into Yeast Mitochondria—When ^{35}S-labeled *Neurospora* precursors in a reticulocyte lysate were incubated with mitochondria isolated from yeast cells, import of labeled proteins took place. Using antibodies against subunit V (Fe-S protein) of the *Neurospora* cytochrome bc_1 complex, both bound precursor (M_r = 28,000) and a protein with the apparent molecular weight of the mature subunit (M_r = 25,000) could be immunoprecipitated from the yeast mitochondria following transfer *in vitro*. The proteolytically processed mature subunit had been translocated into the mitochondria, as judged by its resistance to added protease (Fig. 7). The transfer was dependent on the mitochondrial membrane potential, as shown by the inhibition in the presence of valinomycin or antimycin A + oligomycin.

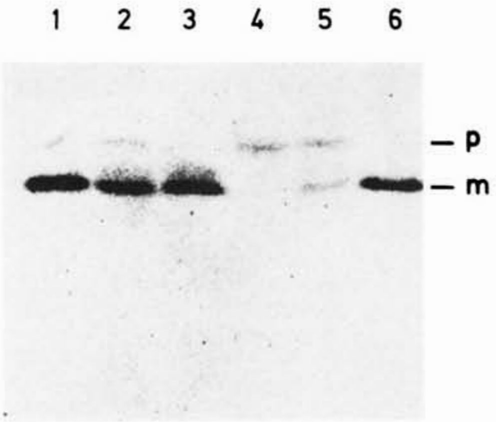

FIG. 7. **Transfer of *Neurospora* subunit V precursor into yeast mitochondria.** *Saccharomyces cerevisiae* (wild type 611-1 B) were grown at 30 °C in 1% yeast extract (Difco), 2% peptone (Difco), 3% ethanol, 3% glycerol (46) and harvested in midlog phase. Five g of cells were resuspended in 2.5 ml of 0.65 M sorbitol, 0.1 mM EDTA, 10 mM Tris-HCl, pH 6.5, and vortexed with 10 ml of glass beads (pore diameter, 0.5 mm) for 5 min at 4 °C (47, 48). After spinning down the glass beads and cell debris (2 × 2,000 × *g*), the mitochondria were pelleted by centrifuging for 10 min at 17,400 × *g*. Mitochondria were suspended in ^{35}S-labeled reticulocyte lysate supernatants supplemented with methionine and sucrose as described for *Neurospora* mitochondria. *1*, subunit V from [^{35}S]sulfate-labeled *Neurospora* mitochondria; *2*, control transfer (1 h, 25 °C); *3*, transfer followed by proteinase K treatment; *4*, transfer in tbe presence of 2 μM valinomycin; *5*, transfer in the presence of 5 μM antimycin A, 10 μM oligomycin; *6*, same as *1*.

DISCUSSION

We have shown in earlier publications that a number of mitochondrial proteins synthesized on cytoplasmic ribosomes are translocated into *Neurospora* mitochondria by a post-translational mechanism (20, 21, 49). Some of these proteins, such as subunit 9 of the oligomycin-sensitive ATPase or citrate synthase, are synthesized as larger precursors with additional sequences that are removed during or after transfer into mitochondria. Other mitochondrial proteins, such as cytochrome *c* (22), the ADP/ATP carrier (31), and the mitochondrial porin[2] are transferred without proteolytic processing. In the experiments reported here, both of these modes of translocation were observed among the various subunits of the cytochrome bc_1 complex. Subunits I, II, IV, V, VII, and VIII were synthesized as precursors with apparent molecular weights larger than those of the respective mature subunits. Subunit VI, however, appeared not to have a precursor with an additional sequence.

Subunit IV (cytochrome c_1) is unusual in that it seems to be processed in two discrete steps. In the first, the precursor (M_r = 38,000) is processed to an intermediate form (M_r = 35,000), which is already in a location or a form that makes it resistant to proteolysis by proteinase K added to intact mitochondria. In the second processing step, the intermediate form is converted to the size of the mature protein (M_r = 31,000); this species is also in a protease-resistant location, presumably the functional site.

Subunits VII and VIII were also unusual because the apparent molecular weights of their precursors were only about 500 larger than those of the respective mature subunits. This would correspond to an additional sequence of only about five amino acids in each case, which is much shorter than those observed on other proteins that are proteolytically processed upon translocation across a membrane. The precursors to cytochrome c_1 and the Fe-S protein have also been studied in yeast (36, 50). Interestingly, the extensions appear to have the same size as in *Neurospora*. In addition, our finding that a *Neurospora* precursor could be imported into yeast mitochondria and was processed to the correct apparent molecular weight shows that the components of the transfer pathway must be very similar in these two species. The transfer of *Neurospora* precursors into rat liver mitochondria has also been observed (42), indicating that proteins involved in import and processing of precursors must be highly conserved in evolution.

For all of the cytochrome bc_1 complex subunits whose transfer was studied, the import and proteolytic processing of the precursors *in vitro* required a membrane potential across the inner mitochondrial membrane. We discriminated between the membrane potential and the closely inter-related (40) matrix ATP level by employing conditions under which the level of ATP in the matrix was high and the membrane potential was dissipated. The precursors were able to bind to the mitochondria under these conditions, but they were neither imported nor proteolytically processed. On the other hand, when the matrix ATP was depleted but the membrane potential was not destroyed, the transfer of the subunit proteins was not inhibited. Identical results have recently been obtained with two other integral inner membrane proteins in *Neurospora*: ADP/ATP carrier and subunit 9 of the oligomycin-sensitive ATPase (42). Nelson and Schatz (50) concluded from experiments with whole yeast cells that processing of precursors to the cytochrome bc_1 complex proteins, cytochrome c_1, and the Fe-S protein required ATP in the mitochondrial matrix. Their results showed that rho$^-$ mutants, which should not be able to generate a membrane potential via electron transport or the reversed action of ATP synthase (51, 52), are still able to import these proteins into their mitochondria. It cannot be ruled out, however, that rho$^-$ mitochondria can still generate a small membrane potential sufficient to drive protein transfer, by import of ATP or some other process.

In the case of cytochrome c_1, where the proteolytic processing of the precursor takes place in two separate steps, our experiments *in vivo* showed that both the processing of the precursor to the intermediate form and the conversion of the intermediate form to mature cytochrome c_1 are energy-dependent. Our experiments also showed that the intermediate form is indeed an intermediate in the processing of the precursor to the mature protein and that the intermediate form can be accumulated by cooling the cells to 8 °C. A similar situation exists in yeast, where Schatz and co-workers have shown the existence of an intermediate form of cytochrome c_1 *in vivo*, which accumulates in a heme-deficient mutant (53). When the mutant was given δ-aminolevulinic acid, the intermediate form was converted to mature cytochrome c_1. In order to eliminate the possibility that the intermediate form is simply an apocytochrome c_1, missing the heme group but having an apparently larger molecular weight, we prepared apocytochrome c_1 by a method established for cytochrome *c* (54). In our gel system, this apocytochrome c_1 had an apparent molecular weight equal to or slightly smaller than that of mature cytochrome c_1 (not shown) and therefore could not be the intermediate form.

The transfer of cytochrome *c* into *Neurospora* mitochondria has been studied more extensively (22, 44). The precursor, apocytochrome *c*, binds to specific sites on the mitochondria and is imported when the heme group is attached to form holocytochrome *c*. This process does not require energization of the mitochondria. Excess unlabeled apocytochrome *c* was able to compete with radioactive precursor and inhibit the

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[2] H. Freitag and W. Neupert, unpublished observations.

appearance of radioactive holocytochrome *c* in the mitochondria, but it had no effect on the import and processing of cytochrome c_1 precursor. This observation, together with the difference in energy requirements, lead us to conclude that the pathway by which cytochrome c_1 is transferred is different from that of cytochrome *c*.

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