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A Mutant of *Neurospora crassa* Deficient in Cytochrome *c* Heme Lyase Activity Cannot Import Cytochrome *c* into Mitochondria*

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The nuclear *cyt-2-1* mutant of *Neurospora crassa* is characterized by a gross deficiency of cytochrome *c* (Bertrand, H., and Collins, R. A. (1978) *Mol. Gen. Genet.* 166, 1–13). The mutant produces mRNA that can be translated into apocytochrome *c* *in vitro*. Apocytochrome *c* is also synthesized *in vivo* in *cyt-2-1*, but it is rapidly degraded and thus does not accumulate in the cytosol. Mitochondria from wild-type cells bind apocytochrome *c* made *in vitro* from either wild-type or *cyt-2-1* mRNA and convert it to holocytochrome *c*. This conversion depends on the addition of heme by cytochrome *c* heme lyase and is coupled to translocation of cytochrome *c* into the intermembrane space. Mitochondria from the *cyt-2-1* strain are deficient in the ability to bind apocytochrome *c*. They are also completely devoid of cytochrome *c* heme lyase activity. These defects explain the inability of the *cyt-2-1* mutant to convert apocytochrome *c* to the holo form and to import it into mitochondria.

Cytochrome *c* is imported into *Neurospora crassa* mitochondria by a pathway that significantly differs from that of other proteins. Apocytochrome *c*, the precursor of holocytochrome *c*, is synthesized on free ribosomes in the cytosol and released into a cytosolic pool (Korb and Neupert, 1978). The apocytochrome is then bound at the outer mitochondrial membrane by specific receptors (Hennig and Neupert, 1981; Zimmermann *et al.*, 1981; Hennig *et al.*, 1983). Heme is covalently attached to the apoprotein by the enzyme cytochrome *c* heme lyase which faces the intermembrane space (Hennig and Neupert, 1981; Nicholson *et al.*, 1987). The subsequent conformational change is believed to pull cytochrome *c* completely across the outer membrane into the intermembrane space where it functions as an electron carrier between cytochrome *c* reductase and oxidase. Unlike most other important mitochondrial proteins, it does not contain a cleavable N-terminal targeting prepiece (Zimmermann *et al.*, 1979).

In order to define genetically specific components necessary for the import of cytochrome *c* we have examined the nuclear *cyt-2-1* mutant of *N. crassa*. This mutant was first described by Mitchell *et al.* (1953). The strain is characterized by its slow growth, female infertility, and gross deficiencies of spectrally detectable cytochromes *c* and aa_3 (Mitchell *et al.*, 1953). It has also been shown by immunological methods that the mutant possesses little, if any, fully assembled cytochrome *c* oxidase. This deficiency appears to be related to the presence of a high molecular weight form of a mitochondrially synthesized subunit 1 of cytochrome *c* oxidase in the mutant (Bertrand and Werner, 1979). The latter trait is also characteristic of the extranuclear [*mi-3*] mutant (Bertrand and Werner, 1979; Werner and Bertrand, 1979) and another nuclear mutant *299-1* (Bertrand and Werner, 1979), both of which are deficient only in cytochrome aa_3. Both [*mi-3*] and *cyt-2-1* can be induced to synthesize cytochrome aa_3 by growth in medium containing low levels of antimycin A, an inhibitor of electron transport in the cytochrome bc_1 region of the electron transport chain (Bertrand and Collins, 1978). Furthermore, both [*mi-3*] and *cyt-2-1* also synthesize cytochrome aa_3 when double mutants containing the *cyb-1-1* nuclear gene mutation are constructed (Bertrand and Collins, 1978). Surprisingly, although the *cyb-1-1* mutation normally causes a deficiency of cytochrome *b*, which persists in *cyb-1* [*mi-3*] double mutants, the mitochondria of *cyb-1-1 cyt-2-1* strains are not deficient in cytochrome *b* (Bertrand *et al.*, 1977, Bertrand and Collins, 1978). Based on these observations, models involving regulatory and/or processing systems for the production of cytochrome *c* oxidase were proposed (Bertrand and Collins, 1978; Bertrand and Werner, 1979).

While the above studies demonstrated that the cytochrome aa_3 deficiency of *cyt-2-1* could be relieved under various conditions, it was also shown that these conditions did not alleviate the deficiency of cytochrome *c* in the mutant (Bertrand and Collins, 1978). In this study we have examined various aspects of the cytochrome *c* deficiency in the *cyt-2-1* strain. Our results suggest that the primary defect in the mutant is a deficiency of cytochrome *c* heme lyase activity.

MATERIALS AND METHODS

Strains, Growth, and Labeling of Cells—Strains of *N. crassa* used in this study were the wild-type strains 74-OR23-1*A* (74*A*), 240*A nic-1 al-2* (*nic*240), and a derivative (NCN68) of a *cyt-2-1 pan-2 a* strain, IS-4. Both *nic*240 and IS-4 were obtained from H. Bertrand, University of Regina. The NCN68 strain was derived in the following manner. The wild-type strain 74*A* was crossed with IS-4. One of the single ascospore progeny of this cross, namely NCN44 *cyt-2-1 pan-2 A*, was crossed with a *qa-2 arom-9 inos a* strain obtained from Robert Akins, St. Louis University. NCN68 *cyt-2-1 A* was a single ascospore isolate from this cross. The strain may also carry either the *qa-2* or *arom-9* marker, but not both, since it does not require aromatic amino acids for growth. In all crosses the *cyt-2-1* strains were used as the male parent.

Cultures were grown in Vogel's medium as described previously (Davis and de Serres, 1970). Liquid medium was inoculated at a concentration of 1–5 × 10^6 conidia/ml of medium. Growth was at 25–30 °C. Aeration of liquid cultures was achieved either by shaking on an orbital shaker or by bubbling filter-sterilized air through the medium. Labeling of cells with sodium [^{35}S]sulfate (Du Pont-New England Nuclear, 380 mCi/mmol) was achieved as described by

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Hallermayer *et al.* (1977). Labeling of cells with L-[^{3}H]leucine (Amersham Corp., 60 Ci/mmol) was achieved by growing mycelium to log phase in 100 ml of liquid Vogel's medium, adding 2 mCi of [^{3}H] leucine, and continuing growth for 3 h before harvesting the cells.

Pulse labeling with L-[^{3}H]leucine was basically as described by Zimmermann and Neupert (1983) with modifications. Cultures of *cyt-2-1* were grown at 25 °C for 30–40 h whereupon isotope was added to a concentration of 2 mCi/liter. Labeling was allowed to proceed for 3 min, after which a dose of unlabeled leucine (final concentration 10 mM) was added to the culture. After various times, aliquots of the culture were removed and quickly poured into 2 volumes of ice water containing 10 mM unlabeled leucine and then harvested by filtration. The mycelial pads were immediately placed into a mortar containing liquid N_2. The samples were ground with acid-washed sea sand (1 g for each g wet weight of mycelium) under liquid N_2 in the presence of 3% SDS,[1] 10 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM 2-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride (1.5 ml of buffer/g wet weight of mycelium) and further processed as described for the examination of precursor proteins by immunoprecipitation (Zimmermann and Neupert, 1983). During all labeling experiments, separate cultures started from the same batch of conidia were used for analysis of mitochondrial cytochromes in order to monitor for possible contamination.

Isolation of Mitochondria—Mitochondria used for holocytochrome *c* immunoprecipitation and cytochrome spectra were isolated basically as described previously using either a Tris-HCl (Bertrand and Pittenger, 1969, 1972) or a MOPS-based buffer system (Nicholson *et al.*, 1987). During the isolation of isotopically labeled mitochondria, the mitochondrial pellets were washed once by resuspending in isolation buffer. Mitochondria prepared for use in import and binding experiments were isolated by differential centrifugation as described previously (Pfanner and Neupert, 1985) except that phenylmethylsulfonyl fluoride was omitted when import to a protease-resistant location was tested.

Spectral Analysis of Mitochondrial Cytochromes—Spectral analysis was performed as described by Bertrand and Pittenger (1972).

Isolation of Poly(A)-containing RNA—Total RNA was extracted as described by Reinert *et al.* (1981) with minor modifications. Mycelium from 14-h wild-type cultures or 40-h *cyt-2-1* cultures was harvested by filtration and immediately frozen in liquid nitrogen. While still frozen, the mycelium was ground to a powder with acid-washed, heat-sterilized Ottawa sand. Extraction buffer (4% SDS, 100 mM sodium acetate, 1 mM EDTA acid, pH 5.0) was added, and grinding was continued until a smooth paste was formed. This was quickly poured into corex tubes and an equal volume of phenol/chloroform/isoamyl alcohol (25/24/1) was added. The tubes were mixed by inversion for 15 min and then centrifuged for 10 min at 10,000 rpm in a Sorvall SS-34 centrifuge rotor. The aqueous phase was removed and placed in a clean tube. Protease K (Sigma, type XI) was added to a final concentration of 0.1 mg/ml, and the solution was mixed gently for 60 min at room temperature. Following this, the phenol/chloroform/isoamyl alcohol extraction was repeated two more times. The final aqueous phase was collected, and further steps for isolation of total cellular RNA were as described (Reinert *et al.*, 1981).

For the isolation of poly(A) RNA species, 5–10 mg of total RNA was applied to an oligo(dT)-cellulose (Pharmacia LKB Biotechnology Inc., type 7) column in 10 mM Tris-Cl, pH 7.5, 0.4 M NaCl, 0.1 mM EDTA, 0.5% SDS. After washing the column with the same buffer, the poly(A) RNA was eluted with 2 ml of buffer containing 10 mM Tris-Cl, pH 7.5, 0.1 mM EDTA, 0.05% SDS. The eluate was made 0.3 M in sodium acetate and RNA was precipitated with 2.5 volumes of ethanol. The pellet was dissolved in sterile H_2O and stored at −90 °C at a concentration of 1 mg/ml.

In Vitro Translation—*In vitro* translation was performed using a rabbit reticulocyte system obtained from Bethesda Research Laboratories or with reticulocyte lysates prepared as described (Pelham and Jackson, 1976; Nicholson *et al.*, 1987). The radioactive amino acid used in the system was usually L-[^{35}S]methionine (Amersham Corp., 1270 Ci/mmol). However, for *in vitro* translation products to be used in cytochrome *c* heme lyase assays, L-[^{35}S]cysteine (Amersham Corp., 1355 Ci/mmol) was used.

Antibodies and Immunoprecipitations—For holocytochrome *c* immunoprecipitation, 125 μl of antiserum was added to 3 mg of mitochondrial protein containing 1–2 × 10^6 cpm of ^{3}H or 1 × 10^5 1–3 × 10^5 cpm of ^{35}S. The immunoprecipitation and washing of the immunoprecipitates was as described by Hennig and Neupert (1983) except that the final pellets were dissolved in 100 mM Tris-Cl pH 7.5, 5% SDS, 5% β-mercaptoethanol. The dissolved pellets were boiled for 5 min prior to electrophoresis.

For immunoprecipitation of apocytochrome *c* directly from *in vitro* translation mixtures, the lysates were made 2% in SDS and boiled for 5 min. A volume of this lysate corresponding to 1–2 × 10^6 cpm of incorporated ^{35}S radioactivity, determined as described by Pelham and Jackson (1976), was diluted with 20 volumes of dilution buffer (10 mM Tris-Cl, pH 7.5, 300 mM NaCl, 1 mM EDTA, 2% Triton X-100). Immunoglobulins from 50 μl of antiserum raised against apocytochrome *c* were bound to 8 mg of protein A-Sepharose (Pharmacia, CL-4B) in the presence of 1 ml of dilution buffer for 1 h. The protein A-Sepharose-immunoglobulin complex was washed once in dilution buffer and then added to the diluted *in vitro* translation mixture. After 3 h incubation with gentle shaking at 4 °C, the protein A-Sepharose was pelleted by spinning for 2 min in an Eppendorf microcentrifuge. These pellets were washed 3 times with dilution buffer and once with dilution buffer without the Triton X-100. The final pellet was suspended in 100 mM Tris-Cl, pH 7.5, 5% SDS, 5% β-mercaptoethanol and boiled for 5 min. The protein A-Sepharose was removed by centrifugation, and the supernatant was electrophoresed as described below.

In Vitro Protein Import—Mitochondria (75 μg) were preincubated for 5 min at 25 °C in 150 μl of SME buffer (250 mM sucrose, 10 mM MOPS-KOH, pH 7.2; 2 mM EDTA) and then for 30 min more (only for 10 min more for cytochrome *c* import) following the addition of 50 μl of post-ribosomal supernatant from a rabbit reticulocyte lysate that had been used to translate 2 μg of poly(A) RNA. Mitochondria were re-isolated by centrifugation at 17,400 × *g* for 12 min, washed once with SME buffer, and resuspended at 1 mg of mitochondrial protein/ml SME buffer. The amount of each protein imported to a protease-resistant location was determined as previously optimized. In the case of porin, protease-resistant imported porin was assessed by treatment with 200 μg of proteinase K/ml for 30 min at 0 °C (Freitag *et al.*, 1982). Protease-resistant ATPase $F_1\beta$ was measured following treatment with 15 μg of proteinase K/ml for 30 min at 0 °C (Schleyer and Neupert, 1985). Imported ADP/ATP carrier was assessed by treatment with proteinase K, as described for ATPase $F_1\beta$, followed by detergent solubilization and passage over columns of hydroxylapatite to distinguish mature ADP/ATP carrier (which does not bind) from other species (which do bind) (Schleyer and Neupert, 1984). Protease-resistant imported cytochrome *c* was measured (where indicated) after proteinase K treatment as per ATPase $F_1\beta$. In all cases, digestion was halted by addition of phenylmethylsulfonyl fluoride to a final concentration of 2 mM. The samples were then treated with specific antisera, and the immunoprecipitated proteins were resolved in SDS-polyacrylamide gels.

Electrophoresis, Fluorography, and Determination of Radioactivity in Samples—Electrophoresis of immunoprecipitates to quantify holocytochrome *c* in mitochondria (Fig. 2) or apocytochrome *c* (Fig. 3) from either *in vitro* translation mixtures or from pulse-labeling experiments (Fig. 5) was basically as described (Weiss *et al.*, 1971; Bertrand and Werner, 1977) except that the electrophoresis was carried out vertically using 1.5- or 3-mm-thick gels. The gels were sliced into 1.1-mm slices and placed in plastic scintillation minivials. The proteins were eluted by adding 0.75 ml of electrophoresis buffer to each vial and heating the samples for 6–12 h at 65 °C. Five ml of scintillation fluid (Amersham aqueous counting scintillation fluid) was added and the counts were determined in a Beckman LS7500 liquid scintillation counter. Where appropriate, corrections for spill-over of ^{35}S counts into the ^{3}H counting channel were made prior to plotting the data.

Immunoprecipitates from *in vitro* import experiments were dissociated prior to electrophoresis by incubation at 95 °C for 5 min in 2% SDS, 0.35 M 2-mercaptoethanol, 50 mM Tris-HCl, pH 6.8, 10% glycerol, and 0.005% bromphenol blue. Gels were stained with Coomassie Blue and destained to detect standard molecular weight markers. The gels were then soaked in Amplify (Amersham Corp.) for 30 min prior to drying and fluorography.

Protein Determination—Protein concentrations were determined by either the biuret method (Gornall *et al.*, 1949) or by dye-binding assay (Bradford, 1976).

Cytochrome c Heme Lyase Assay and Scatchard Analysis—The determination of high affinity binding sites for cytochrome *c* was performed as described (Hennig *et al.*, 1983; Nicholson *et al.*, 1987). Cytochrome *c* heme lyase in intact mitochondria was assayed as described in Nicholson *et al.* (1987). To measure heme lyase activity

[1] The abbreviations used are: SDS, sodium dodecyl sulfate; MOPS, 4-morpholinepropanesulfonic acid.

in the absence of the preceding high affinity binding step, mitochondria (2.5 mg of mitochondrial protein/ml) were solubilized with 1% (w/v) octylglucoside by shaking for 5 min at 4 °C. The assay was then performed as for intact mitochondria except that sodium dithionite (1 mg/ml) was used as a reducing agent in place of NADH.

RESULTS

The gross deficiency of spectrally detectable cytochromes aa_3 and *c* that characterize the *cyt-2-1* mutant are shown in Fig. 1. To test for the presence of small amounts of cytochrome *c* protein we examined the mitochondria of the mutant, and of wild-type, that had been labeled by growth in [^{35}S]sulfate, for the presence of cytochrome *c* using antiserum specific for the *N. crassa* holocytochrome. Prior to the addition of antiserum, wild-type mitochondria labeled with [^{3}H]leucine were added to both the wild-type and *cyt-2-1* ^{35}S-labeled mitochondria to provide a control for the immunoprecipitation. As shown in Fig. 2, *A* and *B*, holocytochrome *c* was detectable in wild-type cells labeled with either [^{3}H]leucine or [^{35}S]sulfate. However, virtually no holocytochrome *c* was found in *cyt-2-1* mitochondria.

Since the mitochondria of *cyt-2-1* appeared to be devoid of holocytochrome *c*, it was of interest to determine if the mutant produced mRNA coding for the apocytochrome. Poly(A)-containing RNA isolated from both wild-type and *cyt-2-1* cultures was translated in a rabbit reticulocyte system containing [^{35}S]methionine and examined for the presence of immunoprecipitable apocytochrome *c*. As shown in Fig. 3, *in vitro* translation of poly(A)-containing RNA from either strain gave rise to immunologically detectable amounts of apocytochrome *c*. We have not precisely quantitated the amount of cytochrome *c* mRNA present in the mutant, but it is apparent that the levels are approximately equal to or slightly higher than the levels in wild-type cells. The latter is based on the observation that the total number of radioactive counts immunoprecipitated as apocytochrome *c* from *in vitro* translations was always slightly greater, in relation to the total counts incorporated, if the system was charged with poly(A) RNA isolated from *cyt-2-1* as opposed to poly(A) RNA from wild-type cells (not shown).

The observation that the *cyt-2-1* mutant produced the mRNA for cytochrome *c* while the mitochondria of the mutant

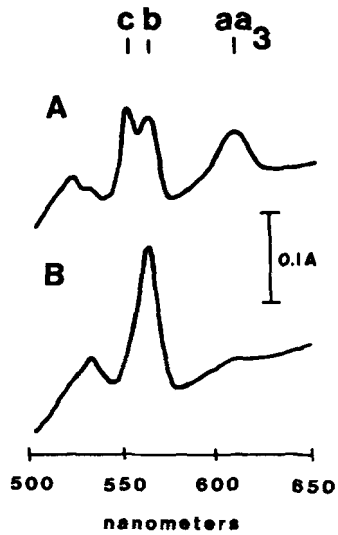

FIG. 1. **Mitochondrial cytochrome difference spectra.** Reduced *versus* oxidized difference spectra were recorded from lysed mitochondria as described under "Materials and Methods." *A*, wild-type; and *B*, *cyt-2-1*. The positions of the α-bands of cytochromes aa_3, *b*, and *c* are indicated.

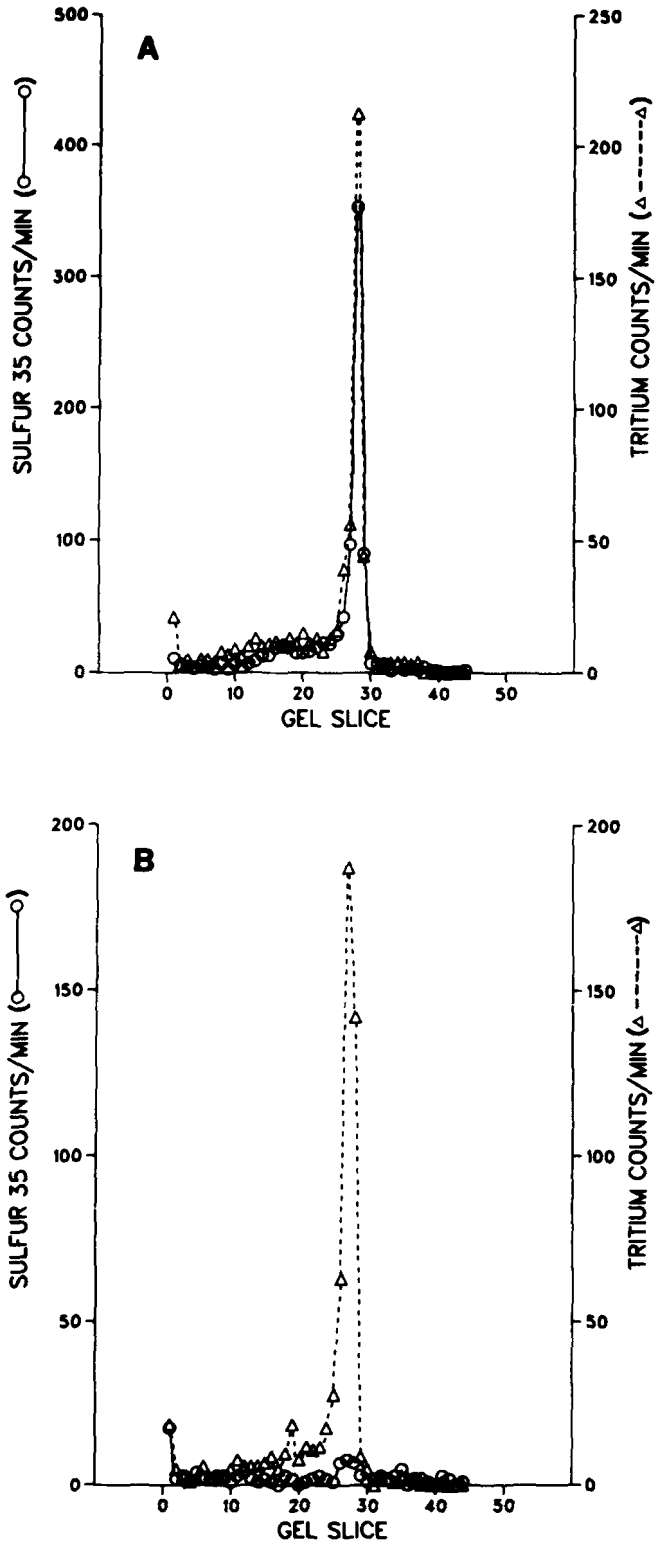

FIG. 2. **Analysis of holocytochrome c from wild-type and *cyt-2-1* mitochondria.** *A*, mitochondria isolated from wild-type cells grown in the presence of sodium [^{35}S]sulfate were mixed with wild-type mitochondria labeled with [^{3}H]leucine. The mitochondria were then solubilized and treated with antibodies to holocytochrome *c*. The immunoprecipitate was washed, solubilized, and then subjected to SDS gel electrophoresis. The gel was sliced and the ^{3}H and ^{35}S radioactivity in each slice was determined. *B*, as in *A*, except that the ^{35}S-labeled mitochondria were isolated from the *cyt-2-1* mutant.

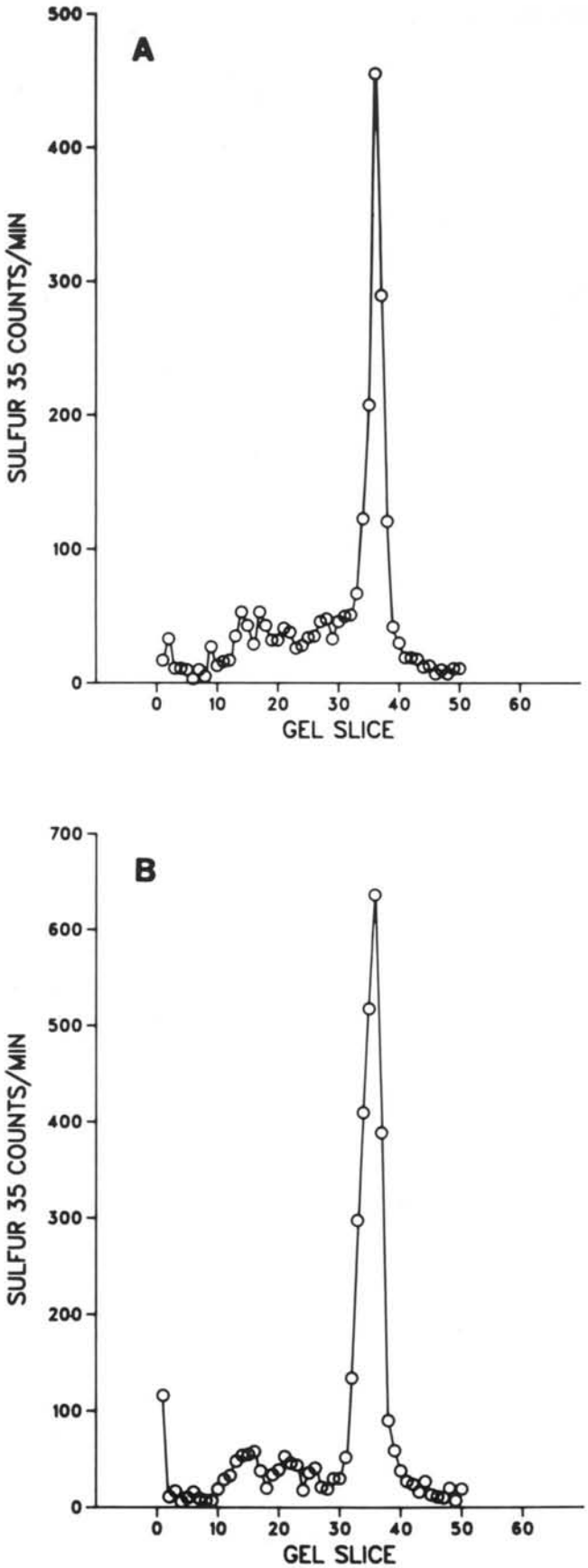

FIG. 3. **Translatable apocytochrome *c* mRNA in wild-type and *cyt-2-1* cells.** Rabbit reticulocyte lysates were programmed with poly(A)-containing RNA isolated from wild-type (*A*) or *cyt-2-1* (*B*) cells. Following *in vitro* translation, the lysates were dissociated in SDS-containing buffer for 5 min at 95 °C, diluted with buffer containing Triton X-100, and then mixed with antibodies to apocytochrome *c* bound to protein A-sepharose. The immunoprecipitates were washed, solubilized, and then resolved by SDS gel electrophoresis. Gels were sliced, and the ^{35}S radioactivity in each slice was determined.

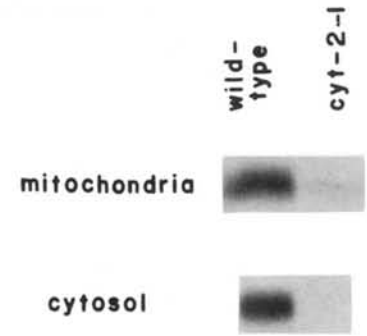

FIG. 4. **Cytochrome *c* in mitochondria and in the cytosol fraction of wild-type and *cyt-2-1* cells.** Cell cultures were labeled by growth in the presence of $^{35}SO_4$, and total cytochrome *c* was immunoprecipitated from the indicated cell fractions with a mixture of antibodies recognizing both holo- and apocytochrome *c*. The immunoprecipitates were washed, solubilized, and resolved by SDS gel electrophoresis. The gels were dried, and bands were visualized by fluorography. The resulting cytochrome *c* bands are shown.

were devoid of the cytochrome suggested three main possibilities as the cause of the cytochrome *c* deficiency in *cyt-2-1* cells. First, the mutant might be deficient in its ability to bind apocytochrome *c* to the receptors found at the outer mitochondrial membrane (Zimmermann *et al.*, 1981; Hennig and Neupert, 1981; Hennig *et al.*, 1983). Second, the mitochondria of the mutant might be deficient in cytochrome *c* heme lyase activity, which is necessary for covalent attachment of the heme group to the apocytochrome and its transfer into the intermembrane space (Hennig and Neupert, 1981; Nicholson *et al.*, 1987). Finally, it was possible that the mRNA for apocytochrome *c* in *cyt-2-1* was not efficiently translated *in vivo*.

To investigate the latter possibility, cultures of wild-type and the mutant were grown in the presence of [^{35}S]sulfate, and cytochrome *c* was immunoprecipitated from both the mitochondria and cytosol of the two strains. As shown in Fig. 4, both the mitochondria and cytosolic fractions of wild-type contain considerable amounts of cytochrome *c*. It is likely that most of the cytochrome detected in the cytosol of wild-type is due to leakage of the holocytochrome from mitochondria during the isolation process. It has been shown previously that apocytochrome *c* exists only transiently in the cytosol of wild-type cells before being transported into mitochondria (Hennig and Neupert, 1981). A small amount of cytochrome *c* is associated with the mitochondria of *cyt-2-1*. Presumably this is due to the presence of apocytochrome *c* bound to mitochondria since the earlier experiments had shown *cyt-2-1* mitochondria to be almost completely devoid of the holocytochrome (Fig. 2). Surprisingly, virtually no free apocytochrome *c* could be detected in the cytosol of the mutant suggesting that the protein is either inefficiently translated and/or rapidly degraded in *cyt-2-1*. In an attempt to distinguish between these possibilities, a pulse-chase labeling experiment was performed on *cyt-2-1* using [^{3}H]leucine. The results of this experiment support the view that apocytochrome *c* is in fact translated in the mutant but is then degraded quite rapidly. As shown in Fig. 5, the amount of the pulse labeled product is significantly reduced 15 min after the addition of chase and has almost completely disappeared after 60 min of chase. The slight increase in apocytochrome *c* that

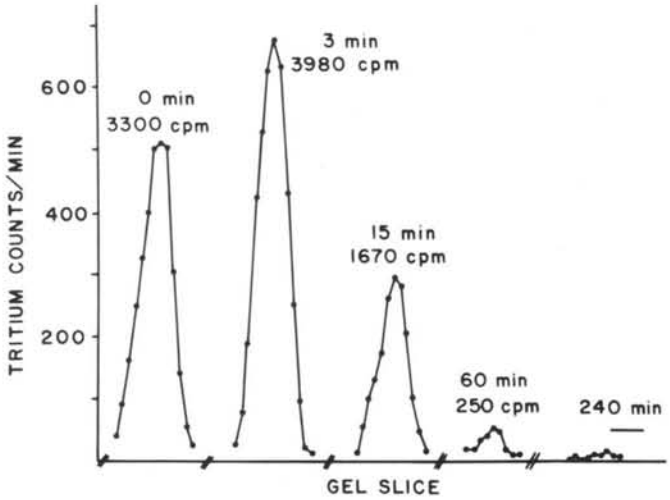

FIG. 5. **Labeling of apocytochrome *c* in *cyt-2-1* cells in a pulse-chase experiment.** [^{3}H]Leucine (2 mCi/liter) was added to a culture of *cyt-2-1* cells following 30 h of growth (start of pulse label). Three min after the addition of radiolabel, the culture was made 10 mM in unlabeled leucine (start of chase). Samples were removed from the culture following further incubation for the times indicated. Total cytochrome *c* was immunoprecipitated from lysed whole cell fractions. After washing, the immunoprecipitates were solubilized and resolved by SDS gel electrophoresis. The gels were sliced, and the radioactivity in each slice was determined. The same amount of cells was used from each of the different chase periods. For each time point, the radioactivity in the peaks corresponding to apocytochrome *c* is shown, and the total number of counts/min in each peak is given.

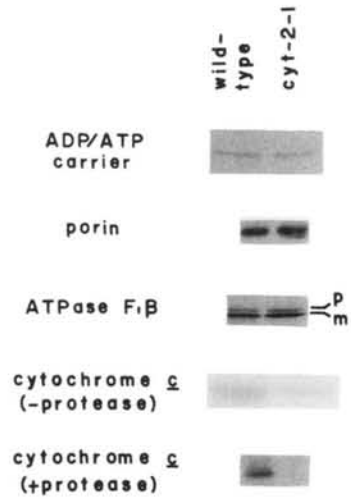

FIG. 6. **Import of various precursor proteins into mitochondria isolated from wild-type and *cyt-2-1* cells.** Conditions for import were as described under "Materials and Methods." Following the import reactions, mitochondria were washed, solubilized, and subjected to immunoprecipitation with antibodies specific to the proteins indicated on the figure. The immunoprecipitates were solubilized and resolved by SDS gel electrophoresis. The gels were dried, and bands were visualized by fluorography. The bands corresponding to the proteins indicated in the figure are shown. In the case of cytochrome *c*, + (or −) *protease* indicates that mitochondria were (or were not) subjected to protease treatment to remove unimported cytochrome *c* prior to solubilization and immunoprecipitation. In the case of ATPase $F_1\beta$, *p* and *m* indicate precursor and mature forms of the protein, respectively.

occurs in the sample taken 3 min after the addition of chase is likely due to a lag in the time of transporting the cold leucine into the cells and/or the completion of translation of nascent [^{3}H]leucine-containing apocytochrome *c* molecules during the initial moments of the chase.

Since the *cyt-2-1* mutant appeared to be capable of producing apocytochrome *c*, mitochondria isolated from the *cyt-2-1* mutant were compared to wild-type for their ability to bind and import apocytochrome *c* as compared to various other mitochondrial proteins. The *in vitro* translation products of poly(A) RNA isolated from wild-type *N. crassa* cells were incubated with mitochondria isolated from either wild-type or *cyt-2-1* cultures. Following the incubation period, the mitochondria were solubilized and treated with antisera to various mitochondrial proteins. As shown in Fig. 6, the *cyt-2-1* mitochondria were approximately equal to wild-type in their ability to import the ADP/ATP carrier, porin, and the β subunit of F_1-ATPase. However, the amount of cytochrome *c* associated with the *cyt-2-1* mitochondria is less than the amount associated with wild-type mitochondria. Similar results were obtained when *in vitro* translation products synthesized from poly(A) RNA isolated from *cyt-2-1* were incubated with either wild-type or *cyt-2-1* mitochondria (not shown). The antisera and conditions used in the above experiments were unable to differentiate between apo- and holocytochrome *c*. However, considering that, *in vivo*, *cyt-2-1* mitochondria do not contain holocytochrome *c* (Fig. 1), it seemed likely that the cytochrome *c* found to be associated with the *cyt-2-1* mitochondria in the *in vitro* import experiments was apocytochome *c* bound to the surface of the outer mitochondrial membrane. In support of this interpretation, it could be shown that protease treatment following the import experiment effectively removed cytochrome *c* from the *cyt-2-1* mitochondria but not from wild-type (Fig. 6). Furthermore, it is likely that the low level of apocytochrome *c* associated with the mitochondria of *cyt-2-1* is due to nonspecific binding

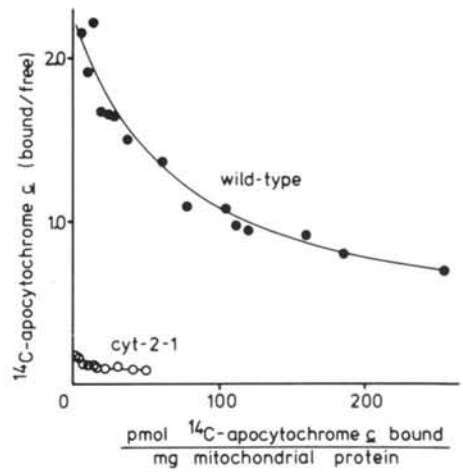

FIG. 7. **Scatchard analysis of apocytochrome *c* binding to wild-type (●) and *cyt-2-1* (○) mitochondria.** Mitochondria were isolated and incubated with varying concentrations of ^{14}C-labeled apocytochrome *c* for 25 min at 25 °C. Following the incubation, the mitochondria were reisolated by centrifugation and the amount of free radioactivity was determined by counting aliquots of the supernatant. The amount of bound radioactivity was determined by counting the mitochondrial pellet following an additional wash and solubilization in SDS-containing buffer.

since, when bovine serum albumin is included in the buffer system (as is the case in the Scatchard analysis, see below, Fig. 7), almost no apocytochrome *c* binds to the mutant mitochondria.

To elucidate the nature of the binding of apocytochrome *c* to *cyt-2-1* mitochondria, the mitochondria of the mutant were examined by Scatchard analysis (Weder *et al.*, 1974). As shown in Fig. 7, the mutant appears to be deficient in the number of high affinity binding sites for apocytochrome *c*,

TABLE I

Heme lyase activity in wild-type and cyt-2-1 mitochondria

Sample	Cytochrome *c* heme lyase activity			
	−NADH	+NADH	$-Na_2S_2O_4$	$+Na_2S_2O_4$
	fmol holocytochrome c formed · min^{-1} · mg^{-1}			
Intact mitochondria				
Wild-type	0.063	0.946		
cyt-2-1	0.031	0.025		
Octyl glucoside lysed mitochondria				
Wild-type			0.042	0.964
cyt-2-1			0.019	0.008

containing only about 5% of the number found on wild-type mitochondria (approximately 4.7 *versus* 85 pmol/mg mitochondrial protein, respectively). The association constants (K_a) determined in two separate experiments for the high affinity binding of apocytochrome *c* to wild-type mitochondria were: 6.4 and 5.5 × 10^7 M^{-1}.

The possibility that apocytochrome *c* could not be converted to holocytochrome *c* by *cyt-2-1* mitochondria and then transferred across the membrane was investigated by assaying *cyt-2-1* mitochondria for the presence of cytochrome *c* heme lyase activity. *In vitro* translation products labeled with L-[^{35}S] cysteine were obtained using poly(A) RNA isolated from wild-type *N. crassa* in a rabbit reticulocyte lysate. These products were incubated with either intact or solubilized mitochondria isolated from wild-type or *cyt-2-1* cultures. Following the incubation period, cytochrome *c* was immunoprecipitated using antiserum that did not distinguish between the precursor and mature form. The immunoprecipitates were processed as described previously for the heme lyase assay (Nicholson *et al.*, 1987) and, following tryptic digestion, were analyzed by high pressure liquid chromatography to distinguish between peptides characteristic of apo- and holocytochrome *c*. The assay is based on the observation that the tryptic peptide of cytochrome *c* consisting of amino acids 18–31, which contains the heme attachment sites (cysteines 18 and 21), is eluted from the column at different times depending on whether or not the heme group is attached (Nicholson *et al.*, 1987). The results of the assay are shown in Table I. In intact mitochondria, the mutant exhibits less than 3% of the activity found in wild-type mitochondria. However, since the assay using whole mitochondria could be influenced by the deficiency of high affinity binding sites on *cyt-2-1* mitochondria, the assay was repeated using mitochondria solubilized with octyl glucoside. Under these conditions, virtually identical results were obtained. The mutant possessed less than 1% of the activity found in solubilized wild-type mitochondria.

DISCUSSION

The cytochrome *c* deficient *cyt-2-1* mutant produces the mRNA for apocytochrome *c* and is able to translate the mRNA into apocytochrome *c in vivo*. However, the mutant is unable to convert the apoprotein to the holo form because of a gross deficiency of cytochrome *c* heme lyase. Apocytochrome *c* does not accumulate in the cytosol due to its rapid degradation. A deficiency of high affinity binding sites in the mutant could also contribute to the lack of holocytochrome *c*. Conceivably, this alone could account for the absence of measurable cytochrome *c* heme lyase activity in *cyt-2-1* mitochondria, since high affinity binding must, presumably, precede the heme lyase step in intact mitochondria. However cytochrome *c* heme lyase activity was also absent in detergent-solubilized preparations of *cyt-2-1* mitochondria where the binding step was circumvented.

There are a number of possibilities which might explain the low number of high affinity binding sites observed on *cyt-2-1* mitochondria. First, the amount of receptor protein synthesized in the mutant might be reduced as the result of a regulatory event related to the deficiency of heme lyase. Conversely, the primary defect in the mutant might actually affect the gene encoding the receptor protein, and the deficiency of heme lyase is a regulatory consequence of this defect. A third possibility is that cytochrome *c* heme lyase is required to enhance the efficiency of, or mediate, the binding step. Indeed, preliminary experiments show that heme lyase is directly involved in the binding of apocytochrome *c* to mitochondria. Therefore, our working hypothesis is that the primary defect of the mutant lies in heme lyase.

Recently, it has been shown that the *cyc-3* mutants of *Saccharomyces cerevisiae* are also deficient in cytochrome *c* heme lyase activity (Dumont *et al.*, 1987), although the ability of *cyc-3* mitochondria to bind apocytochrome *c* was not tested. These mutants lack both of the forms of cytochrome *c* that occur in yeast; *i.e.* iso-1- and iso-2-cytochrome *c* (Sherman *et al.*, 1965; Matner and Sherman, 1982). Interestingly, the mRNAs for both isoforms of the protein are present in normal amounts in *cyc-3* strains, but the amount of apo-iso-1-cytochrome *c* protein found in the mutants is very low or absent while the amount of the apo-iso-2 form is quite high (Matner and Sherman, 1982; Laz *et al.*, 1984). It has been suggested that the absence of the apo-iso-1 form in yeast is due either to its rapid degradation or some form of regulation at the post-transcriptional level (Matner and Sherman, 1982; Laz *et al.*, 1984). Thus, the behavior of the cytochrome *c* precursor in *Neurospora* and the iso-1-cytochrome *c* precursor in yeast may be analogous in the *cyt-2-1* and *cyc-3* mutants, respectively.

The reason for the deficiency of cytochrome aa_3 in *cyt-2-1* is not immediately obvious in view of the fact that the primary lesion in the mutant appears to affect cytochrome *c* heme lyase. However, it should be noted that in this respect *cyt-2-1* is again analogous to the *cyc-3* mutants of yeast, since certain alleles of the latter also cause a deficiency of cytochrome aa_3, particularly under glucose repressing conditions (Reilly and Sherman, 1965; Sherman *et al.*, 1965). It is likely that in both species the cytochrome aa_3 deficiency is a secondary manifestation of the cytochrome *c* deficiency since strains of yeast that are severely deficient in both the iso-1 and iso-2 forms of cytochrome *c*, due to mutations in both the *cyc-1* and *cyc-7* loci, respectively, are also deficient in cytochrome aa_3 (Downie *et al.*, 1977a, 1977b). Similarly, the cytochrome *c*-deficient *cyc-1-2* mutant of *N. crassa*, which has been recently shown to directly affect the cytochrome *c* protein (Stuart *et al.*, 1987), is also deficient in cytochrome aa_3 when grown at 37 °C.[2] These observations lead to the obvious suggestion that a functional cytochrome *c* is required for efficient expression and/or assembly of cytochrome *c* oxidase. However, this interpretation may be too simplistic since *cyt-2-1 cyb-1-1* double mutants of *Neurospora* are still deficient in cytochrome *c*, and presumably cytochrome *c* heme lyase, but do contain cytochrome aa_3 (Bertrand and Collins, 1978).

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[2] H. Bertrand, personal communication.

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