Cytochrome c₁ of Bakers’ Yeast

II. SYNTHESIS ON CYTOPLASMIC RIBOSOMES AND INFLUENCE OF OXYGEN AND HEME ON ACCUMULATION OF THE APOPROTEIN*

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In the preceding paper (Ross, E., and Schatz, G. (1976) J. Biol. Chem. 251, 1991–1996) yeast cytochrome c₁ was characterized as a 31,000 dalton polypeptide with a covalently bound heme group. In order to determine the site of translation of this heme-carrying polypeptide, yeast cells were labeled with ['H]leucine under the following conditions: (a) in the absence of inhibitors, (b) in the presence of acriflavine (an inhibitor of mitochondrial translation), or (c) in the presence of cycloheximide (an inhibitor of cytoplasmic translation). The incorporation of radioactivity into the hemeprotein was measured by immunoprecipitating it from mitochondrial extracts and analyzing it by dodecyl sulfate-polyacrylamide gel electrophoresis. Label was incorporated into the cytochrome c₁ apoprotein only in the presence of acriflavine or in the absence of inhibitor, but not in the presence of cycloheximide. Cytochrome c₁ is thus a cytoplasmic translation product.

This conclusion was further supported by the demonstration that a cytoplasmic petite mutant lacking mitochondrial protein synthesis still contained holocytochrome c₁ that was indistinguishable from cytochrome c₁ of wild type yeast with respect to molecular weight, absorption spectrum, the presence of a covalently bound heme group, and antigenic properties. Cytochrome c₁ in the mitochondria of the cytoplasmic petite mutant is firmly bound to the membrane, and its concentration approaches that typical of wild type mitochondria. However, its lability to proteolysis appeared to be increased. A mitochondrial translation product may thus be necessary for the correct conformation or orientation of cytochrome c₁ in the mitochondrial inner membrane.

Accumulation of cytochrome c₁ protein in mitochondria is dependent on the availability of heme. This was shown with a δ-aminolevulinic acid synthetase-deficient yeast mutant which lacks heme and any light-absorbing peaks attributable to cytochromes. Mitochondria from mutant cells grown without added δ-aminolevulinic acid contained at least 20 times less protein immunoprecipitable by cytochrome c₁-antisera than mitochondria from cells grown in the presence of the heme precursor. Similarly, the respiration-deficient promitochondria of anaerobically grown wild type cells are almost completely devoid of material cross-reacting with cytochrome c₁-antisera.

A 105,000 × g supernatant of aerobically grown wild type cells contains a 29,000 dalton polypeptide that is precipitated by cytochrome c₁-antiserum but not by nonimmune serum. This polypeptide is also present in high speed supernatants from the heme-deficient mutant or from anaerobically grown wild type cells. The possible identity of this polypeptide with soluble apocytochrome c₁ is being investigated.

In the preceding paper we have described the purification of cytochrome c₁ from the yeast Saccharomyces cerevisiae (1).

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The availability of the purified cytochrome and the information on its molecular properties allowed us to study its biosynthesis by methods which were not limited to absorption spectroscopy of the holocytochrome. In the present paper we describe experiments in which cytochrome c₁ was isolated by immunoprecipitation from yeast cells that had been labeled with radioactive leucine in the presence of inhibitors of cytoplasmic or mitochondrial protein synthesis.

In a complementary approach, we report the accumulation of cytochrome c₁ in an extrachromosomal petite mutant lacking mitochondrial protein synthesis. Since previous investigators (4–6) failed to detect the spectral absorbance bands of
cytochrome $c_1$ in the petite mutant, we initially speculated (2) that the assembly or integration of cytochrome $c_1$ might require mitochondrial translation. However, Claissé and Pajot (7) have recently reported that an absorption peak of petite mitochondria which had usually been attributed to "cytochrome $b_1$" (4) was indistinguishable from that of cytochrome $c_1$ of wild type yeast cells. These careful spectroscopic studies clearly invalidated earlier claims about the absence of cytochrome $c_1$ absorption bands in extrachromosomal petite mutants, but the chemical identity of the cytochrome $c_1$ like pigment remained open. We now identify this pigment as cytochrome $c_1$ on the basis of its molecular weight, its antigenic properties, its absorption spectrum, and its covalently bound heme group. Although we find no difference between cytochrome $c_1$ from the wild type or the petite mutant in vivo, cytochrome $c_1$ from the petite mutant is much more labile to proteolysis (cf. Ref. 1) than is cytochrome $c_1$ from wild type cells. This suggests that some mitochondrial translation product may be associated with cytochrome $c_1$ in wild type cells.

In addition, we have investigated the effects of anaerobiosis and heme deprivation on cytochrome $c_1$ biogenesis. When Saccharomyces cerevisiae adapts to anaerobiosis, its mitochondria are converted to promitochondria which do not respire, lack the absorbance bands of the respiratory cytochromes, and lack succinate- and cytochrome $c$-oxidase activities (8). Promitochondria have also been shown to lack several protein subunits of cytochrome $c$ oxidase (9), and it has been suggested that this deficiency may reflect control of their translation or accumulation by oxygen (10-12). In this paper we report that cytochrome $c_1$, protein cannot be detected immunochromically in promitochondria from anaerobically grown yeast. Similarly, mitochondria from mutant cells which are heme-deficient due to a lesion in δ-aminolevulinic acid synthase (13) also lack immunochromically detectable apocytochrome $c_1$. 

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Cell Growth**—Unless noted otherwise, the wild type strain was D273-10B (ATCC 24657; α PET ρ+). The isogenic cytoplasmic petite mutant D273-10B-1 (α PET ρ-) had been derived from the wild type strain by treatment with acriflavine (14). This strain has previously been shown to lack mitochondrial ribosomes (15) and mitochondrial protein synthesis (16, 17). These two strains were grown in the semisynthetic medium described by Ono et al. (18) containing 0.8% galactose and the concentration of yeast extract noted in the text. The heme-requiring mutant X2180-38 (a gal-2 pet p+), and the corresponding prototrophic strain X2180 (a gal-2 PET ρ+) were obtained from D. Sprinson and E. G. Golub (Columbia University) (13). The strains were grown in semisynthetic medium containing 1% glucose since the gal-2 marker precluded growth with galactose; where noted, diisopropyl fluorophosphate was added immediately after disrupting the cells or after the preparation of spheroplasts. Rapid preparation of spheroplasts was performed as described (1).

**Materials**—L-[4,5-3H]Leucine (6 Ci mmol−1), L-[U-14C]leucine (312 mCi mmol−1), δ-aminolevulinic acid (33 Ci mmol−1), δ-aminolevulinic acid (33 Ci mmol−1), 8-amino[2,3-3H]leucine (317 mCi mmol−1), and 8-amino[2,3-3H]leucine (317 mCi mmol−1) were obtained from New England Nuclear, and L-leucine from Sigma. Other materials were purchased from the sources listed in the preceding paper (1).

**RESULTS**

**Immunochromic Identification and Isolation of Cytochrome $c_1$**—Immunoprecipitation has proved to be very useful for studying the synthesis of specific membrane proteins (22, 25). In order to identify the site of synthesis of cytochrome $c_1$, we therefore decided to prepare an antiserum against the purified yeast cytochrome $c_1$. The initial injection contained 5 mg of purified cytochrome $c_1$ (1), and each booster injection contained 1 mg of purified cytochrome $c_1$ according to Ebner et al. (22), except that extraction of mitochondria prior to immunoprecipitation was done as follows. Mitochondria were prepared according to Schatz and Kováč (19), modified to include 0.5 mm phenylmethlysulfonyl fluoride in all solutions used after the preparations of spheroplasts. The mitochondria were converted to submitochondrial particles by sonication in 0.1 M NaP/O.9 mm EDTA, pH 7.5. The particles were collected by centrifugation at 35,000 rpm for 30 min in a SpincNo. 50 rotor and suspended to 10 mg ml−1 in the same buffer. Sodium cholate was added as a 20% solution to a final concentration of 3%, 144 mg of solid ammonium sulfate was added to each ml of suspension, and the mixture was stirred for 60 min. The insoluble residue was removed by centrifugation at 35,000 rpm for 30 min in a SpincNo. 50 rotor and the supernatant was dialyzed against 10 mM NaP/O 1 M NaCl/0.5% cholate/0.5% EDTA, pH 7.0. The dialyzed extract was cleared of any precipitate by centrifugation as above. The clear extract generally contained 40 to 50% of the protein originally present in the submitochondrial particles. When cytochrome $c_1$ was immunoprecipitated from extracts of whole spheroplasts, the spheroplasts were prepared according to Schatz and Kováč (19), but lysed by homogenization in 0.1 M NaP/O, pH 7.5/0.5 mM EDTA containing 0.5 mM phenylmethlysulfonyl fluoride and 57 μM diisopropyl fluorophosphate. Debris was removed by centrifugation at 40,000 rpm for 10 min in a Sorval SS-34 rotor. The supernatant was then extracted with cholate and (NH₄)₂SO₄ exactly as described for submitochondrial particles.

**Other Methods**—Quantitation of cytochromes and submitochondrial particles was performed according to the method of von Jagow et al. (23) using the spectrophotometric procedures described in the previous paper (1). This is essentially the method used by Claissé and Pajot (7) in their determination of cytochrome $c_1$. Interference coefficients for cytochromes $c$ and $c_1$ were determined with the purified yeast cytochromes. Published procedures were used for the purification of yeast cytochrome $c$ (24) and yeast cytochrome $c_1$ (11). Other procedures were described in the preceding paper (1).

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sulfate-polyacrylamide gel electrophoresis, the result shown in Fig. 2 is obtained. The 31,000 dalton cytochrome c1 protein is in the predominant band. The 18,500 dalton polypeptide which often co-purifies with cytochrome c1 (1) is not immunoprecipitated under these conditions. However, there is a certain amount of high molecular weight radioactive contaminants, whose total amount is usually somewhat less than the radioactivity present in the cytochrome c1 peak. It cannot be determined if co-precipitation of impurities represents an artifact of immunoprecipitation or contaminating antibodies in the serum. Since the serum is relatively weak, both explanations are plausible. Nevertheless, the antisera permits the rapid detection and quantitation of cytochrome c1 in appropriately labeled cell fractions.

**Site of Synthesis of Cytochrome c1**—We have now confirmed that cytochrome c1 is translated on cytoplasmic ribosomes (2). In these early experiments yeast cells were labeled with [3H]leucine under nongrowing conditions and radioactivity was found in a purified cytochrome c1 fraction. Labeling of this fraction was blocked by cycloheximide (an inhibitor of cytoplasmic protein synthesis), but not by acriflavin (an inhibitor of mitochondrial protein synthesis). However, as pointed out earlier, it remained to be shown that the measured radioactivity was indeed associated with the cytochrome c1 heme protein rather than with contaminants (2, 26).

By combining immunoprecipitation and gel electrophoresis we have now confirmed that the cytochrome c1 heme protein is synthesized on cytoplasmic ribosomes. Yeast cells which were first grown for approximately 10 generations in [14C]leucine to uniformly label all protein were harvested and labeled with [3H]leucine under one of the following conditions: (a) in the absence of inhibitor, (b) in the presence of acriflavin, or (c) in the presence of cycloheximide. After incubation, the mitochondria were isolated (21) from each aliquot and subjected to immunoprecipitation with anti-cytochrome c1 antiserum as described under “Experimental Procedures.” The radioactive polypeptides present in the immunoprecipitates were analyzed by dodecyl sulfate-polyacrylamide gel electrophoresis, and the relative amount of [3H]leucine incorporated into cytochrome c1 protein was calculated by integration of the area under the cytochrome c1 heme protein peak (cf. Fig. 2 and Table I). The amount of radioactivity incorporated into the cytochrome c1 heme protein under each of the three conditions is shown in Table I.
Since acriflavin does not inhibit incorporation of leucine into cytochrome $c_1$ heme protein, whereas cycloheximide inhibits completely, we conclude that the cytochrome $c_1$ heme protein is synthesized on cytoplasmic ribosomes.

**Detection of Cytochrome $c_1$ in a Cytoplasmic Petite Mutant**

If cytochrome $c_1$ is a cytoplasmic translation product, then it might be expected to be present in cytoplasmic petite mutants which have lost mitochondrial protein synthesis (16, 27). While several groups had reported the absence of cytochrome $c_1$ absorption bands in these mutants (4-6), Classie and Pajot (7) have recently observed that mitochondria from cytoplasmic petite mutants contain a pigment with the absorption characteristics of cytochrome $c_1$. We have reinvestigated the presence of cytochrome $c_1$ in cytoplasmic petite mutants by biochemical and immunological techniques.

The cytoplasmic petite mutant D273-10B-1 was grown in the presence of $\text{[^3H]}$leucine and its mitochondria were mixed with mitochondria from wild type cells which had been grown in $\text{[^14C]}$leucine. Extracts of the mixed mitochondria were then subjected to immunoprecipitation with anticytochrome $c_1$ antiserum and the immunoprecipitate was analyzed by dodecyl sulfate-polyacrylamide gel electrophoresis. The result of one experiment is shown in Fig. 3. It is clear that the immunoprecipitate from the cytoplasmic petite mutant contains cytochrome $c_1$ protein. By taking into account the different amounts of radioactivity initially present in the two types of mitochondria and by integrating the radioactivity present in the cytochrome $c_1$ peak of wild type and mutant cells, it can be calculated that the mutant mitochondria contain 73% of the cytochrome $c_1$ protein present in the wild type. Two additional experiments yielded values between 70 and 80%. The experiment depicted in Fig. 3 does not exclude the possibility that the tritium-labeled cytochrome $c_1$ protein immunoprecipitated from the petite mutant represents heme-free apocytochrome $c_1$. In order to rule out this possibility the heme moieties of cytochromes in wild type and petite mutant cells were labeled with the radioactive heme precursor $\delta$-amino$[^3H]$levulinic acid. Wild type and petite mutant cells were grown separately for about 10 generations in the presence of the precursor and cytochrome $c_1$ was immunoprecipitated from mitochondrial extracts. Electrophoresis of the immunoprecipitates in dodecyl sulfate-polyacrylamide gels yielded the radioactivity patterns shown in Fig. 4. In the wild type immunoprecipitate, the cytochrome $c_1$ heme protein is the predominant band, and the low amount of label in other regions of the gels suggests that the precursor was not significantly converted to amino acids. The radioactivity pattern of the immunoprecipitate from the petite mutant is essentially identical to that of the wild type. Since heme not covalently bound to protein is released upon dissociation in dodecyl sulfate and migrates close to the dye front (1), the result of Fig. 4 shows that cytoplasmic petite mutants contain holocytochrome $c_1$ whose heme is covalently attached to the cytochrome $c_1$ apoprotein. In both Figs. 3 and 4, the cytochrome $c_1$ heme protein which is immunoprecipitated from petite mitochondria has a slightly higher electrophoretic mobility than that of the wild type. Since cytochrome $c_1$ is labile to proteolysis (1), it seemed likely that the increased mobility results from attack by intracellular proteases. It was of interest to discover whether this proteolysis reflected release of proteases during the isolation of mitochondria or whether cytochrome $c_1$ of cytoplasmic petite mutants is modified by

![Fig. 3](http://www.jbc.org/)

**Fig. 3.** Presence of cytochrome $c_1$ in a cytoplasmic petite mutant. The cytoplasmic petite mutant D273-10B-1 and the isogenic wild type strain D273-10B were grown in semisynthetic medium containing 0.2% yeast extract and either 5 mCi liter$^{-1}$ of [4,5-$^3$H]leucine or 0.5 mCi liter$^{-1}$ of [U-$^{14}$C]leucine, respectively. Mitochondria were prepared from each batch of cells and the mitochondria were mixed and extracted. Cytochrome $c_1$ was immunoprecipitated from the extract and the immunoprecipitate was analyzed by dodecyl sulfate-polyacrylamide gel electrophoresis. Dotted line, $^3$H, solid line, $^{14}$C.

![Fig. 4](http://www.jbc.org/)

**Fig. 4.** Labeling of the heme moiety of cytochrome $c_1$ with $\delta$-amino-$[^3H]$levulinic acid. The wild type (D273-10B) or the cytoplasmic petite mutant (D273-10B-1) was grown in semisynthetic medium containing 0.1% yeast extract and 0.5 mCi liter$^{-1}$ of $\delta$-amino[2,3-$^3$H]levulinic acid. Cytochrome $c_1$ was immunoprecipitated from extracts of the wild type or mutant mitochondria and analyzed by dodecyl sulfate-polyacrylamide gel electrophoresis.
proteases in vivo. In order to decide between these alternatives, we exploited the possibility (1) of identifying cytochrome $c_1$ directly in lysed cells. Cells of the cytoplasmic petite mutant and the wild type were grown in $^3$H- and $^14$C-labeled aminolevulinic acid, respectively, and converted to spheroplasts; the spheroplasts were mixed, solubilized with dodecyl sulfate dissociation buffer at 100°C in the presence of protease inhibitors, and analyzed by dodecyl sulfate-polyacrylamide gel electrophoresis (1). With both mutant and wild type, the radioactivity pattern illustrated in Fig. 5 clearly shows the distinct 31,000-dalton peak attributable to cytochrome $c_1$. The cytoplasmic petite mutation does thus not alter the molecular weight of cytochrome $c_1$ in vivo, but merely renders the cytochrome more susceptible to proteolytic attack upon cell disruption. A further experiment has shown that this proteolysis occurs to an appreciable extent only after the mitochondria are solubilized with cholate.Cytochrome $c_1$ was immunoprecipitated from a mitochondrial extract prepared from pooled $\delta$-amino-$^3$HJlevulinic acid-labeled wild type cells and $\delta$-amino-$^3$HJlevulinic acid-labeled petite cells. Aliquots of the spheroplasts, mitochondria, submitochondrial particles, dialyzed mitochondrial extracts, and immunoprecipitated cytochrome $c_1$ were analyzed by dodecyl sulfate gel electrophoresis. Only cytochrome $c_1$ from the petite mutant was shortened to the 29,000-dalton species, and this shortening was observed only in the extract and immunoprecipitated fractions.

Since we had detected cytochrome $c_1$ in the petite mutant by chemical and immunochemical methods, we decided to re-examine the spectral evidence which had been cited to indicate the absence of the cytochrome in the petite mutant. Typical low temperature reduced minus oxidized difference spectra of submitochondrial particles from wild type and petite cells are shown in Fig. 6. The spectra are simplified by employing ascorbate as reductant (so as not to reduce cytochrome $b$) and

![Image](http://www.jbc.org/)

Fig. 5. Determination of the in vivo molecular weight of cytochrome $c_1$ in a cytoplasmic petite mutant. The wild type D273-10B and the cytoplasmic petite mutant D273-10B-1 were grown in semisynthetic medium containing 0.1% yeast extract and either 2 $\mu$Ci ml$^{-1}$ of $\delta$-amino[$^3$H]levulinic acid or 2 $\mu$Ci ml$^{-1}$ of $\delta$-amino[$^14$C]levulinic acid, respectively. Cells were converted to spheroplasts (see Ref. 1 and "Experimental Procedures"). The mixed spheroplasts were lysed in dodecyl sulfate dissociation buffer (20) containing 1.9 mM phenylmethylsulfonylfluoride and 57 $\mu$M diisopropyl fluorophosphate and were analyzed by dodecyl sulfate-polyacrylamide gel electrophoresis. Dotted line, $^14$C; solid line, $^3$H.

![Image](http://www.jbc.org/)

Fig. 6. Reduced minus oxidized difference spectra of mitochondria from wild type yeast and a cytoplasmic petite mutant. The wild type (D273-10B) or the cytoplasmic petite mutant (D273-10B-1) was grown in 0.3% galactose/1% yeast extract/2% peptone, and the mitochondria were prepared from the broken cells as described (21). The mitochondria were suspended in 10 mM sodium phosphate/0.5 mM EDTA/0.15 M NaCl, pH 7.5, sonicated for 20 s, and centrifuged for 20 min at 35,000 rpm in a Spincno No. 50 rotor. The resulting submitochondrial particles were sonicated and washed three more times in the same buffer. Further washing as above or washing in 0.25 M NaCl caused a less than 5% further decrease in the cytochrome $c$ content of the particles. Spectra were measured at $-196^\circ$ as described (1). Wild type particles were measured at a final protein concentration of 1.0 mg ml$^{-1}$ and petite particles at 4.4 mg ml$^{-1}$. The reductant was ascorbate and the oxidant was ferricyanide. The calibration bar represents 0.05 absorbance unit for the wild type and 0.02 for the petite mutant. In this experiment, the calculated (23) cytochrome $c_1$ content of the petite mutant particles was 89% that of the wild type.
c1—Since heme is an essential functional component of cytochrome c1, it was of interest to know whether the synthesis (or the accumulation) of the apocytochrome was regulated by the availability of heme within the cell. We have approached this question with the heme-requiring yeast mutant X2180-38 which lacks δ-aminolevulinic acid synthetase (13). When grown in the absence of δ-aminolevulinic acid, the mutant cannot synthesize porphyrins, lacks all mitochondrial cytochromes, and is respiration-deficient; it also requires ergosterol as well as oleic acid for growth since the synthesis of these essential lipids is mediated by heme proteins. However, when grown in the presence of δ-aminolevulinic acid, the mutant is phenotypically wild type.

The mutant X2180-38 was grown in a δ-aminolevulinic acid deficient medium in the presence of [3H]leucine and converted to spheroplasts. These were then mixed with spheroplasts from the same mutant grown in the presence of δ-aminolevulinic acid and [14C]leucine. The mixed spheroplasts were lysed, the mitochondria were isolated and extracted, and cytochrome c1 was immunoprecipitated from the combined extract as described under “Experimental Procedures.” The electrophoretic analysis of the immunoprecipitate is given in Fig. 7. While mitochondria from mutant cells which were rendered heme-replete by growth with δ-aminolevulinic acid clearly contain cytochrome c1, the immunoprecipitate from mitochondria of cells grown in the absence of δ-aminolevulinic acid contains little, if any, cytochrome c1 protein. Even if all of the rather diffuse 14C radioactivity in the 29,000 to 31,000 dalton region is assumed to be apocytochrome c1, the heme-deficient cells contain at most 9% of the cytochrome c1 protein present in wild type cells or in cells of mutant X2180-38 grown in the presence of δ-aminolevulinic acid. In other experiments, values as low as 5% were found. Since these are definitely upper limits, it is possible that mitochondria from heme-deficient cells are completely devoid of apocytochrome c1.

These experiments indicate that the heme-carrying polypeptide of cytochrome c1 is not significantly accumulated in the mitochondrial membrane unless heme is present. It is, however, possible that apocytochrome c1 is translated in the absence of heme, but that it is not properly integrated into the membrane. To test this possibility, we assayed for apocytochrome c1 in whole cell extracts of the mutant X2180-38 grown in the absence of δ-aminolevulinic acid. Cells of the mutant grown in lipid-supplemented medium containing [3H]leucine were mixed with cells of the wild type X2180 grown in the same medium containing [14C]leucine. The cells were converted to spheroplasts, and a cholate extract prepared from a hypotonic lysate of the spheroplasts was subjected to immunoprecipitation with anticytochrome c1 serum. Fig. 8 shows the dodecyl sulfate-polyacrylamide gel electrophoretic analysis of the immunoprecipitated protein. The protein pattern for wild type or mutant cells is similar, with the major peak at the proper molecular weight for partially proteolyzed cytochrome c1 (1). The proteolysis might be expected, since the extraction of whole cells frees protease activity normally contained in vacuoles (29-31). It thus appears possible that the protein which was immunoprecipitated from the mutant in this experiment was apocytochrome c1, since it cross-reacted with cytochrome c1-specific antiserum and since it exhibited the expected molecular weight. This possibility will be further discussed below.

Synthesis of Cytochrome c1 during Anaerobiosis—During anaerobiosis, the mitochondria of Saccharomyces cerevisiae dedifferentiate into respiration-deficient promitochondria which lack the characteristic spectra of the respiratory cytochromes (8). We used immunoprecipitation to assay for the presence of cytochrome c1 in whole cell extracts of wild type cells grown anaerobically with [3H]leucine and of mitochondria from cells grown aerobically with [14C]leucine. An electrophoretic analysis of one immunoprecipitate is shown in Fig. 9. No cytochrome c1 was found in the promitochondria at a level of

Fig. 7. Lack of cytochrome c1 in mitochondria of a heme-requiring mutant. The δ-aminolevulinic acid synthetase-deficient mutant X2180-38 was grown in semisynthetic medium containing 0.2% yeast extract, 1.0% glucose, and either 30 μg/ml of δ-aminolevulinic acid and 0.45 μCi/ml of [14C]leucine or 12 μg/ml of ergosterol, 2.5 mg/ml of Tween 80 and 10 μCi/ml of [4,5-3H]leucine. Mitochondria from each batch of cells were pooled and cytochrome c1 was immunoprecipitated from an extract of the mitochondria. Solid line, 14C. Dashed line, 3H.

Fig. 8. Assay for apocytochrome c1 in whole cell extracts of a heme-deficient mutant. The mutant X2180-38 was grown in lipid-supplemented semisynthetic medium containing L-[4,5-3H]leucine, and the isogenic wild type strain X2180 was grown in the same medium containing L-[U-14C]leucine. Spheroplasts were prepared from each batch of cells (see Ref. 19 and “Experimental Procedures”), pooled, lysed in 100 mM NaPO4/0.6 mM EDTA, pH 7.0, and extracted with cholate as described under “Experimental Procedures.” All solutions used after preparation of spheroplasts contained 1 mM phenylmethylsulfonylfluoride and 67 μM diisopropyl fluorophosphate. Proteins which were precipitated by cytochrome c1-specific antiserum were analyzed by dodecyl sulfate-polyacrylamide gel electrophoresis. Solid line, 14C, dashed line, 3H.
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detection of less than 10% of aerobic mitochondria. It is therefore unlikely that cytochrome c₁ or apocytochrome c₁ accumulate in the promitochondria of anaerobically grown yeast.

Since the situation during anaerobiosis could be analogous to that of heme deprivation, we searched for cytochrome c₁ protein in extracts of whole cells. We grew wild type cells anaerobically in the presence of [³H]leucine, converted them to spheroplasts, and immunoprecipitated any cross-reacting material with cytochrome c₁-specific antiserum. Fig. 10, a dodecyl sulfate electrophoretic analysis of the immunoprecipitate shows that a cytochrome c₁-like protein is found in anaerobically grown yeast, even though it cannot be detected in promitochondria. The total amount of the protein present is roughly one-tenth that found in aerobically grown cells.

DISCUSSION

Earlier studies in different laboratories have shown that the biogenesis of the ubiquinone-cytochrome c reductase complex of the respiratory chain is controlled by mitochondrial and cytoplasmic protein synthesis (26, 32-34, 36), heme synthesis (13, 35), oxygen (8), and catabolite repression (33, 34). We have begun to study the biogenesis of this complex by concentrating on a relatively well defined component, cytochrome c₁. In the previous paper (1), we reported the purification of yeast cytochrome c₁ by classical techniques and described some properties of the cytochrome. In this paper, we have shown that cytochrome c₁ can also be isolated from mitochondria by immunoprecipitation. This technique has proved very useful for studying the biogenesis of cytochrome c₁; if carried out in the presence of protease inhibitors, it has also allowed us to isolate the cytochrome c₁, heme protein with minimal, if any, proteolytic alteration. The extreme lability of this heme protein towards protease makes this much more difficult if classical multistep procedures are used.

While our data show that cytochrome c₁ is synthesized on cytoplasmic ribosomes, it appears relevant that cytochrome c₁ in cytoplasmic petite mutants is significantly more susceptible to proteolysis during isolation than cytochrome c₁ from the corresponding wild type cells. Since this proteolysis will effect only the cytochrome from the petite mutant in a mixture of petite and wild type mitochondria, and since it occurs only after the mitochondria have been lysed with cholate, it is unlikely that the proteolysis is an artifact arising from elevated protease activity in the petite mutant. Rather, mitochondrial translation may be a prerequisite for the proper incorporation or orientation of cytochrome c₁ into the ubiquinone-cytochrome c reductase complex by furnishing cytochrome c₁-binding polypeptides such as (perhaps) cytochrome b₁ (32, 36). It has already been reported that some of the cytoplasmically made subunits of cytochrome c oxidase (22) and of the ATPase complex (37) are only weakly bound to the mitochondrial inner membrane if the corresponding mitochondrially made subunits are missing.

A second major point of this study is the observation that either anaerobiosis or heme deprivation block the accumulation of apocytochrome c₁ in the mitochondrial inner membrane. Since the synthesis of protoporphyrin IX appears to require molecular oxygen (38), the effect of anaerobiosis may actually reflect a lack of heme. However, the oxygen requirement of heme synthesis in yeast is not yet entirely clear since promitochondria do contain cytochrome b₁ (8, 39, 40) whose prosthetic group is probably iron protoporphyrin IX.

It could also be argued that the defective mitochondrial organelles mentioned above contained apocytochrome c₁ which did not cross-react with the antisera that had been raised against the holo cytochrome. While we cannot completely exclude this possibility, the following experiments (not documented here) argue against it. (a) The antisera were not specifically directed against “native” holocytochrome c₁, but also immunoprecipitated cytochrome c₁ that had been acetylated with [³H]acetic anhydride and partially denatured by dodecyl sulfate. (b) When yeast cells were grown anaerobically in the presence of [³H]leucine and adapted to oxygen under nongrowing conditions in the presence of [¹⁴C]leucine, the [¹⁴C]/[³H] ratio of cytochrome c₁ immunoprecipitated from the adapted mitochondria was much higher than that of the total cellular proteins. According to this “chase” experiment, respiratory adaptation involves de novo synthesis of cytochrome c₁ rather than the addition of heme to pre-existing apocytochrome c₁.

Deficiency of oxygen (9) or heme (10) also causes the loss of some,

*J. Saltzgaber and G. Schatz, unpublished observation.
but not all subunits of cytochrome c oxidase from yeast mitochondria. As in the case of cytochrome c1 it is not known whether the subunits are no longer synthesized or whether they are improperly assembled and rapidly degraded by proteases. If the latter case is correct, the loss of some subunits, but not of others, could simply mean that some subunits are more susceptible to proteolytic degradation than others. This could perhaps also explain why heme-deficient Escherichia coli cells still accumulate their apocytochromes (41, 42) whereas bacteriochlorophyll-deficient Rhodopseudomonas spheroides cells appear to lack several reaction center proteins (43).

The identity of the "cytochrome c1-like protein" detected in whole cell extracts of heme-deficient or anaerobically grown yeast cells remains an intriguing enigma. On the one hand, this polypeptide is specifically immunoprecipitated by cytochrome c1-specific antisera but not by preimmune sera and exhibits the molecular weight of partly proteolyzed cytochrome c1. These facts, and the localization of the protein in the high speed supernatants (not shown) suggest that we have isolated an apoprotein precursor of cytochrome c1. On the other hand, this protein is also found in high speed supernatants of aerobiologically grown wild type cells. Furthermore, "chase" experiments with oxygen-adapting yeast cells (cf. above) failed to reveal a role of this protein as a cytochrome c1 precursor. Even though this question remains open, the soluble cross-reacting material is of sufficient interest to justify further scrutiny.

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