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

(Frank E. Nargang‡, Mariola E. Drygaś, Patrick L. Kwong‡, Donald W. Nicholson§, and Walter Neupert§)

*From the ‡Department of Genetics, University of Alberta, Edmonton, Alberta T6G 2E9, Canada and the §Institut für Physiologische Chemie, Physikalische Biochemie und Zellbiologie der Universität München, Goethestrasse 33, 8000 München 2, Federal Republic of Germany

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.* 169, 1–13). The mutant produces mRNA that can be translated into apocytochrome c in vitro. Apocytochrome c is also synthesized in vitro 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 c is then bound at the outer mitochondrial membrane by specific receptors (Hennig and Neupert, 1981; Zimmermann et al., 1981; Hennig et al., 1988). 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. (1983). The strain is characterized by its slow growth, female infertility, and gross deficiencies of spectrally detectable cytochromes c and aa3 (Mitchell et al., 1983). 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 cyt-298-1 (Bertrand and Werner, 1979), both of which are deficient only in cytochrome c. Both [mi-3] and cyt-2-1 can be induced to synthesize cytochrome aa3 by growth in medium containing low levels of antymycin 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 aa3 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 aa3 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-OR23-1A (74A), 240A nic-1 al-2 (nic240), and a derivative (NCN68) of a cyt-2-1 pan-2 a strain, IS-4. Both nic240 and IS-4 were obtained from H. Bertrand, University of Regina. The NCN68 strain was derived in the following manner. The wild-type strain 74A 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-2-3 aro-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 aro-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 x 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
be used in cytochrome c concentration of 2 mCi/liter. Labeling was allowed to proceed for 3 h, 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\textsubscript{2}. The samples were ground with acid-washed sea sand (1 g for each g wet weight of mycelium) under liquid nitrogen. While still frozen, the mycelium was ground to a powder with acid-washed, heat-stabilized 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/chlorform/isomyl 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 30 min. Following treatment, the phenol/chlorform/isomyl 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 buffer. 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 5 volumes of ethanol. The pellet was dissolved in sterile H\textsubscript{2}O and stored at -20 °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]methylmethionine (Amersham Corp., 1270 Ci/mmol). However, for in vitro translation products to be used in cytochrome c heme assay studies, L-[^{35}S]cysteine (Amersham Corp., 1555 Ci/mmol) was used.

Immunoprecipitations—For holocytochrome c immunoprecipitations, 125 μl of antiserum was added to 3 mg of mitochondrial protein containing 1-2 x 10\textsuperscript{6} cpm of heme or 1 x 10\textsuperscript{6} 1-3 x 10\textsuperscript{6} 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 10 min. After the corresponding to 1-7 x 10\textsuperscript{6} cpm incorporated ^{35}S radioactivity, as determined as described by Pelham and Jackson (1976), was diluted with 20 volumes of dilution buffer (10 mM Tris-Cl, pH 7.5, 500 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 (20 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 μM protease-resistant imported porin. After 30 min, mitochondria were re-isolated by centrifugation at 17,400 x 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 measured by treatment with 200 μg of protease K/ml for 30 min at 0 °C (Freitag et al., 1982). Protease-resistant ATPase F\textsubscript{1,2} was measured following treatment with 15 μg of protease K/ml for 30 min at 0 °C (Schleyer and Neupert, 1988). Imported ADP/ATP carrier was assayed by treatment with proteinase K, as described for ATPase F\textsubscript{1,2}, followed by detergent solubilization and passage over columns of hydroxyapatite 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 protease K treatment as per ATPase F\textsubscript{1,2}.

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 anti sera, and the immunoprecipitated proteins were resolved in SDS-polyacrylamide gels.

Electrophoresis, Fluorography, and Determination of Radioactivity in the 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 vials. 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 LS9000 liquid scintillation counter. Where appropriate, corrections for spill-over of ^{35}S counts into ^{3}H channeling were made prior to plotting the data.

Immunoprecipitates from in vitro import experiments were disassociated prior to electrophoresis by incubation at 95 °C for 5 min in 2% SDS, 0.35 M 2-mercaptoethanol, 50 mM Tris-Cl, pH 6.8, 10% glycerol, and 0.005% bromphenol blue. Gels were stained with Coumassie 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 the biuret method (Gornall et al., 1949) or by dye-binding assay (Bradford, 1976).

Cytochrome c Heme Lysate Assay and Scatchard Analysis—The determination of high affinity binding sites for cytochrome c was performed as described (Hennig et al., 1985; Nicholson et al., 1987). Cytochrome c heme lysates in intact mitochondria was assayed as described in Nicholson et al. (1987). To measure heme lysate activity...
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 [35S]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 [3H]leucine were added to both the wild-type and cyt-2-1 [35S]-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 [3H]leucine or [35S]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 [35S]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 $\alpha$-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 [35S]sulfate were mixed with wild-type mitochondria labeled with [3H]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 [35S]-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. The gels were dried, and bands were visualized by fluorography. The resulting cytochrome c bands are shown.

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 [35S]sulfate, 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; Henning and Neupert, 1981; Henning 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 (Henning 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 [35S]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 (Henning 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 [3H]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
cytochrome c was immunoprecipitated from lysed whole cell fractions. The culture following further incubation for the times indicated was subjected to a pulse-chase experiment. Three min after the addition of radiolabel, the culture was made 10% of the cells were labeled with [3H]leucine (2 mCi/liter), and the radioactivity in each slice was determined. The amount of cells was used to calculate the total number of counts/min in each peak is given.

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 ATPase F1β. 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 apocytochrome 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 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**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cytochrome c heme lyase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−NADH +NADH</td>
</tr>
<tr>
<td>Intact mitochondria</td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>0.063</td>
</tr>
<tr>
<td>cyt-2-1</td>
<td>0.031</td>
</tr>
<tr>
<td>Octyl glucoside lysed</td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>0.042</td>
</tr>
<tr>
<td>cyt-2-1</td>
<td>0.019</td>
</tr>
</tbody>
</table>

containing only about 5% of the number found on wild-type mitochondria (approximately 4.7 versus 86 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 × 10⁷ 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 [³⁵S]cysteine were obtained using poly(A) RNA isolated from wild-type Neurospora 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 trypic 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 cysteine 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 holoprotein form because of a gross deficiency of apocytochrome 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 aa₃ 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 aa₃. Moreover, the behaviour of the cytochrome aa₃ and the iso-1-cytochrome c protein in the mutant might be influenced by the deficiency of the heme lyase, which was not tested.

**Acknowledgments**—We are grateful to Drs. Dan Gietz and Richard Zimmerman for helpful discussions and to Dr. Kenneth Morgan for assistance with computerized representation of data. Gabi Zimmerman provided excellent technical assistance. We also thank Dr. Bernd Hennig for providing us with a computer program for graphic-parameter analysis of Scatchard plots and Christoph Hergersberg for his help during the binding experiments.

*H. Bertrand, personal communication.*
REFERENCES