Cyc2p, a Membrane-bound Flavoprotein Involved in the Maturation of Mitochondrial c-Type Cytochromes* 8

Delphine G. Bernard‡, Sophie Quevillon-Cheruel†, Sabeeha Merchant‡, Bernard Guiard‡1, and Patrice P. Hamel1,2
From the 1Department of Chemistry and Biochemistry, UCLA, Los Angeles, California 90095-1569, 6Centre de Génétique Moléculaire, CNRS, 91198 Gif-sur-Yvette, France and 7Institut de Biochimie et de Biophysique Moléculaire et Cellulaire, Université Paris-Sud, Orsay 91405, France

Mitochondrial apocytochrome c and c1 are converted to their holoforms in the intermembrane space by attachment of heme to the cysteines of the CXXCH motif through the activity of assembly factors cytochrome c heme lyase and cytochrome c1 heme lyase (CCHL and CC1HL). The maintenance of apocytochrome sulphydryls and heme substrates in a reduced state is critical for the ligation of heme. Factors that control the redox chemistry of the heme attachment reaction to apocytochrome c are known in bacteria and plastids but not in mitochondria. We have explored the function of Cyc2p, a candidate redox cytochrome c assembly component in yeast mitochondria. We show that Cyc2p is required for the activity of CCHL toward apocytochrome c and c1, and becomes essential for the heme attachment to apocytochrome c1 carrying a CAPCH instead of CAACH heme binding site. A redox function for Cyc2p in the heme lyase reaction is suggested from 1) the presence of a non- covalently bound FAD molecule in the C-terminal domain of Cyc2p, 2) the localization of Cyc2p in the inner membrane with the FAD binding domain exposed to the intermembrane space, and 3) the ability of recombinant Cyc2p to carry the NADPH-dependent reduction of ferricyanide. We postulate that, in vivo, Cyc2p interacts with CCHL and is involved in the reduction of heme prior to its ligation to apocytochrome c by CCHL.

Cytochromes define a large and structurally diverse class of heme containing proteins that are active in electron transfer (1, 2) or catalysis of oxidoreduction reactions. Since they were first recognized in the 1920s, cytochromes have been the focus of intense investigation from mechanism to structure and now to the details of their maturation. Type c cytochromes are a distinct subgroup of cytochromes characterized by a covalently attached protoporphyrin IX prosthetic group that is linked via thioether bonds to cysteine residues in a CXXCH motif (where X can represent any amino acid except cysteine), also referred to as a heme binding site (1–3). Besides their well recognized role in bioenergetic redox chemistry, c-type cytochromes can also be recruited to function in antioxidative pathways, signaling, and regulation (4). This diversity in terms of function is paralleled by a diversity in terms of assembly pathways and a surprising finding was the discovery that three pathways (Systems I–III) for the biogenesis of c-type cytochromes have evolved in bacteria, plastids, and mitochondria (for reviews, see Refs. 1, 2, and 4–6). The definition of the three systems is based on the occurrence of specific assembly components that are unique to each maturation pathway (4). The basis for such a diversity is not obvious, because it appears that the biochemistry to complete holocytochrome c formation is the same regardless of the assembly system under study (4). The biochemical requirements for holocytochrome c formation can be divided into functions needed for the transport and delivery of heme, the reduction of apoprotein cysteinyl thiol and heme co-factor prior to the heme ligation reaction, and the catalysis of thioether bond formation (4). This view was substantiated by the genetic and biochemical analysis of Systems I and II in bacteria and plastids, which led to the identification of multiple assembly factors with proposed or established activity in the transport and chaperoning of heme (4, 7–9), the provision of reducing equivalents for maintenance of reduced apocytochromes/heme substrates (4, 10–15), and the heme ligation reaction (8, 9, 16).

Mitochondrial c-type cytochromes from fungi, green algae, and animals are assembled through System III, but despite saturating genetic screens in fungal experimental models such as Saccharomyces cerevisiae (reviewed in Refs. 17 and 18), the mechanisms of heme delivery and how redoxantants are supplied to the site of assembly have escaped identification and remain so far completely unknown in this pathway. The composition of System III appears unexpectedly simple in the context of the known biochemical requirements for compartmentalized holocytochrome c formation. Either a pair of related proteins, the so-called cytochrome c and cytochrome c1 heme lyase (CCHL and CC1HL), or a single cytochrome c heme lyase, also named holocytochrome c synthase, is needed to attach heme to the apoforms of soluble cytochrome c and membrane-bound cytochrome c1 in the mitochondrial IMS (19, 20). The occurrence of distinct CCHL and CC1HL, originally described to display strict specificity toward their respective apocytochrome c and c1 substrates (19, 21, 22), seems to be restricted to fungi and green algae (23). A recent re-investigation of the heme lyase substrate specificity in yeast showed that whereas CC1HL activity toward cytochrome c1 is strict, CCHL is able to act on both apocytochrome c and c1 substrates (23). On the other hand, the single heme lyase (HCCS) present in animals is able to assemble both cytochrome c and c1 (23). Despite considerable effort to orient the reconstitution of the cytochrome c and c1 heme lyase reaction in isolated mitochondria (19, 24–29) or with partially purified enzyme (27, 30), and despite the demonstration that heme and apocytochromes c and c1 substrates interact with the CCHL and CC1HL diversity in terms function is paralleled by a diversity in terms of assembly pathways and a surprising finding was the discovery that three pathways (Systems I–III) for the biogenesis of c-type cytochromes have evolved in bacteria, plastids, and mitochondria (for reviews, see Refs. 1, 2, and 4–6). The definition of the three systems is based on the occurrence of specific assembly components that are unique to each maturation pathway (4). The basis for such a diversity is not obvious, because it appears that the biochemistry to complete holocytochrome c formation is the same regardless of the assembly system under study (4). The biochemical requirements for holocytochrome c formation can be divided into functions needed for the transport and delivery of heme, the reduction of apoprotein cysteinyl thiol and heme co-factor prior to the heme ligation reaction, and the catalysis of thioether bond formation (4). This view was substantiated by the genetic and biochemical analysis of Systems I and II in bacteria and plastids, which led to the identification of multiple assembly factors with proposed or established activity in the transport and chaperoning of heme (4, 7–9), the provision of reducing equivalents for maintenance of reduced apocytochromes/heme substrates (4, 10–15), and the heme ligation reaction (8, 9, 16).
(18, 28, 31–33), the enzymology of the heme attachment reaction is currently not known.

The existence of well defined reductant delivery mechanisms, candidate heme transporter, and chaperone in Systems I and II suggests that cyc2 mutations do not solely affect cytochrome c but also impact other unrelated mitochondrial processes led to the perception that Cyc2p acts as a "general" factor for mitochondrial biogenesis (35, 36). The recent reisolation of the CYC2 chromosomal locus by transformation of the W303-1B strain. Yeast cells were transformed by the lithium acetate method. The YPH6-9C is a spore issued from the cross between UV34 and YPH1.

**Construction, Manipulation, and Growth of Yeast Strains**

**TABLE ONE**

| Genotype and sources of yeast strains |
|--------------------------------------|
| **Strain** | **Genotype** | **Source** |
| W303-1A | a ade2-1 his3-11,15 leu2-3,112 trpl-1 ura3-1 can1-100 | R. Rothstein* |
| W303-1B | a ade2-1 his3-11,15 leu2-3,112 trpl-1 ura3-1 can1-100 | R. Rothstein* |
| SMY1 | a ade2-1 his3-11,15 leu2-3,112 trpl-1 ura3-1 can1-100 cyt2::his5* | Ref. 23 |
| YPH1 | a ade2-1 his3-11,15 leu2-3,112 trpl-1 ura3-1 can1-100 cyc2::3ph | Ref. 23 |
| UV48 | a ade2-1 his3-11,15 leu2-3,112 trpl-1 ura3-1 can1-100 CYT1-48 cyt2::his5* | Ref. 23 |
| UV34 | a ade2-1 his3-11,15 leu2-3,112 trpl-1 ura3-1 can1-100 CYT1-34 cyt2::his5* | Ref. 23 |
| YCT1-7D | a ade2-1 his3-11,15 leu2-3,112 trpl-1 ura3-1 can1-100 CYT1-34 cyc2::3ph | This study* |
| YDR8 | a ade2-1 his3-11,15 leu2-3,112 trpl-1 ura3-1 can1-100 CYT1-48 cyc2::3ph | This study* |
| YPH10-8A | a ade2-1 his3-11,15 leu2-3,112 trpl-1 ura3-1 can1-100 CYT1-48 | This study* |
| YPH6-9C | a ade2-1 his3-11,15 leu2-3,112 trpl-1 ura3-1 can1-100 CYT1-34 cyc2::3ph | This study* |
| YPH71-14B | a ade2-1 his3-11,15 leu2-3,112 trpl-1 ura3-1 can1-100 CYT1-34 | This study* |
| YDB2 | a ade2-1 his3-11,15 leu2-3,112 trpl-1 ura3-1 can1-100 CYC2-(HA)-HIS3 | This study* |

*Department of Human Genetics, Columbia University.

**Enzymatic Activities**—NAD(P)H-ferricyanide reductase activities were assayed at 25 °C in 50 mM Tris-Cl, pH 7.5, in the presence of 400 mM ferricyanide and 2.5 mM recombinant soluble Cyc2p-His6. The reduction of ferricyanide was monitored by measuring the decrease of absorbance at 340 nm in a Cary 400 spectrophotometer. Kinetic param-
A Cytochrome c Assembly Flavoprotein

eries were determined by measuring initial velocities at different sub-
strate concentrations. Rates were calculated using a Δε of 1.04 mm−1
cm−1 for the conversion of ferricyanide. NADH and NADPH were
obtained from Sigma.

Identification of the Flavin Cofactor—Recombinant Cyc2p-His6 pro-
tein was denatured in 4.5 M guanidine chloride and analyzed by reversed
phase HPLC using negative electrospray and coupled to mass spectrom-
etry. Pure FAD and FMN, obtained from Sigma, were treated in the
same fashion and used as mass standards.

Production of Antibodies—For generation of anti-Cyc2p and anti-
CCHL antisera, rabbits were injected with native Cyc2p (prepared as
described above) or denatured CCHL prepared according to QIAex-
press purification protocol (Qiagen), respectively. As expected, both
anti-Cyc2p and anti-CCHL antibodies immunoreact against mitocondri-
dal proteins of ~40 and 33 kDa, respectively (see Fig. 3; data not
shown).

Protein Preparation and Analysis—Mitochondria were purified from
yeast grown in galactose medium as described earlier (45), and the mito-
chondrial protein concentration was determined using the Bradford
reagent (Sigma) or the Coomassie protein assay reagent (Pierce). Pro-
tein samples were analyzed through lithium dodecyl sulfate-PAGE
(4 °C) or SDS-PAGE (room temperature) and subsequently immobi-
lized by electrophoresis to polyvinylidene difluoride or nitrocellulose
membranes (0.45 mm). For heme staining of mitochondrial c-type cyto-
chromes, samples were reduced with dithiothreitol on ice for 30 min
and separated by lithium dodecyl sulfate-PAGE at 4 °C (46). The heme-
associated peroxidase activity was revealed directly on membrane-
transferred c-type cytochromes by the enhanced chemiluminescence
method from Pierce. Polyclonal antibodies raised against CCHL, Cyc2p
(this work), cytochrome b2, AAC, Hsp60 (Dr. C. Meisinger, Freiburg,
Germany), and cytochrome c1 (Dr. C. Lemaire, Gif-sur-Yvette, France),
and monoclonal antibody against HA epitope (Santa Cruz Biotechnol-
ogy, Inc., Santa Cruz, CA) and porin (Molecular Probes, Inc., Eugene,
OR) were used for immunodetection of immobilized proteins. Bound
antibodies were detected by horseradish peroxidase-conjugated sec-
ondary antibodies.

Biochemical Methods—Hypotonic swelling of mitochondria, sub-
sequent treatment with protease K, and carbonate extraction were per-
formed as described (47), except that trichloroacetic precipitation was
omitted. When indicated, mitochondria were treated with 30 or 60
mg/ml protease K for 20 min on ice. After the addition of phenylmethyl-
sulfonyl fluoride and 10-min incubation on ice, mitochondria were
reisolated after centrifugation at 15,000 × g for 30 min and resuspended
in sample buffer for SDS-PAGE analysis. Mitochondria were sonicated
on ice (Bioblock Scientific, 3 × 30 s, duty cycle 40%, output 5), and the
soluble fraction was separated from the membrane fraction by centri-
fugation at 100,000 × g for 1 h at 4 °C. For carbonate extraction, mito-
chondria were treated with 0.1 M Na2CO3, pH 11.5 or 10.8 (adjusted
with HCl), incubated for 20 min on ice, and subjected to centrifugation
at 100,000 × g for 1 h at 4 °C. Total (T), pellet (P), and supernatant (S)
fractions were then analyzed by SDS-PAGE. For submitochondrial frac-
tionation, 8 mg of mitochondria were resuspended in 4 ml of swelling
buffer (20 mM Hapes/KOH, pH 7.4, 0.5 mM EDTA, and 1 mM phenyl-
methylsulfonyl fluoride). After 30 min incubation on ice, sucrose was
added to 0.45 M final concentration, and mitochondria were further
incubated for 10 min on ice. After sonication (Bioblock Scientific, 3 ×
30 s, duty cycle 80%), the remaining intact mitochondria and large frag-
ments were removed by centrifugation (15,000 × g, 10 min). The super-
natant was centrifuged at 200,000 × g for 45 min at 4 °C. The pellet was
resuspended in 300 µl of buffer containing 5 mM Hapes/KOH, pH 7.4,

10 mM KCl, 1 mM phenylmethylsulfonyl fluoride. After a clarifying spin
(15,000 × g, 10 min), the supernatant was loaded onto a discontinuous
sucrose gradient (made with 1.4 ml of 55%, 5.1 ml of 46%, 2.3 ml of 38%,
and 1.4 ml of 29% (w/v) sucrose in 10 mM KCl, 1 mM phenylmethyl-
sulfonyl fluoride). After a clarifying spin (100,000 × g, 16 h, 4 °C), 700-ml fractions
were collected from bottom to top.

RESULTS

Cyc2p Is Required for the Activity of CCHL toward Cytochrome c and
Cytochrome c1—Cyc2p was originally described as an accessory cyto-
chrome c assembly factor on the basis that strains carrying mutations in
the CYC2 gene accumulate at least 10% of the wild type complement
of holocytochrome c (34–36). A Δcycc2 mutant displays a very slight
respiration-deficient growth phenotype, consistent with the observation
that only levels of holocytochrome c below 5% of the normal level will
impact appreciably the respiratory growth. The partial deficiency in
holocytochrome c cannot be attributed to loss of its cognate assembly
factor, since the abundance of CCHL does not appear to be affected by
the absence of Cyc2p (Fig. 1). Note that the accumulation of wild type
level of holocytochrome c1 in the Δcycc2 mutant indicates that Cyc2p is
not required for the CC;HL-dependent assembly of cytochrome c1
(Fig. 1).

We have previously established that CCHL exhibits intrinsic activity
toward apocytochrome c1 and is able to promote low level of holocy-
тоchrome c1 formation in the Δcc;hl mutant (Fig. 1) (see Ref. 23). The fact
that the Δcycc2 Δcc;hl mutant is no longer able to assemble holocyto-
ochrome c1 indicates that Cyc2p is also required for the activity of CCHL
toward apocytochrome c1 (Fig. 1) and accounts for the synthetic respi-
ration-deficient phenotype of the Δcc;hl Δcycc2 mutant (Fig. 1) (see Ref.
23). Immunoblotting analysis showed that, similar to the Δcycc2 mutant,
the accumulation of CCHL is not modified in the absence of both Cyc2p
and CC;HL. This latter result solidifies the view that Cyc2p modulates the
activity of CCHL in the assembly process (23). We concluded that
Cyc2p is required for the CCHL-dependent assembly of cytochrome c
and cytochrome c1 but not for the assembly of cytochrome c1 when
alyzed by CC;HL.

Cyc2p Becomes Essential for Respiration When Cytochrome c1 Carries
a Mutant CAPCH Heme Binding Site—A previous hunt for suppressors
for which holocytochrome c1 assembly was enhanced in the absence of

FIGURE 1. Cyc2p is required for CCHL function. Heme stain of mitochondrial c-type cytochromes. 70 µg of mitochondrial protein from wild type (WT) (W303-1B), SMY1 (Δcc;hl), YCT1-7D (Δcycc2 Δcc;hl), and YPH1 (Δcycc2) strains grown at 28 °C in galactose medium were analyzed for the abundance of the holoforms of cytochrome c and cyto-
ochrome c1. Dithiothreitol-treated samples were separated by electrophoresis in a 12%
lithium dodecyl sulfate-polyacrylamide gel at 4 °C. The level of holocytochromes c and c1 was assessed by heme staining. CCHL and cytochrome b2 (loading control) were detected by immunodecoration.
CC1HL uncovered nuclear dominant mutations in the CYT1 gene (23). During the genetic analysis of the suppressed strains, we discovered that the CYT1–34 and CYT1–48 suppressor mutations that both map to the heme binding site of cytochrome c1 (22) displayed a distinct respiratory phenotype in combination with a Δcyt2 allele (not shown). Whereas an alanine to proline substitution in CYT1–34 (CAPCH) results in a complete respiratory block in a Δcyt2 background, the same alanine, when mutated to aspartic acid in CYT1–48 (CADCH), does not cause any visible phenotype (Fig. 2). The loss of respiratory competence in the Δcyt2 cyt1–34 is a synthetic phenotype, because a strain carrying the CYT1–34 mutation in another otherwise wild type context is respiration-proficient and only slightly affected for growth compared with a wild type strain (Fig. 2). We also verified that transformation of the Δcyt2 cyt1–34 mutant with a plasmid carrying the wild type CYT1–34 gene restores the ability to grow on respiratory substrates (not shown).

Spectral and heme staining analyses showed that there is a dramatic loss of holocytochrome c1 in the Δcyt2 cyt1–34 mutant (Fig. 3) but no visible change in the steady state abundance of both CCHL and CC1HL (Fig. 3B; data not shown). Consistent with the slow respiratory growth phenotype, decreased levels of holocytochrome c1 are detected in a strain carrying the CYT1–34 mutation in another otherwise wild type background (Fig. 3). As expected, the CYT1–48 mutation does not affect the assembly of holocytochrome c1 and could not be distinguished from the CYT1 allele, either in an otherwise wild type background or when combined with the absence of Cyc2p (Fig. 3). We conclude from this study that Cyc2p becomes essential for the assembly of cytochrome c1 with a CAPCH heme binding site.

Cyc2p Is a Flavoprotein with a Redox Active Co-factor—We have hypothesized that Cyc2p might be a flavoprotein, because sequence analysis revealed an FAD binding fold typical of the ferredoxin reductase family (48) in the C-terminal part of the protein (supplemental Fig. 1). In order to test this hypothesis, we engineered a hexahistidinyl-tagged version of the C-terminal domain of the protein for expression in E. coli. This domain was chosen, because it is predicted to be soluble and includes the entire FAD binding fold (supplemental Fig. 1). The recombinant protein was found to be mostly soluble when produced at 20 °C and could be further purified to homogeneity using metal chelate chromatography (data not shown). Spectroscopic analysis was performed on purified Cyc2p-His₆ to detect absorbance peaks at around 382 and 454 nm, indicative of bound FAD. Bound FAD exhibits small but distinct spectroscopic differences from free FAD (49–51). As shown in Fig. 4A, the recombinant Cyc2p-His₆ displays a spectrum typical of a flavoprotein with a maximum absorbance peak shifted by 6 nm compared with free FAD. Because FAD and FMN are not easily distinguishable on the basis of their spectroscopic properties, we decided to confirm the identity of the flavin group by mass spectroscopy.

As the predicted FAD-binding fold present in Cyc2p suggests noncovalent binding of the cofactor (48), purified Cyc2p-His₆ was treated with 4.5 M guanidine chloride in order to liberate the flavin group. The mass of the cofactor was then determined by mass spectrometric analysis after purification via HPLC and compared with the mass of pure FAD or pure FMN in order to discriminate between the two flavin cofactors. From the data obtained by mass spectrometry, we were able to establish unambiguously that the cofactor bound to the C-terminal domain of Cyc2p is FAD (Fig. 4B). The cofactor to protein molar ratio was found to be ~1:1 indicative of a stoichiometry of one bound molecule of FAD per molecule of Cyc2p (not shown).

The presence of a flavin molecule in Cyc2p prompted us to examine the functionality of the cofactor in an in vitro assay. Our experimental design was driven by the proposed model that Cyc2p operates in a reducing pathway in cytochrome c assembly and therefore exhibits redox activity (23). A standard demonstration of the redox activity of a

---

**A Cytochrome c Assembly Flavoprotein**

---

**FIGURE 2.** Respiratory growth of cytochrome c assembly mutants. Dilution series of wild type (WT) (W303-1A), cyt1–34 (YPH71-14B), cyt1–48 (YPH10-8A), Δcyt2 (YPH11), ΔΔcyt2 cyt1–34 (YPH6-9C), ΔΔcyt2 cyt1–48 (YPHDB8), ΔΔcyt2 (SMY1), ΔΔΔcyt2 cyt1–34 (UV34), and ΔΔΔcyt2 cyt1–48 (UV48) strains were grown on medium containing fermentable (glucose) or respiratory (glycerol) substrates and incubated for 3 days at 28 or 36 °C.

**FIGURE 3.** Loss of holocytochrome c1 assembly in the ΔΔcyt2 cyt1–34 mutant. A, cytochrome c absorption spectra. Low temperature absorption spectra of cells (see Fig. 2) grown in galactose were recorded with a Cary 400 spectrophotometer as already described (65). The arrows indicate the absorption maxima of the a bands of cytochromes c (546 nm), c1 (552 nm), b (558 nm), and a + a₃ (602 nm). Note that cytochromes a₉c and cytochrome c oxidase are spectrally detectable in the ΔΔcyt2 cyt1–34 mutant that still assembles a residual level of holocytochrome c (see B). Only low levels of holocytochrome c1 are required to maintain assembly of cytochrome c oxidase (66, 67). B, heme stain of mitochondrial c-type cytochromes. 6 µg of mitochondrial protein from cells grown at 28 °C in galactose medium were analyzed for the abundance of the holoforms of cytochrome c and cytochrome c1. Dithiothreitol-treated samples were separated in a 12% lithium dodecyl sulfate-polyacrylamide gel at 4 °C. The level of holocytochrome c and c1 was assessed by heme staining. CCHL, Cyc2p, and cytochrome b₂ (loading control) were detected by immunodetection. WT, wild type.
flavoprotein is the assay for the NAD(P)H-dependent reduction of an artificial electron acceptor like ferricyanide (52, 53). As shown in TABLE TWO, recombinant Cyc2p is able to catalyze the reduction of ferricyanide when NADH or NADPH is used as an electron donor in the reaction. This result is in accord with the presence of a consensus binding site for NAD(P)H in Cyc2p (supplemental Fig. 1). The determination of the kinetic parameters indicates that Cyc2p has the same maximum velocity in the NADH- or NADPH-dependent reduction of ferricyanide but has a much higher affinity for NADPH versus NADH as the donor substrate (TABLE TWO).

Based on our results, we concluded that the C-terminal domain of Cyc2p binds one molecule of FAD co-factor and displays redox activity in an in vitro assay.

Cyc2p Is a Mitochondrial Inner Membrane Protein with FAD Binding Domain in the IMS—One key question in terms of deducing Cyc2p function in the heme lyase pathway is to define its topological arrangement, particularly with respect to the FAD binding domain. The predicted monotopic arrangement with a matrix-side N terminus and IMS-side C-terminal FAD-containing domain is compatible with the known location of CCHL in the IMS (31, 54) and the proposed model for redox function of Cyc2p (23). In order to verify this model experimentally, an HA tag was engineered by PCR at the C terminus of Cyc2p. The strain expressing Cyc2p-HA displayed a wild type cytochrome absorption spectrum, showing that the tagged protein is functional (not shown). Using an anti-HA antibody, we determined that Cyc2p-HA is associated with the membrane fraction in purified mitochondria and co-fractionates with AAC, a mitochondrial inner membrane embedded carrier (Fig. 5A). Cyc2p association to the membrane was further examined by carbonate extraction, a standard protocol that serves for the separation of integral from peripherally associated membrane proteins. Cyc2p could be mostly extracted by carbonate treatment at pH 11.5, whereas cytochrome c1 and AAC, a monotopic and polytopic membrane protein, respectively, which are tightly anchored to the membrane, are resistant to carbonate extraction (Fig. 5B). Interestingly, the complete extractability of CCHL from mitochondrial membranes at pH 10.8 indicates that, like Cyc2p, the protein is peripherally associated to the inner membrane as already reported in former studies (55). By contrast, Cc1HL behaves like an integral membrane protein and is completely resistant to carbonate extraction (data not shown) (55). To identify the

| Substrate | \( V_{\text{max}} \) (\( \mu \text{mol substrate/min/\( \mu \text{mol enzyme} \))} | \( K_m \) (\( \mu \text{M} \)) | \( V_{\text{max}}/K_m \) (\( \text{M}^{-1}\text{M} \)) |
|-----------|---------------------------------|---------------------|-------------------------------|
| NADH      | 0.11                            | \( 3 \times 10^4 \)  | \( 3.7 \times 10^{-6} \)     |
| NADPH     | 0.13                            | \( 10^2 \)        | \( 1.4 \times 10^{-3} \)     |
mitochondrial membranes with which Cyc2p is associated, we performed a mitochondrial subfractionation under conditions that allow separation of the inner and outer membranes. Immunoblotting with anti-porin antibody enabled the identification of the outer membrane enriched fractions (Fig. 5C). Fractions containing the inner membranes were revealed with antibodies against AAC and CCHL, which are known inner membrane resident proteins (Fig. 5C). Immunodetection of Cyc2p-HA in the different fractions shows that its distribution matches closely that of AAC and CCHL. This suggests that like CCHL, Cyc2p is bound to the inner membrane of the mitochondria. To assess sublocalization of the FAD binding domain of Cyc2p, HA epitope exposure studies by limited proteolysis were carried out in isolated mitochondria. The HA tag was found to be accessible to degradation by added proteinase K, similarly to cytochrome b2, an IMS resident protein (Fig. 5D). As a control, we verified that matrix located Hsp60 was protected from proteolytic degradation by proteinase K. This result enabled us to deduce that the FAD binding domain of Cyc2p is exposed to the IMS. Taken together, these results established that Cyc2p is localized in the mitochondrial inner membrane with the FAD binding domain in the IMS.

**DISCUSSION**

Numerous in organello and in vitro studies on the assembly of mitochondrial cytochrome c have established that the chemistry of thioether bond formation can only proceed when the appropriate redox conditions are provided (i.e. apocytochrome c sulfhydryls and heme substrates are maintained reduced prior to the ligation of heme) (24, 29, 56–58), yet the fact that no mitochondrial cytochrome c assembly proteins besides the so-called heme lyases have been identified led to the assumption that in vivo the redox chemistry of the heme lyase reaction was not under the control of dedicated factors and occurred spontaneously in the “reducing” environment of the IMS. In this paper, we have challenged this thinking through the study of Cyc2p, a cytochrome c assembly component. We provide evidence that Cyc2p may fulfill a redox function in the heme lyase reaction based on the finding that the protein is (i) required for the activity of CCHL toward cytochrome c and cytochrome c1, (ii) carries a FAD cofactor and exhibits diaphorase activity in vitro, and (iii) localizes to the inner membrane with the FAD binding site exposed to the IMS.

*Cyc2p, a Partner of CCHL in the Heme Lyase Reaction—* Detailed phenotypic analysis of cyc2 mutants led to the view that Cyc2p is not strictly necessary for the maturation process of mitochondrial c-type cytochromes (34–36). Indeed, in a Δcyc2 mutant, holocytochrome c formation still takes place, albeit less effectively, and holocytochrome c1 assembly proceeds normally (Fig. 3). However, we found that in certain conditions, the activity of Cyc2p becomes critical for the assembly of c-type cytochromes. First, in the absence of CC1HL, CCHL can only assemble cytochrome c1 when Cyc2p is present (Fig. 1). The dependence of CCHL upon Cyc2p does not seem to be restricted to the cytochrome c1 substrate, since holocytochrome c1 accumulation is significantly more affected in a Δcc1hl Δcyc2 strain than in a Δcyc2 mutant (Fig. 3A). Note that in the absence of both CC1HL and Cyc2p, holocytochrome c assembly is not completely abolished, and some residual level of holocytochrome c1 still accumulates (Fig. 1). We have verified that the abundance of CCHL is not modified in a Δcc1hl Δcyc2 mutant, and it is likely that Cyc2p exerts its effect on cytochrome c maturation by modulating the activity of CCHL (Fig. 1). Second, we have also observed that in a Δcc1hl mutant, enhanced activity of CCHL toward apocytochrome c1 through missense mutations in cytochrome c1, or CCHL or overexpression of CCHL is strictly dependent on the participation of Cyc2p (23). Thus, it appears that Cyc2p becomes essential for c-type cytochrome maturation when CCHL is the only lyase promoting the assembly of both holocytochrome c and c1. Third, intriguingly, whereas Cyc2p is not needed for the assembly of cytochrome c1 with a wild type or CADCH heme binding site (CYT1–48), it is absolutely required when cytochrome c1 carries a CAPCH heme binding site (CYT1–34). The concomitant loss of holocytochrome c and c1 assembly in the absence of Cyc2p and the presence of the CYT1–34 mutation is somehow very similar to the phenotype displayed by the Δcc1hl Δcyc2 mutant (Fig. 3A) and suggests that both CCHL and CC1HL activities are severely compromised in the Δcyc2 cyt1–34 strain.
A Cytochrome c Assembly Flavoprotein

We think that the CYT1–34 mutation, which was selected to increase the affinity of CCHL toward cytochrome c, could act by favoring the interaction of apocytochrome c with CCHL to the detriment of its interaction with CCc HL, its cognate lyase. Loss of assembly of both cytochrome c and cytochrome c1 in the Δcytc2 cytl–34 mutant can therefore be explained by the fact that, similarly to the Δccc hl Δcytc2 mutant, Cytc2p is essential when the assembly of both cytochrome c and c1 relies solely on CCHL. This hypothesis is further solidified by the observation that CCc HL, when overexpressed from a multicopy plasmid alleviates the respiratory deficiency of the Δcytc2 cytl–34 mutant (data not shown). We believe that in this situation, overexpression of CCc HL outcompetes CCHL in the interaction with apocytochrome c, which can be converted to its holoform by action of its cognate lyase. As expected, overexpression of CCHL in the Δcytc2 cytl–34 mutant did not restore the respiratory proficiency (data not shown).

At present, there is no straightforward answer as to why the CYT1–48 mutation which, like CYT1–34 was selected to enhance the affinity of CCHL for cytochrome c1, behaves differently than CYT1–34 in the absence of Cytc2p (Fig. 3). One possibility is that the affinity of CCHL for cytochrome c1 is higher when it carries a CAPCH (CYT1–34) instead of a CADCH heme binding (CYT1–48). Notably, the CYT1–34 and CYT1–48 mutations alter the same alanine residue that lies in between the two cysteines of the CXXCH motif. It is known that between cysteine mutations in CXXC motifs of redox proteins such as thioredoxin DsA or protein–disulfide isomerase affect the reactivity in thiol–disulfide exchange (59–61). It is thus conceivable that the CAPCH and CADCH heme binding sites of cytochrome c1 differ as to the reactivity of their cysteine thiols in the heme attachment reaction.

Based on the results presented in this study, we favor a model where Cytc2p is a partner of CCHL and becomes critical for the maturation process of cytochrome c and c1 when CCc HL is absent or unable to act on its cognate substrate. The localization of both CCHL and Cytc2p in the inner membrane with domains of the proteins exposed to the IMS is compatible with such a model (Fig. 6B).

A Putative Heme Reductase Activity for Cytc2p?—Pioneer studies have established that the reduction of heme in the in organello heme lyase reaction is mediated through the action of pyridine nucleotides and a flavin-linked electron transfer (24, 57). However, the identity of the electron donor, whether NADPH or NADH, still remains unclear. Our discovery that Cytc2p harbors a bound FAD in a domain that localizes to the mitochondrial IMS and exhibits NAD(P)H-ferredoxin oxidoreductase activity in vitro suggests that in vivo, Cytc2p could function in reducing heme prior to its ligation by CCHL. The greater affinity of Cytc2p for the NADPH substrate in vitro makes it likely that NADPH acts as the electron donor to Cytc2p in the mitochondrial IMS. Because the requirement for reductants applies to both heme and apocytochrome c substrates, it is conceivable that Cytc2p reduces both the cofactor and the apocytochrome c sulfhydryls in vivo. However, based on the similarity of Cytc2p with cytochrome b5 reductase-like proteins that reduce heme in cytochrome b5 (supplemental Fig. 2), we favor a model where Cytc2p catalyzes the NADPH-dependent reduction of the heme co-factor only (Fig. 6A). In such a model, heme is handled by CCHL through the heme regulatory motif after its transport from the matrix across the inner membrane, maintained reduced through the activity of Cytc2p and ligated to the apocytochrome c substrate by action of CCHL (Fig. 6B). Additional experimental work is required to establish whether heme and/or apocytochrome c are the relevant targets of action of Cytc2p in vivo.

If Cytc2p is active as a heme and/or apocytochrome reductase in cytochrome c maturation, its function is clearly redundant based on the observation that a Δcytc2 mutant is still able to assemble holocytochrome c (Fig. 3). The function performed by Cytc2p appears essential only when the assembly of both cytochrome c and c1 is dependent upon CCHL. It is possible that the activity of Cytc2p becomes limiting for the assembly process when two apocytochrome substrates instead of one need to be converted to their holoforms by CCHL. This hypothesis is supported by our initial discovery that CYC2 acts as a multicopy suppressor of the absence of CCc HL by enhancing holocytochrome c1 formation (23). Other genes carrying a CYC2-like activity could be identified by such a multicopy suppressor approach. However, a saturating multicopy suppressor screen of the Δccc HL mutant fail to identify other genes besides the CYC2 gene (data not shown).

The discovery of a reductase linked to the activity of CCHL also raises the question of the need for a reductase in the CCc HL-dependent assembly of cytochrome c1. Whereas it is clear that Cytc2p exerts no influence on the CCc HL-catalyzed assembly of cytochrome c1, the requirement for NADH and FMN co-factors for the reduction of heme prior to its linkage to apocytochrome c1 in organello (28) speaks for the existence of a distinct system for the reduction of heme. Whether the in vivo redox status of the apocytochrome c1 sulfhydryls is under the control of the same system or a different one is currently unknown.

The recent identification in the Arabidopsis thaliana mitochondria of AtCCMH, a thiol-disulfide oxidoreductase with a postulated function in System I cytochrome c maturation suggests that similar factors might also exist in fungal, animal, and green algal mitochondria that use System III (62, 63). The components of the thiol-metabolizing pathways are not known in the mitochondrial IMS because of the dogma that this compartment is reducing. This topic has now received attention with the discovery that a disulfide exchange system is recruited for the import of a subset of proteins in the mitochondrial IMS (64, 68, 69).

Acknowledgments—We thank S. Gabilly, I. Aboulfath, and B. Dray for technical assistance and Drs. A. Chacinska and C. Meisinger for technical advice. We thank Dr. Denis Pompon for expertise in mass spectrometry and Dr. G. Dujardin for stimulating discussion and critical reading of the manuscript. We are grateful to Dr. Chris Meisinger for the gift of anti-AAC and anti-Hsp60 antibodies and Dr. Lemaire for anti-cytochrome c antisemur.
