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# Cyc2p, a Membrane-bound Flavoprotein Involved in the Maturation of Mitochondrial *c*-Type Cytochromes*[S]

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**Mitochondrial apocytochrome *c* and $c_1$ are converted to their holoforms in the intermembrane space by attachment of heme to the cysteines of the C*XX*CH motif through the activity of assembly factors cytochrome *c* heme lyase and cytochrome $c_1$ heme lyase (CCHL and $CC_1HL$). The maintenance of apocytochrome sulfhydryls 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 $c_1$ and becomes essential for the heme attachment to apocytochrome $c_1$ 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 protoheme IX prosthetic group that is linked via thioether bonds to cysteine residues in a C*XX*CH 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 reductants 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 $c_1$ heme lyase (CCHL[3] and $CC_1HL$), 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 $c_1$ in the mitochondrial IMS (19, 20). The occurrence of distinct CCHL and $CC_1HL$, originally described to display strict specificity toward their respective apocytochrome *c* and $c_1$ substrates (19, 21, 22), seems to be restricted to fungi and green algae (23). A recent reinvestigation of the heme lyase substrate specificity in yeast showed that whereas $CC_1HL$ activity toward cytochrome $c_1$ is strict, CCHL is able to act on both apocytochrome *c* and $c_1$ substrates (23). On the other hand, the single heme lyase (HCCS) present in animals is able to assemble both cytochrome *c* and $c_1$ (23). Despite considerable effort toward the reconstitution of the cytochrome *c* and $c_1$ 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 $c_1$ substrates interact with the CCHL and $CC_1HL$

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1 and 2.

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[3] The abbreviations used are: CCHL, cytochrome *c* heme lyase; $CC_1HL$, cytochrome $c_1$ heme lyase; HA, hemagglutinin; HPLC, high performance liquid chromatography; IMS, intermembrane space; AAC, ADP/ATP carrier.

**TABLE ONE**

**Genotypes and sources of yeast strains**

| Strain | Genotype | Source |
|---|---|---|
| W303-1A | a *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100* | R. Rothstein[a] |
| W303-1B | α *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100* | R. Rothstein[a] |
| SMY1 | *a ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 cyt2::his5*$^+$ | Ref. 23 |
| YPH1 | α *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 cyc2::hph* | Ref. 23 |
| UV48 | *a ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 CYT1-48 cyt2::his5*$^+$ | Ref. 23 |
| UV34 | *a ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 CYT1-34 cyt2::his5*$^+$ | Ref. 23 |
| YCT1-7D | α *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 cyt2::his5*$^+$ *cyc2::hph* | This study[b] |
| YDB8 | *a ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 CYT1-48 cyc2::hph* | This study[c] |
| YPH10-8A | *a ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 CYT1-48* | This study[d] |
| YPH6-9C | *a ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 cyt1-34 cyc2::hph* | This study[e] |
| YPH71-14B | *a ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 CYT1-34* | This study[f] |
| YDB2 | α *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 CYC2-(HA)$_3$-HIS3* | This study[g] |

[a] Department of Human Genetics, Columbia University.
[b] YCT1-7D is a spore issued from the cross between SMY1 and YPH1.
[c] YDB8 was obtained by inactivation of *CYC2* in YPH10-8A.
[d] YPH10-8A is a spore issued from the cross between UV48 and PHT3 (42).
[e] YPH6-9C is a spore issued from the cross between UV34 and YPH1.
[f] YPH71-14B are spores issued from crosses between UV34 and PHT3 (42).
[g] YDB2 was engineered in theW303-1B background.

(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 additional factors might be recruited to complete the maturation of mitochondrial cytochromes *c* in System III. Cyc2p, a mitochondrial protein identified in a screen for yeast mutants deficient for holocytochrome *c* was postulated to be such a factor (34). However, the observation that some *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* gene as a multicopy suppressor of the absence of $CC_1HL$ has stimulated renewed interest in the function of this accessory component in *c*-type cytochrome biogenesis (23). The recognition of an FAD-binding fold in the protein sequence led to the proposal that Cyc2p is a System III assembly factor with a redox function in the maturation of *c*-type cytochromes (23). In this work, we have explored the function of candidate redox component Cyc2p. We discuss the implications of this unique factor in the control of the redox chemistry of the heme lyase reaction.

## EXPERIMENTAL PROCEDURES

*Construction, Manipulation, and Growth of Yeast Strains*—All of the yeast strains used in the course of this study are listed in TABLE ONE. *S. cerevisiae* wild type strains W303-1A and W303-1B are labeled *WT* in Figs. 1–3. Strains carrying null alleles in the *CYC2* or $CC_1HL$[4] genes are labeled Δ*cyc2* and Δ*cc$_1$hl*, respectively, in the figures. Null alleles of yeast genes in the W303 background were constructed using a PCR-based method. The *hphMX4* (37) and *HIS3MX6* (38) modules were chosen to inactivate the wild type copy of the *CYC2* and $CC_1HL$ genes, respectively. A triple histidine tag was engineered at the 3′-end of the *CYC2* gene using plasmid pFA6a-3HA-HIS3MX6 as a template (39) and integrated at the *CYC2* chromosomal locus by transformation of the W303-1B strain. Yeast cells were transformed by the lithium acetate procedure of Schiestl and Gietz (40). The media used for *S. cerevisiae* have been described elsewhere (41–43). Glucose or galactose was used as fermentable carbon source, and glycerol, glycerol/ethanol, ethanol, or lactate was used as respiratory carbon source.

*Plasmid Construction*—DNA manipulations were carried out following published procedures (44). A DNA fragment encoding amino acids $Glu^{53}$–$Ser^{366}$ of Cyc2p was amplified using W303-1A genomic DNA as a template and the following NheI and XhoI engineered oligonucleotides as primers: 5′-GCTAGCTAGCGAGGAGGGAAAACAAACATGAGTTATCTCC-3′; 5′-CCGCTCGAGTGAAATTTATACACATTATCGGAGTTC-3′.

The NheI/XhoI-digested PCR product was cloned into the NheI-XhoI sites of the hexahistidinyl tag vector pET-24a (Novagen). In the same way, the *CCHL* open reading frame was amplified using W303-1A genomic DNA as template and the following NdeI and XhoI engineered oligonucleotides as primers: 5′-GGAATTCCATATGGGTTGGTTTTGGGCAGATCAAAAAAC-3′; 5′-CCGCTCGAGAGGGGCGGAGGACGAAGAGGACGGACC-3′.

The NdeI/XhoI-digested PCR product was cloned into the NdeI-XhoI sites of pET-24a. *Escherichia coli* strain BL21-CodonPlus(DE3)-RIL (Novagen) was used for the expression of recombinant $His_6$-tagged Cyc2p and CCHL proteins.

*Purification of Native Cyc2p-His$_6$—E. coli* BL21 transformants carrying the pET-24a plasmid expressing the Cyc2p-$His_6$ protein were grown at 37 °C to an $A_{600}$ of 0.5 and induced at 20 °C with 1 mM isopropyl 1-thio-β-D-galactopyranoside. Cells were collected 6 h after induction and disrupted by sonication. In these conditions, Cyc2p was found to be mainly soluble in the supernatant after a 15,000 × *g*, 30-min centrifugation. The presence of the hexahistidinyl tag at the carboxyl terminus of the protein allowed rapid purification on nickel-agarose under native conditions in a buffer consisting of 200 mM NaCl, 20 mM Tris-Cl, pH 7.5. The protein was bound to nickel-agarose (Qiagen), eluted with 400 mM imidazole, and further purified on a Superdex75 column. The concentration of the purified enzyme was measured by absorbance at 280 nm, and the purity was assessed by SDS-PAGE.

*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-$His_6$. The reduction of ferricyanide was monitored by measuring the decrease of absorbance at 340 nm in a Cary 400 spectrophotometer. Kinetic param-

[4] For simplification, the *CYC3* gene specifying CCHL and the *CYT2* gene encoding $CC_1HL$ will be referred to as *CCHL* and *CC$_1$HL* genes, respectively, throughout this work.

eters were determined by measuring initial velocities at different substrate concentrations. Rates were calculated using a $\Delta\varepsilon$ 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-$His_6$ protein was denatured in 4.5 M guanidine chloride and analyzed by reversed phase HPLC using negative electrospray and coupled to mass spectrometry. 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 QIAexpress purification protocol (Qiagen), respectively. As expected, both anti-Cyc2p and anti-CCHL antibodies immunoreact against mitochondrial 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 mitochondrial protein concentration was determined using the Bradford reagent (Sigma) or the Coomassie protein assay reagent (Pierce). Protein samples were analyzed through lithium dodecyl sulfate-PAGE (4 °C) or SDS-PAGE (room temperature) and subsequently immobilized by electrotransfer to polyvinylidene difluoride or nitrocellulose membranes (0.45 mm). For heme staining of mitochondrial *c* type cytochromes, 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 $b_2$, AAC, Hsp60 (Dr. C. Meisinger, Freiburg, Germany), and cytochrome $c_1$ (Dr. C. Lemaire, Gif-sur-Yvette, France), and monoclonal antibody against HA epitope (Santa Cruz Biotechnology, 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 secondary antibodies.

*Biochemical Methods*—Hypotonic swelling of mitochondria, subsequent treatment with proteinase K, and carbonate extraction were performed as described (47), except that trichloroacetic precipitation was omitted. When indicated, mitochondria were treated with 30 or 60 mg/ml proteinase K for 20 min on ice. After the addition of phenylmethylsulfonyl 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 centrifugation at 100,000 × *g* for 1 h at 4 °C. For carbonate extraction, mitochondria were treated with 0.1 M $Na_2CO_3$, 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 fractionation, 8 mg of mitochondria were resuspended in 4 ml of swelling buffer (20 mM Hepes/KOH, pH 7.4, 0.5 mM EDTA, and 1 mM phenylmethylsulfonyl 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 fragments were removed by centrifugation (15,000 × *g*, 10 min). The supernatant was centrifuged at 200,000 × *g* for 45 min at 4 °C. The pellet was resuspended in 300 μl of buffer containing 5 mM Hepes/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, 5 mM Hepes/KOH, pH 7.4). After centrifugation (100,000 × *g*, 16 h, 4 °C), 700-ml fractions were collected from bottom to top.

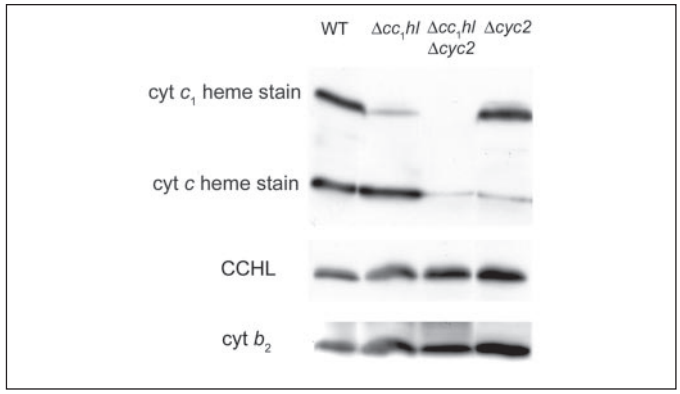


FIGURE 1. **Cyc2p is required for CCHL function.** Heme stain of mitochondrial *c*-type cytochromes. 70 μg of mitochondrial protein from wild type (*WT*) (W303-1B), SMY1 (Δ$cc_1hl$), YCT1-7D (Δ*cyc2* Δ$cc_1hl$), and YPH1 (Δ*cyc2*) strains grown at 28 °C in galactose medium were analyzed for the abundance of the holoforms of cytochrome *c* and cytochrome $c_1$. Dithiothreitol-treated samples were separated by electrophoresis in a 12% lithium dodecyl sulfate-polyacrylamide gel at 4 °C. The level of holocytochromes *c* and $c_1$ was assessed by heme staining. CCHL and cytochrome $b_2$ (loading control) were detected by immunodecoration.

## RESULTS

*Cyc2p Is Required for the Activity of CCHL toward Cytochrome c and Cytochrome $c_1$*—Cyc2p was originally described as an accessory cytochrome *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 Δ*cyc2* 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 $c_1$ in the Δ*cyc2* mutant indicates that Cyc2p is not required for the $CC_1HL$-dependent assembly of cytochrome $c_1$ (Fig. 1).

We have previously established that CCHL exhibits intrinsic activity toward apocytochrome $c_1$ and is able to promote low level of holocytochrome $c_1$ formation in the Δ$cc_1hl$ mutant (Fig. 1) (see Ref. 23). The fact that the Δ*cyc2* Δ$cc_1hl$ mutant is no longer able to assemble holocytochrome $c_1$ indicates that Cyc2p is also required for the activity of CCHL toward apocytochrome $c_1$ (Fig. 1) and accounts for the synthetic respiration-deficient phenotype of the Δ$cc_1hl$ Δ*cyc2* mutant (Fig. 1) (see Ref. 23). Immunoblotting analysis showed that, similar to the Δ*cyc2* mutant, the accumulation of CCHL is not modified in the absence of both Cyc2p and $CC_1HL$. 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 $c_1$ but not for the assembly of cytochrome $c_1$ when catalyzed by $CC_1HL$.

*Cyc2p Becomes Essential for Respiration When Cytochrome $c_1$ Carries a Mutant CAPCH Heme Binding Site*—A previous hunt for suppressors for which holocytochrome $c_1$ assembly was enhanced in the absence of

$CC_1HL$ uncovered nuclear dominant mutations in the *CYT1*[5] 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 $c_1$ (23) displayed a distinct respiratory phenotype in combination with a Δ*cyc2* allele (not shown). Whereas an alanine to proline substitution in *CYT1–34* (CA**P**CH) results in a complete respiratory block in a Δ*cyc2* background, the same alanine, when mutated to aspartic acid in *CYT1–48* (CA**D**CH), does not cause any visible phenotype (Fig. 2). The loss of respiratory competence in the Δ*cyc2 cyt1–34* is a synthetic phenotype, because a strain carrying the *CYT1–34* mutation in an 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 Δ*cyc2 cyt1–34* mutant with a plasmid carrying the wild type *CYT1* or *CYC2* 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 $c_1$ assembly in the Δ*cyc2 cyt1–34* mutant (Fig. 3) but no visible change in the steady state abundance of both CCHL and $CC_1HL$ (Fig. 3*B*; data not shown). Consistent with the slow respiratory growth phenotype, decreased levels of holocytochrome $c_1$ are detected in a strain carrying the *CYT1–34* mutation in an otherwise wild type background (Fig. 3). As expected, the *CYT1–48* mutation does not affect the assembly of holocytochrome $c_1$ 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 concluded from this study that Cyc2p becomes essential for the assembly of cytochrome $c_1$ 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-$His_6$ 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. 4*A*, the recombinant Cyc2p-$His_6$ 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-$His_6$ 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. 4*B*). 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

[5] *CYT1* encodes apocytochrome $c_1$.

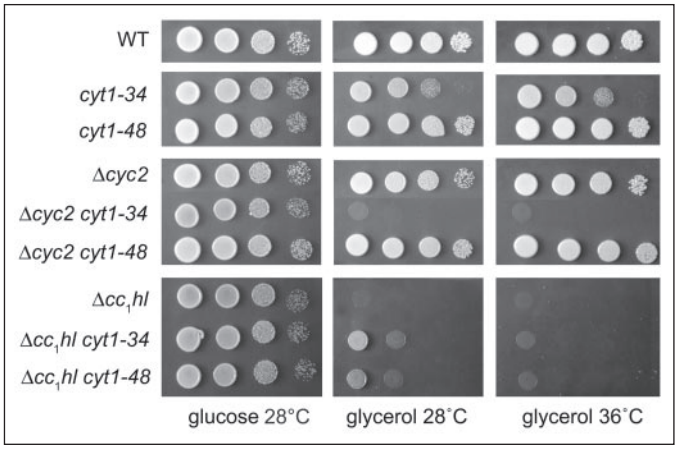


FIGURE 2. **Respiratory growth of cytochrome *c* assembly mutants.** Dilution series of wild type (*WT*) (W303-1A), *cyt1–34* (YPH71-14B), *cyt1–48* (YPH10-8A), Δ*cyc2* (YPH1), Δ*cyc2 cyt1–34* (YPH6-9C), Δ*cyc2 cyt1–48* (YDB8), Δ$cc_1hl$ (SMY1), Δ$cc_1hl$ *cyt1–34* (UV34), and Δ$cc_1hl$ *cyt1–48* (UV48) strains were grown on medium containing fermentable (glucose) or respiratory (glycerol) substrates and incubated for 3 days at 28 or 36 °C.

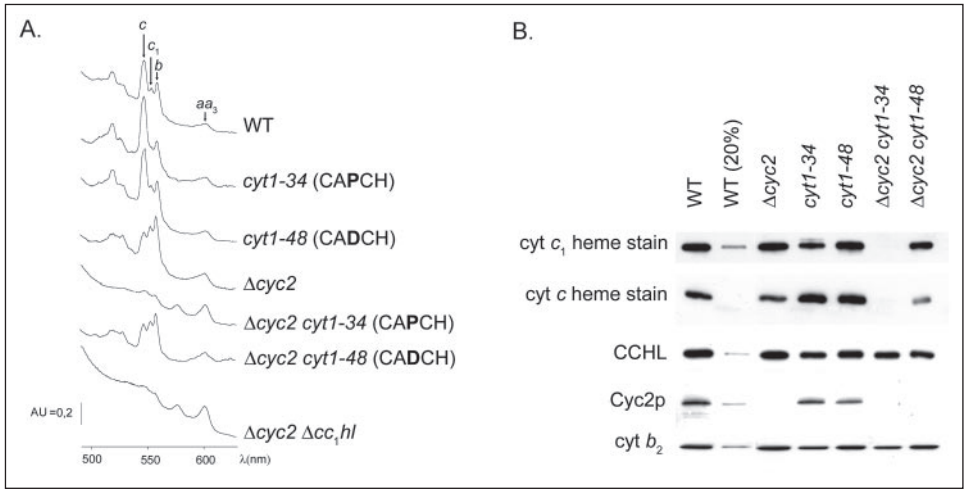


FIGURE 3. **Loss of holocytochrome $c_1$ assembly in the Δ*cyc2 cyt1–34* mutant.** *A*, cytochrome absorption spectra. Low temperature absorption spectra of cells (see Fig. 2) grown in galactose were recorded with a Cary 400 spectrophotometer as already described (65). The *arrows* indicate the absorption maxima of the a bands of cytochromes *c* (546 nm), $c_1$ (552 nm), *b* (558 nm), and $a + a_3$ (602 nm). Note that cytochromes $aa_3$ of cytochrome *c* oxidase are spectrally detectable in the Δ*cyc2 cyt1–34* mutant that still assembles a residual level of holocytochrome *c* (see *B*). Only low levels of holocytochrome *c* 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 $c_1$. Dithiothreitol-treated samples were separated in a 12% lithium dodecyl sulfate-polyacrylamide gel at 4 °C. The level of holocytochrome *c* and $c_1$ was assessed by heme staining. CCHL, Cyc2p, and cytochrome $b_2$ (loading control) were detected by immunodetection. *WT*, wild type.

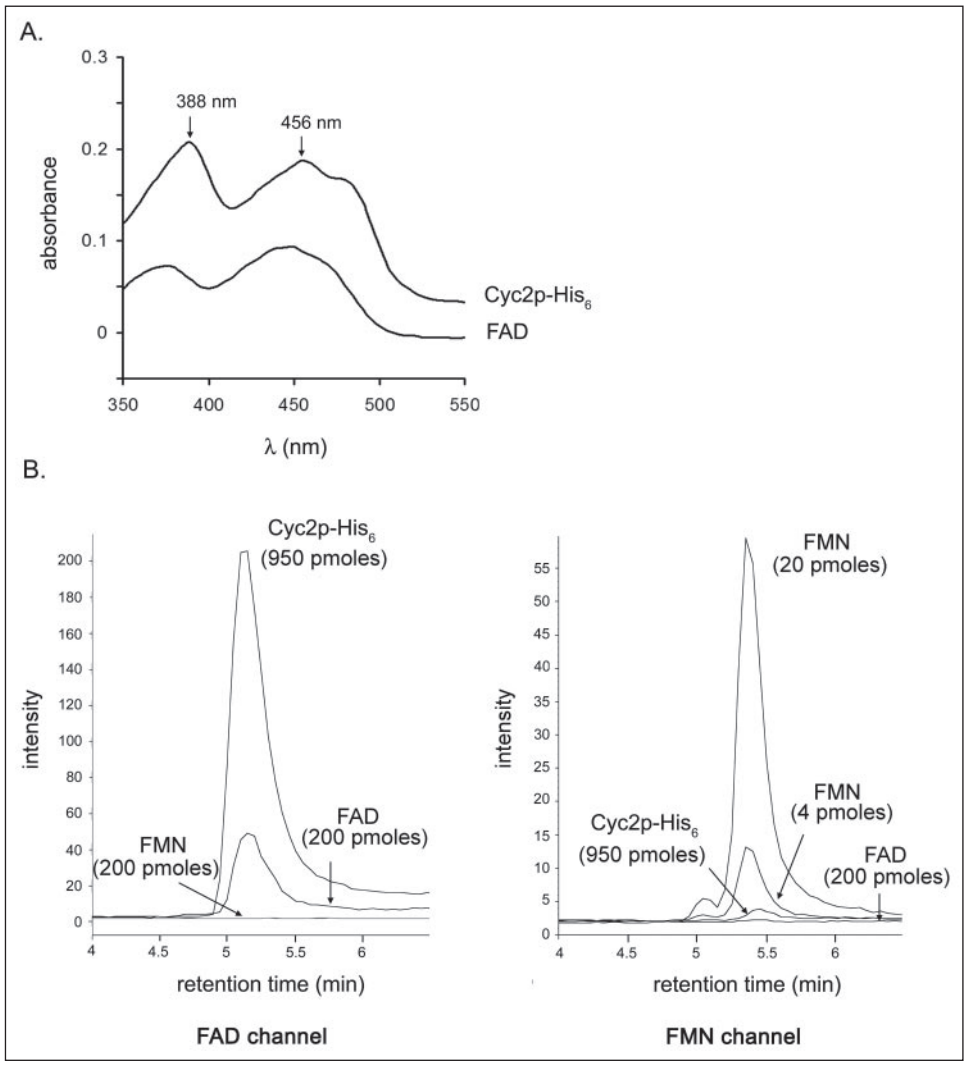


FIGURE 4. **Cyc2p is a FAD-binding protein.** *A*, Cyc2p exhibits the spectrum of a flavoprotein. The absorbance spectrum was recorded in the range of 350–550 nm using purified Cyc2p-$His_6$ or pure FAD. *B*, identification of the cofactor by reverse phase HPLC coupled to mass spectrometry. A solution of Cyc2p-$His_6$ was treated with guanidine 4.5 M in order to liberate the flavin cofactor. The mass of the cofactor was then determined by mass spectrometry analysis and compared with the mass of pure FAD or pure FMN. FAD channel and FMN channel indicate the peak intensity at molar mass = 784.29 and 455.4 g/mol, respectively. The quantity of protein or flavin injected is indicated on the *graph*.

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).

**TABLE TWO**

**Initial rate kinetic parameters obtained for the ferricyanide reductase activity of the recombinant soluble Cyc2p**

| Substrate | $V_{max}$ | $K_m$ | $V_{max}/K_m$ |
|---|---|---|---|
| | *μmol substrate/min/nmol enzyme* | *μM* | |
| NADH | 0.11 | $3 \times 10^4$ | $3.7 \times 10^{-6}$ |
| NADPH | 0.13 | $10^2$ | $1.4 \times 10^{-3}$ |

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. 5*A*). 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 $c_1$ and AAC, a monotopic and polytopic membrane protein, respectively, which are tightly anchored to the membrane, are resistant to carbonate extraction (Fig. 5*B*). 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, $CC_1HL$ behaves like an integral membrane protein and is completely resistant to carbonate extraction (data not shown) (55). To identify the

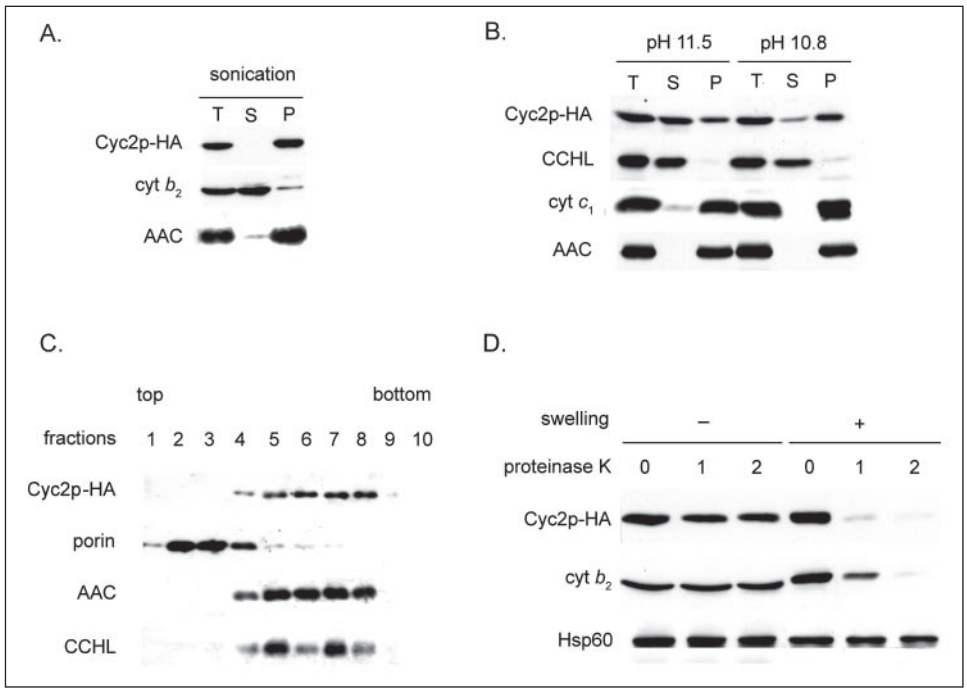


FIGURE 5. **Sublocalization and topological study of Cyc2p in mitochondria.** *A*, Cyc2p is a membrane-bound protein. Mitochondria from yeast cells expressing HA-tagged Cyc2p were sonicated in the presence of 500 mM NaCl. Samples were left untreated (*T*) or subjected to ultracentrifugation at 100,000 × *g* (*S*, supernatant; *P*, pellet) and then analyzed by SDS-PAGE and immunoblotting. *B*, Cyc2p is partially extractable by sodium carbonate. Mitochondria from yeast cells expressing HA-tagged Cyc2p were treated at pH 11.5 or 10.8, followed by an ultracentrifugation at 100,000 × *g* for 1 h. Samples were analyzed by SDS-PAGE and immunoblotting. *C*, Cyc2p is associated with the mitochondrial inner membrane. Mitochondrial outer and inner membrane vesicles were separated on a sucrose gradient, and fractions were analyzed by SDS-PAGE and immunoblotting. *D*, the C-terminal domain of Cyc2p is exposed to the IMS. Mitochondria from yeast cells expressing HA-tagged Cyc2p were either directly treated with proteinase K or subjected to hypotonic swelling before proteinase K treatment (*1*, 30 mg/ml; *2*, 60 mg/ml). Samples were analyzed by SDS-PAGE and immunoblotting.

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. 5*C*). Fractions containing the inner membranes were revealed with antibodies against AAC and CCHL, which are known inner membrane resident proteins (Fig. 5*C*). 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 mitoplasts. The HA tag was found to be accessible to degradation by added proteinase K, similarly to cytochrome $b_2$, an IMS resident protein (Fig. 5*D*). 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 $c_1$, (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 Δ*cyc2* mutant, holocytochrome *c* formation still takes place, albeit less effectively, and holocytochrome $c_1$ 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 $CC_1HL$, CCHL can only assemble cytochrome $c_1$ when Cyc2p is present (Fig. 1). The dependence of CCHL upon Cyc2p does not seem to be restricted to the cytochrome $c_1$ substrate, since holocytochrome *c* accumulation is significantly more affected in a Δ$cc_1hl$ Δ*cyc2* strain than in a Δ*cyc2* mutant (Fig. 3*A*). Note that in the absence of both $CC_1HL$ and Cyc2p, holocytochrome *c* assembly is not completely abolished, and some residual level of holocytochrome *c* still accumulates (Fig. 1). We have verified that the abundance of CCHL is not modified in a Δ$cc_1hl$ Δ*cyc2* 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 Δ$cc_1hl$ mutant, enhanced activity of CCHL toward apocytochrome $c_1$ through missense mutations in cytochrome $c_1$ 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 $c_1$. Third, intriguingly, whereas Cyc2p is not needed for the assembly of cytochrome $c_1$ with a wild type or CADCH heme binding site (*CYT1–48*), it is absolutely required when cytochrome $c_1$ carries a CAPCH heme binding site (*CYT1–34*). The concomitant loss of holocytochrome *c* and $c_1$ assembly in the absence of Cyc2p and the presence of the *CYT1–34* mutation is somehow very similar to the phenotype displayed by the Δ$cc_1hl$ Δ*cyc2* mutant (Fig. 3*A*) and suggests that both CCHL and $CC_1HL$ activities are severely compromised in the Δ*cyc2 cyt1–34* strain.

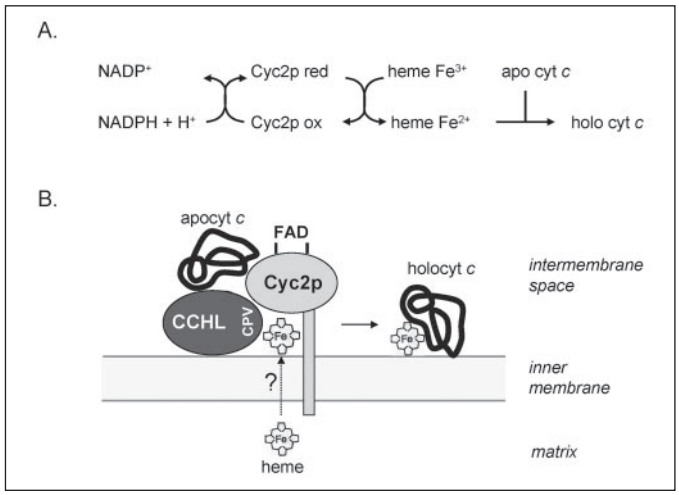


FIGURE 6. **Functional model for Cyc2p in the mitochondria.** *A*, schematic representation of Cyc2p activity. *B*, CCHL and Cyc2p cooperate in the heme lyase reaction in the mitochondrial IMS. Heme is transported from its site of synthesis in the matrix across the mitochondrial inner membrane by an unknown mechanism (*?*). Once emerged from the inner membrane, heme is handled by CCHL via the heme regulatory motifs (*CPV*) and maintained reduced by the activity of membrane-bound flavoprotein Cyc2p. We postulate that yet to be identified components are required to maintain the apocytochrome *c* sulfhydryls reduced prior to the heme ligation by CCHL.

We think that the *CYT1–34* mutation, which was selected to increase the affinity of CCHL toward cytochrome $c_1$ (23), could act by favoring the interaction of apocytochrome $c_1$ with CCHL to the detriment of its interaction with $CC_1HL$, its cognate lyase. Loss of assembly of both cytochrome *c* and $c_1$ in the Δ*cyc2 cyt1–34* mutant can therefore be explained by the fact that, similarly to the Δ*cc*$_1$*hl* Δ*cyc2* mutant, Cyc2p is essential when the assembly of both cytochrome *c* and $c_1$ relies solely on CCHL. This hypothesis is further solidified by the observation that *CC*$_1$*HL* when overexpressed from a multicopy plasmid alleviates the respiratory deficiency of the Δ*cyc2 cyt1–34* mutant (data not shown). We believe that in this situation, overexpression of $CC_1HL$ outcompetes CCHL in the interaction with apocytochrome $c_1$, which can be converted to its holoform by action of its cognate lyase. As expected, overexpression of CCHL in the Δ*cyc2 cyt1–34* 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 $c_1$, behaves differently than *CYT1–34* in the absence of Cyc2p (Fig. 3). One possibility is that the affinity of CCHL for cytochrome $c_1$ 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 C*XX*CH motif. It is known that between cysteine mutations in C*XX*C motifs of redox proteins such as thioredoxin DsbA 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 $c_1$ 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 Cyc2p is a partner of CCHL and becomes critical for the maturation process of cytochrome *c* and $c_1$ when $CC_1HL$ is absent or unable to act on its cognate substrate. The localization of both CCHL and Cyc2p in the inner membrane with domains of the proteins exposed to the IMS is compatible with such a model (Fig. 6*B*).

*A Putative Heme Reductase Activity for Cyc2p?*—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 Cyc2p harbors a bound FAD in a domain that localizes to the mitochondrial IMS and exhibits NAD(P)H-ferricyanide oxidoreductase activity *in vitro* suggests that *in vivo*, Cyc2p could function in reducing heme prior to its ligation by CCHL. The greater affinity of Cyc2p for the NADPH substrate *in vitro* makes it likely that NADPH acts as the electron donor to Cyc2p in the mitochondrial IMS. Because the requirement for reductants applies to both heme and apocytochrome *c* substrates, it is conceivable that Cyc2p reduces both the cofactor and the apocytochrome *c* sulfhydryls *in vivo*. However, based on the similarity of Cyc2p with cytochrome $b_5$ reductase-like proteins that reduce heme in cytochrome $b_5$ (supplemental Fig. 2), we favor a model where Cyc2p catalyzes the NADPH-dependent reduction of the heme co-factor only (Fig. 6*A*). 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 Cyc2p and ligated to the apocytochrome *c* substrate by action of CCHL (Fig. 6*B*). Additional experimental work is required to establish whether heme and/or apocytochrome *c* are the relevant targets of action of Cyc2p *in vivo*.

If Cyc2p is active as a heme and/or apocytochrome reductase in cytochrome *c* maturation, its function is clearly redundant based on the observation that a Δ*cyc2* mutant is still able to assemble holocytochrome *c* (Fig. 3). The function performed by Cyc2p appears essential only when the assembly of both cytochrome *c* and $c_1$ is dependent upon CCHL. It is possible that the activity of Cyc2p 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 $CC_1HL$ by enhancing holocytochrome $c_1$ 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 Δ*cc*$_1$*hl* mutant fail to identify other genes beside 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 $CC_1HL$-dependent assembly of cytochrome $c_1$. Whereas it is clear that Cyc2p exerts no influence on the $CC_1HL$-catalyzed assembly of cytochrome $c_1$, the requirement for NADH and FMN co-factors for the reduction of heme prior to its linkage to apocytochrome $c_1$ *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 $c_1$ 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).

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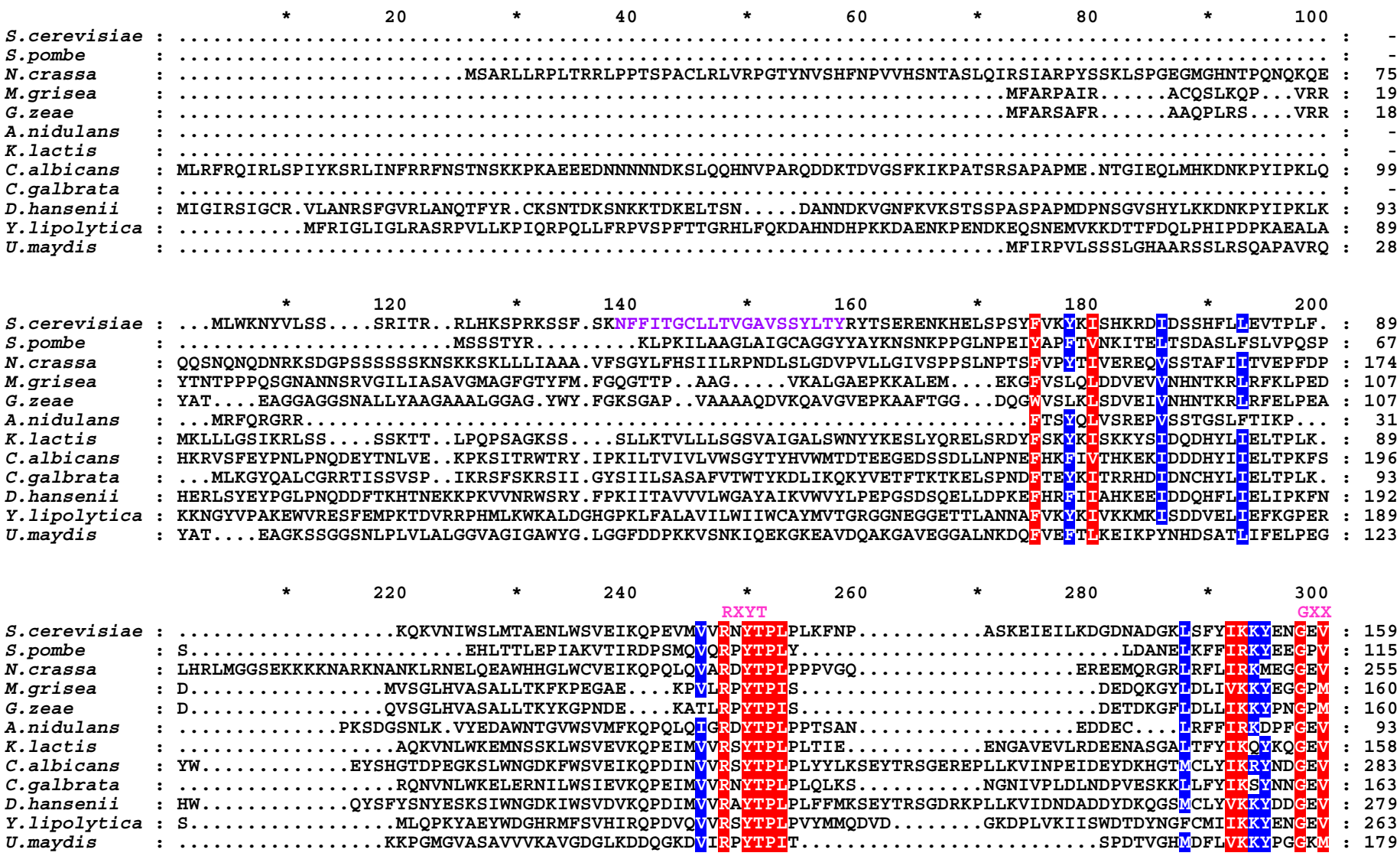
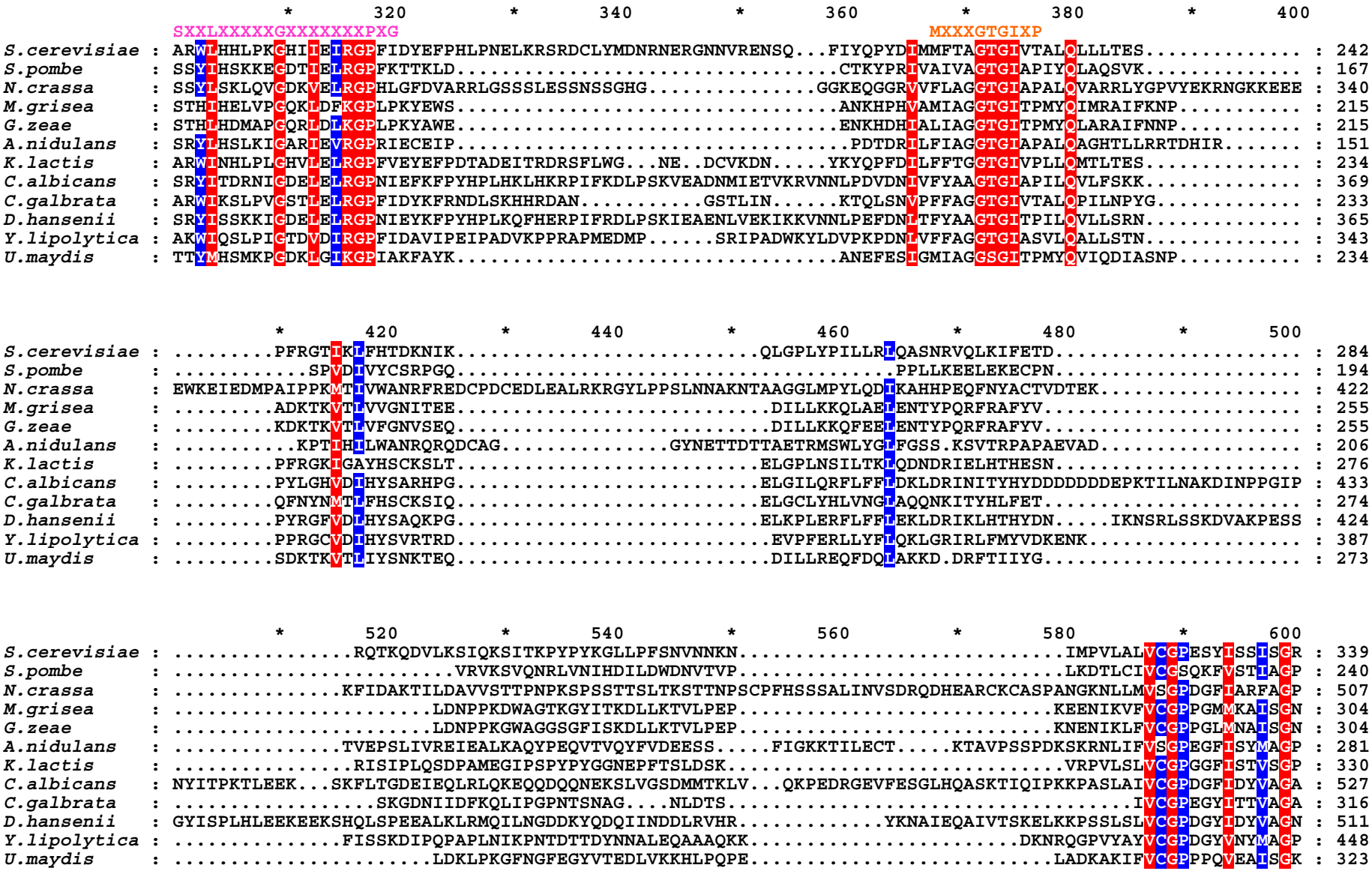
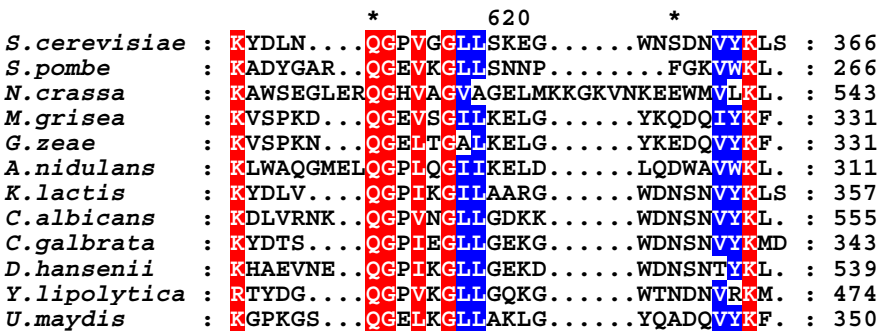
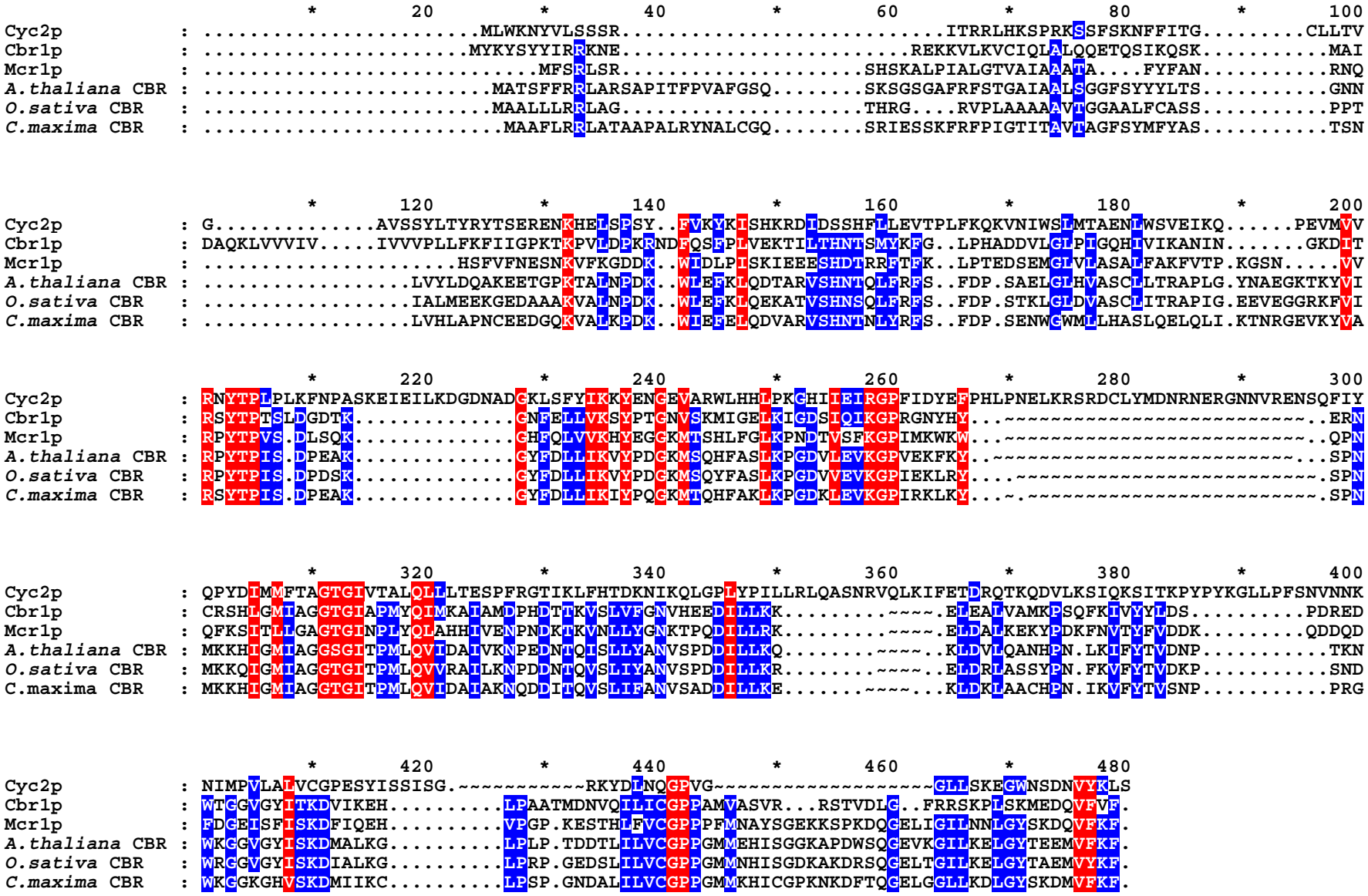

RXYT
GXX
S.cerevisiae
S.pombe
N.crassa
M.grisea
G.zeae
A.nidulans
K.lactis
C.albicans
C.galbrata
D.hansenii
Y.lipolytica
U.maydis


SXXLXXXXXGXXXXXXXPXG
MXXXGTGIXP
S.cerevisiae : ARWLHHLPKGHIIEIRGPFIDYEFPHLPNELKRSRDCLYMDNRNERGNNVRENSQ...FIYQPYDIMMFTAGTGIVTALQLLLTES..... : 242
S.pombe : SSYIHSKKEGDTIELRGPFKTTKLD.....CTKYPRIVAIVAGTGIAPIYQLAQSVK..... : 167
N.crassa : SSYLSKLQVGDKVELRGPHLGFDVARRLGSSSLESSNSSGHG.....GGKEQGGRVVFLAGGTGIAPALQVARRLYGPVYEKRNGKKEEE : 340
M.grisea : STHIHELVPGQKLDFKGPLPKYEWS.....ANKHPHVAMIAGGTGITPMYQIMRAIFKNP..... : 215
G.zeae : STHLHDMAPGQRLDLKGPLPKYAWE.....ENKHDHIALIAGGTGITPMYQLARAIFNNP..... : 215
A.nidulans : SRYLHSLKIGARIEVRGPRIECEIP.....PDTDRILFIAGGTGIAPALQAGHTLLRRTDHIR..... : 151
K.lactis : ARWINHLPLGHVLELRGPFVEYEYEFPDTADEITRDRSFLWG...NE..DCVKDN.....YKYQPFDILFFTGGTGIVPLLQMTLTES..... : 234
C.albicans : SRYITDRNIGDELELRGPNIEFKFPYHPLHKLHKRPIFKDLPSKVEADNMIETVKRVNNLPDVDNIVFYAAGTGIAPILQVLFSKK..... : 369
C.galbrata : ARWIKSLPVGSTLELRGPFIDYKFRNDLSKHHRDAN.....GSTLIN.....KTQLSNVPFFAGGTGIVTALQPILNPYG..... : 233
D.hansenii : SRYISSKKIGDELELRGPNIEYKFPYHPLKQFHERPIFRDLPSKIEAENLVEKIKKVNNLPEFDNLTFYAAGTGITPILQVLLSRN..... : 365
Y.lipolytica : AKWIQSLPIGTDVDIRGPFIDAVIPEIPADVKPPRAPMEDMP.....SRIPADWKYLDVPKPDNLVFFAGGTGIASVLQALLSTN..... : 343
U.maydis : TTYMHSMKPGDKLGIKGPIAKFAYK.....ANEFESIGMIAGGSGITPMYQVIQDIASNP..... : 234
S.cerevisiae : .....PFRGTIKLFHTDKNIK.....QLGPLYPILLRLQASNRVQLKIFETD..... : 284
S.pombe : .....SPVDIVYCSRPGQ.....PPLLKEELEKECPN..... : 194
N.crassa : EWKEIEDMPAIPPKMTIVWANRFREDCPDCEDLEALRKRGYLPPSLNNAKNTAAGGLMPYLQDIKAHHPEQFNYACTVDTEK..... : 422
M.grisea : .....ADKTKVTLVVGNITEE.....DILLKKQLAELENTYPQRFRAFYV..... : 255
G.zeae : .....KDKTKVTLVFGNVSEQ.....DILLKKQFEELENTYPQRFRAFYV..... : 255
A.nidulans : .....KPTIHILWANRQRQDCAG.....GYNETTDTTAETRMSWLYGLFGSS.KSVTRPAPAEVAD..... : 206
K.lactis : .....PFRGKIGAYHSCKSLT.....ELGPLNSILTKLQDNDRIELHTHESN..... : 276
C.albicans : .....PYLGHVDIHYSARHPG.....ELGILQRFLFFLDKLDRINITYHYDDDDDDDEPKTILNAKDINPPGIP : 433
C.galbrata : .....QFNYNMTLFHSCKSIQ.....ELGCLYHLVNGLAQQNKITYHLFET..... : 274
D.hansenii : .....PYRGFVDIHYSAQKPG.....ELKPLERFLFFLEKLDRIKLHTHYDN.....IKNSRLSSKDVAKPESS : 424
Y.lipolytica : .....PPRGCVDIHYSVRTRD.....EVPFERLLYFLQKLGRIRLFMYVDKENK..... : 387
U.maydis : .....SDKTKVTLIYSNKTEQ.....DILLREQFDQLAKKD.DRFTIIYG..... : 273
S.cerevisiae : .....RQTKQDVLKSIQKSITKPYPYKGLLPFSNVNNKN.....IMPVLALVCGPESYISSISGR : 339
S.pombe : .....VRVKSVQNRLVNIHDILDWDNVTVP.....LKDTLCIVCGSQKFVSTIAGP : 240
N.crassa : .....KFIDAKTILDAVVSTTPNPKSPSSTTSLTKSTTNPSCPFHSSSALINVSDRQDHEARCKCASPANGKNLLMVSGPDGFIARFAGP : 507
M.grisea : .....LDNPPKDWAGTKGYITKDLLKTVLPEP.....KEENIKVFVCGPPGMMKAISGN : 304
G.zeae : .....LDNPPKGWAGGSGFISKDLLKTVLPEP.....KNENIKLFVCGPPGLMNAISGN : 304
A.nidulans : .....TVEPSLIVREIEALKAQYPEQVTVQYFVDEESS.....FIGKKTILECT.....KTAVPSSPDKSKRNLIFVSGPEGFISYMAGP : 281
K.lactis : .....RISIPLQSDPAMEGIPSPYPYGGNEPFTSLDSK.....VRPVLSLVCGPGGFISTVSGP : 330
C.albicans : NYITPKTLEEK...SKFLTGDEIEQLRLQKEQQDQQNEKSLVGSDMMTKLV...QKPEDRGEVFESGLHQASKTIQIPKKPASLAIVCGPDGFIDYVAGA : 527
C.galbrata : .....SKGDNIIDFKQLIPGPNTSNAG....NLDTS.....IVCGPEGYITTVAGA : 316
D.hansenii : GYISPLHLEEKEEKSHQLSPEEALKLRMQILNGDDKYQDQIINDDLRVHR.....YKNAIEQAIVTSKELKKPSSLSLVCGPDGYIDYVAGN : 511
Y.lipolytica : .....FISSKDIPQPAPLNIKPNTDTTDYNNALEQAAAQKK.....DKNRQGPVYAYVCGPDGYVNYMAGP : 448
U.maydis : .....LDKLPKGFNGFEGYVTEDLVKKHLPQPE.....LADKAKIFVCGPPPQVEAISGK : 323

Bernard *et al.* **Figure 1 Supplemental material**




Cyc2p
Cbr1p
Mcr1p
A.thaliana CBR
O.sativa CBR
C.maxima CBR
C.maxima CBR