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c-Type Cytochrome Assembly in *Saccharomyces cerevisiae*: A Key Residue for Apocytochrome c_1/Lyase Interaction

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ABSTRACT

The electron transport chains in the membranes of bacteria and organelles generate proton-motive force essential for ATP production. The *c*-type cytochromes, defined by the covalent attachment of heme to a C*XX*CH motif, are key electron carriers in these energy-transducing membranes. In mitochondria, cytochromes *c* and c_1 are assembled by the cytochrome *c* heme lyases (CCHL and CC_1HL) and by Cyc2p, a putative redox protein. A cytochrome c_1 mutant with a CAPCH heme-binding site instead of the wild-type CAACH is strictly dependent upon Cyc2p for assembly. In this context, we found that overexpression of CC_1HL, as well as mutations of the proline in the CAPCH site to H, L, S, or T residues, can bypass the absence of Cyc2p. The P mutation was postulated to shift the C*XX*CH motif to an oxidized form, which must be reduced in a Cyc2p-dependent reaction before heme ligation. However, measurement of the redox midpoint potential of apocytochrome c_1 indicates that neither the P nor the T residues impact the thermodynamic propensity of the C*XX*CH motif to occur in a disulfide *vs.* dithiol form. We show instead that the identity of the second intervening residue in the C*XX*CH motif is key in determining the CCHL-dependent *vs.* CC_1HL-dependent assembly of holocytochrome c_1. We also provide evidence that Cyc2p is dedicated to the CCHL pathway and is not required for the CC_1HL-dependent assembly of cytochrome c_1.

THE *c*-type cytochromes, also referred to as cytochrome *c*, represent a universal class of heme-containing proteins that function as electron carriers in the energy-transducing pathways of bacteria, plastids, and mitochondria (Thöny-Meyer 1997; Nakamoto *et al.* 2000; Bonnard *et al.* 2010). Because cytochromes *c* carry a heme covalently attached to a C*XX*CH motif, they constitute an attractive object of study to address the question of cofactor protein assembly. The biochemical requirements for cytochrome *c* assembly were deduced from *in vivo* and *in vitro* studies, and the conclusion is that both apocytochromes *c* and heme are transported independently across at least one biological membrane and maintained as reduced prior to catalysis of the heme attachment reaction (Allen *et al.* 2003; Hamel *et al.* 2009; Kranz *et al.* 2009; Sanders *et al.* 2010). Bacterial cytochromes *c* are assembled in the periplasmic space, a compartment where cysteine pairs in proteins form disulfide bonds in reactions catalyzed by dedicated enzymes (Inaba 2009; Kadokura and Beckwith 2010). The current thinking holds that a *c*-type apocytochrome is a substrate of the disulfide bond-forming pathway, which introduces an intramolecular disulfide between the two cysteines of the C*XX*CH sequence (Allen *et al.* 2003; Sanders *et al.* 2010). This disulfide needs to be reduced to a dithiol to provide free sulfhydryls for the heme ligation. Consistent with this view is the fact that groups of specific oxido-reductases that constitute a transmembrane dithiol-disulfide relay from the cytosol to the periplasmic space have been shown to function as *c*-type cytochrome assembly factors (Allen *et al.* 2003; Kadokura *et al.* 2003; Mapller and Hederstedt 2006; Sanders *et al.* 2010). The proposal that the components of this pathway control the *in vivo* redox status of the C*XX*CH sulfhydryls has been inferred from the presence of motifs in their protein sequences that are consistent with a function in redox chemistry and also from the demonstration that their recombinant forms participate in dithiol–disulfide exchange reactions (Monika *et al.* 1997; Setterdahl *et al.* 2000). Moreover, the ability of exogenous thiol compounds to bypass the lack of these factors *in vivo* substantiates the view that the redox components have a disulfide-reducing activity in the pathway (*e.g.*, Sambongi and Ferguson 1994; Fabianek *et al.* 1998; Beckett *et al.* 2000; Deshmukh

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et al. 2000; Bardischewsky and Friedrich 2001; Erlendsson and Hederstedt 2002; Erlendsson *et al.* 2003; Feissner *et al.* 2005; Turkarslan *et al.* 2008).

While the role of these pathways is well established in bacteria, much less is known about the components that catalyze thiol/disulfide chemistry in the mitochondrial intermembrane space (IMS), which is topologically equivalent to the bacterial periplasm. By analogy with the bacterial pathways, the participation of redox-active factors that catalyze thiol formation is expected, as the mitochondrial IMS houses two *c*-type cytochromes, the soluble cytochrome *c* and the membrane-bound cytochrome c_1, both of which function in respiration. In fungi, heme attachment to apocytochromes *c* and c_1 is dependent upon the IMS resident cytochrome *c* and c_1 heme lyases, CCHL and CC_1HL, although the exact role of these lyases in the assembly process is still unclear (Dumont *et al.* 1987; Zollner *et al.* 1992). Conversion of apocytochrome to holocytochrome *c* depends only on CCHL, while apocytochrome c_1 can be acted upon by both CCHL and CC_1HL (Matner and Sherman 1982; Dumont *et al.* 1987; Stuart *et al.* 1990; Zollner *et al.* 1992; Bernard *et al.* 2003). In animals, apoforms of cytochromes *c* and c_1 are assembled by a unique heme lyase, HCCS, which carries both the CCHL and CC_1HL activities (Prakash *et al.* 2002; Schwarz and Cox 2002; Bernard *et al.* 2003).

Cyc2p, a component first described as a mitochondrial biogenesis factor in yeast (Matner and Sherman 1982; Dumont *et al.* 1993; Pearce *et al.* 1998; Sanchez *et al.* 2001), was recently rediscovered in the context of cytochrome c_1 maturation (Bernard *et al.* 2003). Cyc2p is located at the mitochondrial inner membrane with its C-terminal domain containing a non-covalently bound FAD exposed to the IMS (Bernard *et al.* 2005). A redox function for Cyc2p is likely based on the finding that a recombinant form of the molecule exhibits a NAD(P)H-dependent reductase activity (Bernard *et al.* 2005). However, as Cyc2p activity is not essential for the maturation process, a functional redundancy was postulated based on the fact that a *cyc2*-null mutant still assembles holoforms of cytochromes *c* and c_1 (Bernard *et al.* 2005). The absolute requirement of Cyc2p was revealed via genetic analysis of the *cyc2*-null *cyt1-34* combination that displays a synthetic respiratory-deficient phenotype with loss of holocytochrome c_1 assembly (Bernard *et al.* 2005). The *cyt1-34* mutation maps to the gene encoding cytochrome c_1 and results in a CAPCH heme-binding site replacing the wild-type CAACH site (Bernard *et al.* 2005). The synthetic interaction is specific for the *cyt1-34* allele carrying the A-to-P mutation and is not observed in a *cyc2*-null *cyt1-48* strain carrying an A-to-D mutation at the heme-binding site of apocytochrome c_1 (Bernard *et al.* 2005). The fact that Cyc2p becomes essential when the cytochrome c_1 heme-binding site carries an A-to-P mutation suggests that the C*XX*CH motif could be the target of Cyc2p action *in vivo*. One possible interpretation for this observation is that the P residue alters the reactivity of the cysteinyl thiols to redox chemistry so that the apocytochrome c_1 CAPCH heme-binding site occurs in an oxidized (disulfide) form, which must be reduced in a Cyc2p-dependent reaction before heme attachment can occur.

In this article, we have undertaken a genetic approach to elucidate this pathway and searched for suppressors that alleviate the respiratory deficiency of the *cyc2*-null *cyt1-34* strain. Either overexpression of CC_1HL or replacement of the P mutation in the heme-binding site by H, L, S, or T residues restore the assembly of holocytochrome c_1. *In vitro* measurement of redox potential of apoforms of CA(A/P/T)CH cytochrome c_1 indicates that there is no change in the thermodynamic stability of the disulfide at the C*XX*CH motif that could account for the Cyc2p-dependent assembly of cytochrome c_1. Genetic studies reveal that the replacement of the second A residue at the CAACH motif by H, L, P, S, and T residues is key in determining the conversion of apocytochrome c_1 to its corresponding holoform via the CCHL and/or CC_1HL-dependent pathway. We also demonstrate that Cyc2p is a component dedicated to the CCHL pathway and is not required for the CC_1HL-dependent assembly of cytochrome c_1. We propose that the CAPCH cytochrome c_1 is strictly dependent upon CCHL and Cyc2p for its assembly but becomes a substrate of CC_1HL upon overexpression of CC_1HL or in the presence of H, L, S, or T mutations.

MATERIALS AND METHODS

Construction, manipulation, and growth of yeast strains: All of the yeast strains used in the course of this study are listed in Table 1. YDM1 and YDM2 strains carry the *cyt1::kan* allele and originate from the parental SMY1 (*MAT***a**) and YCT1-11C (*MAT*α) Δ*cc$_1$hl* strains, respectively. PCR amplification of the *cyt1::kan* cassette was performed using oligonucleotide sequences (5′-AACTGGATCCATAGACTATCTAAG-3′ and 5′-GACACTATTGAAGTGAGACG-3′) and the genomic DNA of the *cyt1::kan* strain from the ResGen knockout collection. Yeast cells were transformed by the lithium acetate procedure (Schiestl and Gietz 1989) or by the one-step technique (Chen *et al.* 1992). Genetic crosses were performed as described in Dujardin *et al.* (1980). Media used for *Saccharomyces cerevisiae* contain glucose or galactose as fermentable substrates and glycerol, ethanol, ethanol/glycerol, or lactate as respiratory substrates and were described elsewhere (Dujardin *et al.* 1980; Hamel *et al.* 1998; Saint-Georges *et al.* 2002).

Isolation of multicopy and genetic suppressors: The multicopy wild-type genomic libraries constructed in the *URA3*-based pFL44L vector (Bach *et al.* 1979) (a generous gift from F. Lacroute) and in the *LEU2*-based Yep24 vector (Carlson and Botstein 1982) (a generous gift from M. Carlson) were used to search for suppressor genes able to alleviate the respiratory deficiency of the *cyc2*-null *cyt1-34* strain. Transformants were plated on medium lacking uracil or leucine, replicated on lactate medium to select for respiratory proficiency, and incubated for 15 days at 28°. Plasmids retrieved through the multicopy suppressor screen

TABLE 1

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]
PHT3	**a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 cyt1::LEU2*	Hamel *et al.* (1998)
YPH71-14B	**a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 cyt1-34*	Bernard *et al.* (2005)
YPH10-8A	**a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 cyt1-48*	Bernard *et al.* (2005)
YPH1	α *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 cyc2::hph*	Bernard *et al.* (2003)
YPH6-9C	**a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 cyt1-34 cyc2::hph*	Bernard *et al.* (2005)
YDB8	**a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 cyt1-48, cyc2::hph*	Bernard *et al.* (2005)
SMY1	**a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 cyt2::his5*$^+$	Bernard *et al.* (2003)
YCT1-11C	α *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 cyt2::his5*$^+$	Bernard *et al.* (2003)
UV34	**a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 cyt1-34 cyt2::his5*$^+$	Bernard *et al.* (2003)
UV48	**a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 cyt1-48 cyt2::his5*$^+$	Bernard *et al.* (2003)
SSP4,5,8,10	**a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 cyt1-35 cyc2::hph*	This study
SSP6	α *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100, cyt1-36 cyc2::hph*	This study
SSP1,2	**a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 cyt1-37 cyc2::hph*	This study
SSP3,7,9,11,12	α *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 cyt1-38 cyc2::hph*	This study
YDM1	**a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 cyt1::kan cyt2::his5*$^+$	This study
YDM2	α *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 cyt1::kan cyt2::his5*$^+$	This study
YDM3-2B	**a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 cyt1-35*	This study[b]
YDM2-2C	**a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 cyt1-36*	This study[c]
YDM1-2A	**a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 cyt1-37*	This study[d]
YDM4-2A	**a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 cyt1-38*	This study[e]
YDM3-1X	α *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 cyt1-35 cyt2::his5*$^+$	This study[f]
YDM2-1X	**a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 cyt1-36 cyt2::his5*$^+$	This study[g]
YDM1-1B	α *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 cyt1-37 cyt2::his5*$^+$	This study[d]
YDM4-1B	α *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 cyt1-38 cyt2::his5*$^+$	This study[e]

[a] Department of Human Genetics, Columbia University.
[b] Segregant of diploid created by crossing YDM2 with SSP10.
[c] Segregant of diploid created by crossing YDM1 with SSP6.
[d] Segregant of diploid created by crossing YDM2 with SSP2.
[e] Segregant of diploid created by crossing YDM1 with SSP12.
[f] Created by inactivation of *CYT2* in YDM3-2B.
[g] Created by inactivation of *CYT2* in YDM2-2C.

were extracted from yeast transformants and propagated in *Escherichia coli* strains (Hoffman and Winston 1987). Independent spontaneous suppressors bypassing the respiratory deficiency of the Δ*cyc2 cyt1-34* strain were isolated on lactate medium. Cells were grown to stationary phase and plated on respiratory medium, and suppressed strains were isolated after 10–15 days incubation at 28°.

Overexpression and purification of apocytochromes c_1: The *CYT1* gene encoding the soluble form (amino acids 62–263) of wild-type apocytochrome c_1 was amplified by PCR from yeast genomic DNA and cloned into the *Nhe*I/*Xho*I sites of the hexahistidinyl tag plasmid pET-24b(+) (Novagen). The cysteines (C188 and C258) were replaced by serines by site-directed mutagenesis. Mutations in the heme-binding motif (CAPCH and CATCH) were also engineered by site-directed mutagenesis. Apocytochromes c_1 were overexpressed in *E. coli* BL21(DE3) strain in the presence of 1 mm isopropyl-β-D-thiogalactopyranoside for 5 hr at 30°. All recombinant apocytochromes c_1 (wild-type and mutants) carry a C-terminal $(His)_6$-tag, and as a result of the C188,258S substitutions, the only cysteines present are the two associated with the heme-binding site. Purification of His-tagged proteins was performed under denaturing conditions. Cells were harvested, resuspended in a HEPES buffer (100 mm HEPES, 100 mm NaCl, 0.01% Tween 20, pH 8.0) containing 8 m urea and 50 mm imidazole and incubated at room temperature for 1 hr. The lysate was clarified by centrifugation at 10,000 × *g* for 20 min at room temperature. The supernatant was then applied to the Ni-NTA resin (Qiagen). Protein renaturation and refolding were carried out directly on the column by gradient buffer exchange to eliminate urea. The resin was washed with the HEPES buffer containing 100 mm imidazole, and the proteins were eluted with 250 mm imidazole. Samples were then dialyzed to remove imidazole and concentrated by using a Centriprep filter unit (Millipore). Purity was assessed by SDS–PAGE, and the concentration of the purified protein was measured by recording the absorbance at 260 nm.

Measurement of midpoint redox potentials of apocytochromes c_1: Redox titrations of disulfide/dithiol couples were performed using thiol labeling with 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS; Invitrogen) (Motohashi and Hisabori 2006). Wild-type and CAP/TCH apocytochromes c_1 recombinant proteins (5 μg) were incubated separately under anaerobic conditions at 25° for 5 or 16 hr in 100 mm HEPES (pH 7.0) and various concentrations of reduced and oxidized DTT at a total DTT concentration of 5 mm. Ambient potential (E_h) values for different DTT_{red}/DTT_{ox} ratios were calculated from the two-electron Nernst equation, using the literature value for the midpoint potential of DTT at pH 7.0 (Hutchison and Ort 1995). Incubations in the presence of 50 μm of $CuCl_2$ or 5 mm reduced DTT served to produce fully oxidized or fully reduced

protein controls, respectively. After incubation, samples were treated with 10% trichloroacetic acid (TCA) and allowed to stand on ice for 30 min. Protein precipitates were washed with 1% TCA and then with ice-cold acetone. Samples were resuspended in buffer containing 50 mM HEPES (pH 7.0), 2.5% SDS, and 10 mM AMS and incubated at 37° for 1 hr in an argon atmosphere. Reduced (AMS-labeled) and oxidized (nonlabeled) forms of apocytochromes c_1 were separated by nonreducing SDS–PAGE on a 16% gel, stained with silver nitrate (Rabilloud *et al.* 1994), and quantified using Image J software (National Institutes of Health). Data were plotted and fitted to the Nernst equation for a single redox couple by iteration, using the solver of Excel (Microsoft). The midpoint potential (E_m) was defined as a variable, and its value was taken after the target cell, containing the average quadratic variation between the theoretical curve and the experimental data, converged to its minimum value. Best fits were obtained for an *n*-value of 2 (*i.e.*, for a two-electron redox reaction). All E_m values reported correspond to the average of three independent titrations.

Mitochondrial protein preparation and analysis: Mitochondria were purified from yeast strains grown in galactose medium as previously described (Bernard *et al.* 2005), and the protein concentration was measured using the Bradford reagent (Bio-Rad). For heme staining of mitochondrial *c*-type cytochromes, protein samples were reduced with 50 mM DTT on ice for 1 hr and separated at 4° by lithium dodecyl sulfate (LDS)–PAGE (Dutta and Henry 1990). Detection of holocytochromes *c* and c_1 was performed on polyvinylidene difluoride membrane (PVDF; 0.45 μm) by the enhanced chemiluminescence (ECL) method (SuperSignal West Pico, Pierce) using the heme-associated peroxidase activity of holocytochromes *c* (Vargas *et al.* 1993). For immunoblotting, mitochondrial proteins were separated by SDS–PAGE and subsequently immobilized by electro-transfer to PVDF membranes. Polyclonal antibodies raised against cytochrome *c* (C. Koehler, University of California, Los Angeles), cytochrome c_1 (C. Lemaire, Gif-sur-Yvette, France), CCHL (Bernard *et al.* 2005), CC_1HL (Zollner *et al.* 1992), Cyc2p (Bernard *et al.* 2005), and Coq1 (Gin and Clarke 2005) were used for immunodetection. Bound antibodies were detected by horseradish peroxidase-conjugated secondary antibody (Bio-Rad) and ECL reagent (SuperSignal West Dura, Pierce).

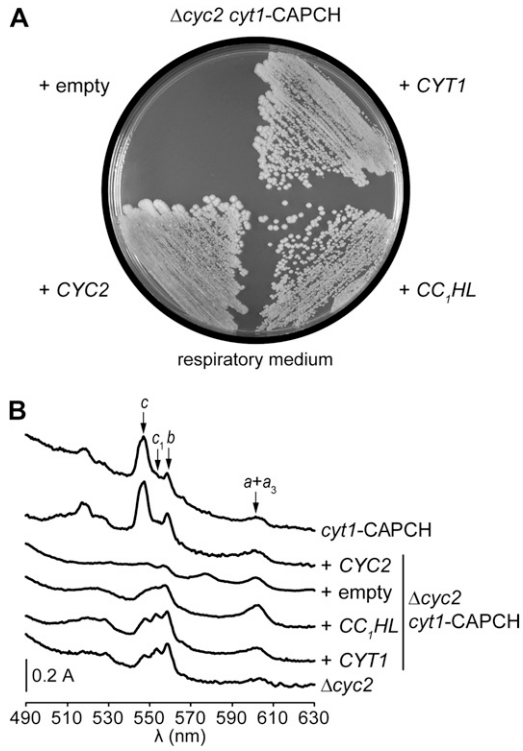

Figure 1.—The *CC_1HL* gene is a multicopy suppressor of the Δ*cyc2 cyt1*-CAPCH mutant. (A) Respiratory growth of the Δ*cyc2 cyt1*-CAPCH transformants. The *cyc2*-null *cyt1*-CAPCH transformants (YPH6-9C) carrying pFL44L (empty), pFL44L-CYT1 (+ *CYT1*), pFL44L-CYC2 (+ *CYC2*), and pFL44L-CC_1HL (+ *CC_1HL*) plasmids were grown on respiratory media containing ethanol/glycerol at 28° for 3 days. The synthetic respiratory-deficient phenotype of the Δ*cyc2 cyt1*-CAPCH mutant cannot be suppressed by overexpression of the *CYC1* or *CYC7* genes encoding the isoforms 1 and 2 of cytochrome *c*, respectively, or by the *CCHL* gene (not shown). (B) Cytochrome absorption spectra of the Δ*cyc2 cyt1*-CAPCH transformants. Low-temperature absorption spectra of cells grown in galactose were recorded with a Cary 400 spectrophotometer as previously described (Claisse *et al.* 1970). The arrows indicate the absorption maxima of the α-bands of cytochromes *c* (546 nm), c_1 (552 nm), *b* (558 nm), and $a + a_3$ (602 nm).

RESULTS

A multicopy suppressor screen of the Δ*cyc2 cyt1*-CAPCH mutant identifies CC_1HL: For convenience, the *cyc2*-null *cyt1-34* mutant will be referred to as Δ*cyc2 cyt1*-CAPCH throughout the entire manuscript. To gain further insight into the function of Cyc2p, we undertook a search for multicopy suppressors able to alleviate the respiratory deficiency of the Δ*cyc2 cyt1*-CAPCH mutant. The respiratory-deficient phenotype of the Δ*cyc2 cyt1*-CAPCH strain is tight and characterized by a dual deficiency in holoforms of cytochrome *c* and c_1 (Figures 1 and 2). Of 500,000 primary transformants, 87 respiratory-competent colonies were selected after a 10-day incubation on medium containing a respiratory substrate. Extended incubation gave rise to colonies in which respiratory proficiency was independent of the presence of the multicopy plasmid and probably caused by suppressor mutations. Diagnostic PCR amplification using *CYC2*- and *CYT1*-specific primers showed that of the 87 transformants, 44 carried a plasmid containing the *CYC2* gene, whereas 36 harbored a plasmid with the cytochrome c_1-encoding gene. Both genes were expected to be recovered, as the Δ*cyc2 cyt1*-CAPCH respiratory-deficient phenotype is synthetic (Figures 1A, 2A, and 3A). The remaining 7 transformants displayed a slower growth phenotype, and PCR amplification failed to reveal the presence of *CYC2* or *CYT1* genes in the transforming plasmids. Sequencing of the insert ends in one multicopy plasmid and subsequent diagnostic PCR revealed that all slow-growing transformants carry the gene encoding CC_1HL. Spectral analysis showed that overexpression of CC_1HL is able to partially restore holocytochromes *c* and c_1 assembly (Figure 1B). This result is consistent with the restoration of the respiratory growth as both cytochromes *c* and c_1 are required for

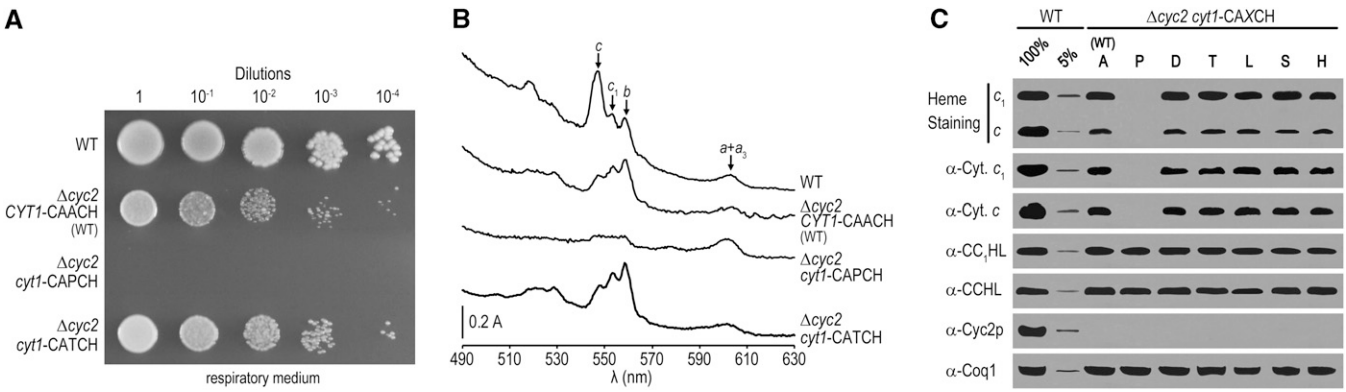

FIGURE 2.—Mutations in cytochrome c_1 heme-binding site suppress the Δ*cyc2 cyt1*-CAPCH mutant. (A) Respiratory growth of the *cyc2*-null mutants. Ten-fold serial dilutions of wild-type (WT) (W303-1A), Δ*cyc2 CYT1*-CAACH (YPH1), Δ*cyc2 cyt1*-CAPCH (YPH6-9C), and Δ*cyc2 cyt1*-CATCH (SSP2) strains were grown on ethanol/glycerol as respiratory substrates and incubated at 28° for 3 days. The respiratory phenotypes of Δ*cyc2 cyt1*-CAXCH strains carrying the H, L, and S mutations in the cytochrome c_1 heme-binding motif are equivalent to that of a Δ*cyc2 cyt1*-CATCH strain (data not shown). (B) Cytochrome absorption spectra of the *cyc2*-null mutants. Spectrophotometric maxima of cytochromes *c*, c_1, *b*, and $a + a_3$ were detected as previously indicated (see Figure 1B). The spectral profiles of Δ*cyc2* strains carrying the *cyt1* suppressor mutations D, H, L, or S are identical to that of a Δ*cyc2 cyt1*-CATCH strain (data not shown). (C) Heme stain and immunodetection of mitochondrial *c*-type cytochromes and assembly proteins in the *cyc2*-null mutants. Mitochondrial proteins (85 μg) purified from cells grown in galactose medium were separated by LDS–PAGE for heme staining or by SDS–PAGE for immunoblotting as described in MATERIALS AND METHODS. The *cyt1*-CADCH and *cyt1*-CAPCH were selected as suppressor mutations of the absence of CC_1HL (BERNARD *et al.* 2003). The Δ*cyc2 cyt1*-CADCH, unlike Δ*cyc2 cyt1*-CAPCH, does not display a respiratory-deficient phenotype (BERNARD *et al.* 2005). Coq1 immunodetection serves as a loading control.

respiration (Figure 1A). As expected, overexpression of *CYT1* in Δ*cyc2 cyt1*-CAPCH resulted in levels of *c*-type cytochromes identical to that of a Δ*cyc2* strain while overexpression of *CYC2* yielded a spectral phenotype identical to the one of the *cyt1*-CAPCH mutant (Figure 1B). We also demonstrated that overexpression of either CCHL- or apocytochrome *c*-encoding genes in Δ*cyc2 cyt1*-CAPCH does not restore cytochrome *c* or c_1 assembly (not shown). We concluded that CC_1HL acts as a multicopy suppressor that can partially compensate for the dual deficiency of the holocytochromes *c* and c_1 in the Δ*cyc2 cyt1*-CAPCH mutant.

Suppressor mutations of the Δ*cyc2 cyt1*-CAPCH mutant map to the cytochrome c_1 heme-binding site: We observed that Δ*cyc2 cyt1*-CAPCH could revert to respiratory proficiency, allowing us to isolate SSP (for *S*uppressor of *S*ynthetic *P*henotype) strains. Spontaneous independent suppressors (SSP1–SSP14) of the Δ*cyc2 cyt1*-CAPCH were isolated and further characterized. All SSP strains were restored to the same extent of respiratory growth (Figure 2A). Genetic analysis indicated that the suppressor mutation is monogenic and linked to the original mutations. Because *CYC2* and *CYT1* are genetically linked and the Δ*cyc2* allele corresponds to a complete deletion of the *CYC2* gene, we reasoned that the suppressor mutation mapped to the cytochrome c_1-encoding gene (*CYT1*). Sequencing of the *CYT1* gene in the different SSP strains showed that a single-nucleotide change resulted in the alteration of the proline in the CAPCH heme-binding site to A, H, L, S, or T residues (Table 2). Note that SSP13 and SSP14 have reverted to a wild-type heme-binding site (CAACH), an expected finding as assembly of wild-type cytochrome c_1 does not require Cyc2p (BERNARD *et al.* 2005) (Figure 2, B and C). Analysis of the holoforms of *c*-type cytochromes of representative SSP strains carrying the CAHCH, CALCH, CASCH, and CATCH heme-binding sites reveals that holocytochrome c_1 accumulation is restored to the wild-type level while the level of holocytochrome *c* is similar to that of the Δ*cyc2* mutant (Figure 2, B and C).

The suppressor mutations do not affect cytochrome c_1 assembly: The observation that the A-to-P mutation results in a slow respiratory growth associated with a decrease in holocytochrome c_1 accumulation (BERNARD *et al.* 2005) (Figure 3) prompted us to assess the impact of the *cyt1*-CAHCH, -CALCH, -CASCH, and -CATCH alleles in an otherwise wild-type background. Strains carrying cytochrome c_1 with a CAHCH, CALCH, CASCH, or CATCH heme-binding site were constructed by genetic crosses and examined for respiratory competence and holocytochrome *c* assembly. All strains were found to have respiratory growth rates similar to that of the wild type (Figure 3A). Spectral and heme stain analyses showed that they were indistinguishable from wild type as far as holocytochrome *c* and c_1 assembly is concerned (Figure 3, B and C). We concluded that, unlike the A-to-P mutation, the A-to -H, -L, -S, and -T substitutions in the heme-binding site have no impact on holocytochrome c_1 assembly.

Redox titrations of apocytochromes c_1 do not indicate a change in the midpoint potential of cysteines at the heme-binding site: One possible scenario to account for the fact that CAPCH cytochrome c_1 assembly is dependent upon Cyc2p is that the A-to-P mutation

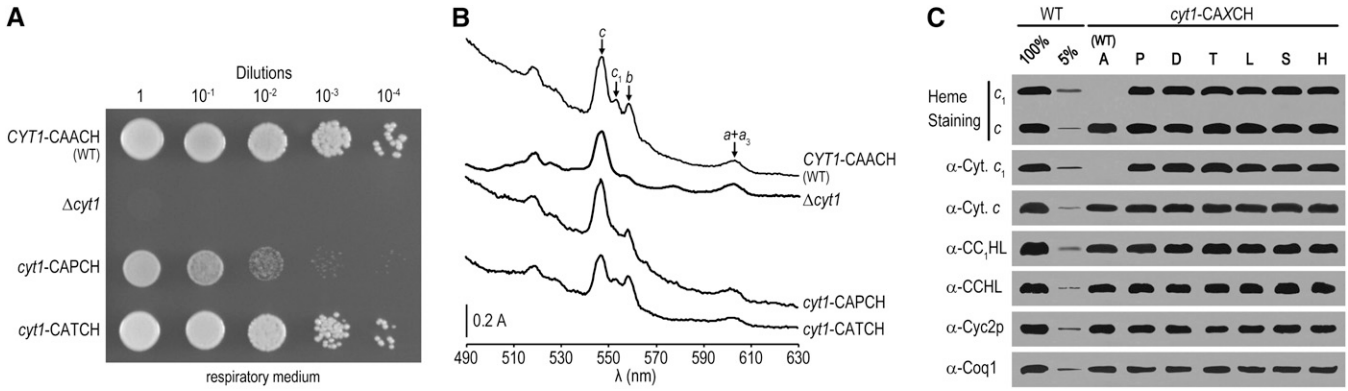

FIGURE 3.—The suppressor mutations at the heme-binding motif do not impact cytochrome c_1 maturation. (A) Respiratory growth of the *cyt1* mutants. Ten-fold serial dilutions of a wild-type (WT) strain carrying the *CYT1*-CAACH allele (W303-1A), Δ*cyt1* (PHT3), *cyt1*-CAPCH (YPH71-14B), and *cyt1*-CATCH (YDM1-2A) strains were grown on respiratory medium containing ethanol/glycerol as described in Figure 2A. The respiratory phenotype of strains harboring *cyt1*-CA(D, L, S, or H)CH mutations is indistinguishable from the *cyt1*-CATCH or wild-type strains (data not shown). (B) Cytochrome absorbance spectra of the *cyt1* mutants. The absorption maxima of cytochromes *c*, c_1, *b*, and $a + a_3$ were measured as previously mentioned (see Figure 1B). Absorbance spectra profiles for strains carrying *cyt1*-CA(D, L, S, or H)CH mutations are comparable to the *cyt1*-CATCH strain (data not shown). (C) Heme stain and immunodetection of mitochondrial *c*-type cytochromes and assembly factors in the *cyt1* mutants. Mitochondrial proteins were prepared, separated, and visualized for heme staining and immunoblotting as described in MATERIALS AND METHODS. Immunoblotting against Coq1 served as a loading control.

alters the redox chemistry of the cysteinyl thiols so that the apocytochrome c_1 heme-binding site is present in a predominantly oxidized, disulfide form. In such a scenario, the cysteine thiols necessary for heme attachment are either not present or present only to a limited extent. To assess whether mutations in the heme-binding motif of cytochrome c_1 might affect the redox chemistry of the cysteinyl thiols of the heme-binding motif in a manner that would make the oxidized disulfide state thermodynamically more stable, we chose to measure the midpoint redox potential (E_m) of recombinant wild-type (CAACH) and mutant (CAPCH and CATCH) apocytochromes c_1. This is the best quantitative measure of the thermodynamic propensity of a cysteine pair to be either in a reduced or in an oxidized state (SEVIER and KAISER 2002; ORTENBERG and BECKWITH 2003). It originally appeared likely that CAPCH apocytochrome c_1 might be characterized by a midpoint potential value that was substantially more negative than that of the wild-type form, given our prediction that the A-to-P mutation produces a higher level of the oxidized form of the apoprotein. To test this hypothesis, redox titrations were carried out using recombinant forms of the apocytochrome c_1 that corresponded to the soluble domain of the protein and did not contain cysteine residues other than the pair present at the heme-binding site. Measurements of redox potentials were carried out using DTT_{red}/DTT_{ox} buffers to poise samples at defined ambient potential (E_h) values, prior to their treatment with the thiol modifier AMS (MOTOHASHI and HISABORI 2006). AMS increases the molecular weight of a protein by 0.5 kDa per thiol modified, allowing the separation of the doubly modified, reduced form from the unmodified oxidized form by electrophoresis on a nonreducing SDS gel (Figure 4A). All titrations gave excellent fits to the Nernst equation for a single two-electron redox component, with E_m values of -266 ± 4 mV, -261 ± 3 mV, and -257 ± 3 mV for the apocytochromes c_1 carrying the CAACH (wild type), CATCH, and CAPCH variations of the heme-binding motif, respectively (Figure 4B). Because there was little difference between the midpoint potentials determined for these three forms of the apocytochrome c_1, we concluded that the A, P, and T residues did not significantly impact the thermodynamic propensity of the heme-binding site to occur in a disulfide *vs.* dithiol form.

The CASCH mutation suppresses the absence of CC_1HL: The *cyt1*-CAPCH allele and the *cyt1*-CADCH mutation were originally isolated in a screen for mutations enhancing the activity of CCHL toward apocytochrome c_1 in the absence of its cognate lyase CC_1HL (BERNARD *et al.* 2003). To test whether H, L, S, and T mutations increase the activity of CCHL toward apocytochrome c_1, we generated the Δ*cc$_1$hl cyt1*-CAHCH, -CALCH, -CASCH, and -CATCH strains. In such strains, CC_1HL is absent, and apocytochrome c_1 conversion to its corresponding holoform is completely dependent upon CCHL. While cytochrome c_1 with a CASCH heme-binding domain could restore the respiratory growth in a Δ*cc$_1$hl* strain, cytochrome c_1 with a CAHCH, CALCH, or CATCH heme-binding domain further diminished the ability of the Δ*cc$_1$hl* strain to grow on a respiratory substrate (Figure 5A). Consistent with the respiratory growth phenotype, the Δ*cc$_1$hl cyt1*-CASCH strain exhibits enhanced level of holocytochrome c_1 while the Δ*cc$_1$hl cyt1*-CATCH strain accumulates lower level of holocytochrome c_1 than the Δ*cc$_1$hl* strain (Figure 5, B and C).

TABLE 2

Sequences of cytochrome c_1 heme-binding sites

Strain	DNA sequence	Heme-binding motif
Wild type	TGT GCC GCC TGC CAT	CAACH
Δ*cyc2 cyt1-34*	TGT GCC CCC TGC CAT	CAPCH
SSP1,2	TGT GCC ACC TGC CAT	CATCH
SSP4,5,8,10	TGT GCC CAC TGC CAT	CAHCH
SSP6	TGT GCC CTC TGC CAT	CALCH
SSP3,7,9,11,12	TGT GCC TCC TGC CAT	CASCH
SSP13,14	TGT GCC GCC TGC CAT	CAACH

Note that the Δ*cc$_1$hl* strain displays a reduced level of respiratory growth that is attributed to the weak activity of CCHL toward apocytochrome c_1 (Bernard *et al.* 2003). We concluded that the S mutation in the heme-binding site increased the activity of CCHL toward apocytochrome c_1 while the H, L, and T residues decreased the activity of CCHL toward its noncognate substrate.

Cyc2p is not required for CC$_1$HL-dependent assembly of cytochrome c_1: Earlier studies have established that the assembly of cytochrome c_1 proceeds normally in the absence of CCHL, an indication that holocytochrome c_1 is synthesized independently via its cognate heme lyase, CC$_1$HL (Matner and Sherman 1982; Dumont *et al.* 1987; Stuart *et al.* 1990; Bernard *et al.* 2003). To assess whether Cyc2p function was also required for the assembly of cytochrome c_1 via CC$_1$HL, we constructed a *cchl*-null *cyc2*-null mutant and monitored the level of *c*-type cytochromes by spectra (Figure 6). As expected, the *cchl*-null *cyc2*-null strain is deficient in holocytochrome *c* due to the lack of its cognate heme lyase but retains a wild-type level of cytochrome c_1, suggesting that CC$_1$HL does not require the activity of Cyc2p for conversion of apocytochrome c_1 to its corresponding holoform.

The function of Cyc2p is clearly redundant based on the fact that a *cyc2*-null mutant is not completely deficient in cytochrome *c* (Figure 2, B and C). Functional redundancy does not involve Mcr1p and Cbr1p, two proteins with mitochondrial localization that exhibit similarity to Cyc2p (Haucke *et al.* 1997; Sickmann *et al.* 2003; Bernard *et al.* 2005) (not shown).

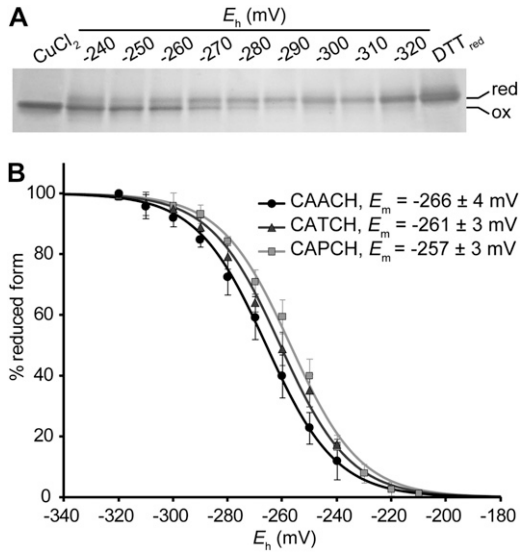

Figure 4.—Determination of the redox midpoint potential of wild-type and mutant apocytochromes c_1. Redox titrations of the dithiol/disulfide couple in apocytochrome c_1 were carried out using DTT redox buffer as described in materials and methods. Redox equilibration (5 μg of apocytochromes c_1 per reaction) was performed under anaerobic conditions prior to the AMS labeling. (A) Separation of oxidized and reduced forms of CAACH apocytochrome c_1 under nonreducing SDS–PAGE. Oxidized (non-modified) and reduced (AMS modified) forms of wild-type proteins carrying a CAACH heme-binding motif were separated on a 16% nonreducing SDS gel and visualized by silver staining. As a control for fully oxidized or fully reduced forms, apocytochrome c_1 was incubated in the presence of either 50 μM $CuCl_2$ or 5 mM DTT_{red}, respectively. (B) Oxidation-reduction titrations of wild-type and mutant apocytochromes c_1. Oxidized (unmodified) and reduced (AMS modified) fractions of apoproteins for different ambient potentials were quantified by using Image J software. Percentages of the reduced form of apocytochrome c_1 with a CAACH (wild type, solid circles), CATCH (darkly shaded triangles), and CAPCH (lightly shaded squares) heme-binding motif were plotted as a function of ambient potential (E_h). Lines represent fits of the data to a two-electron Nernst curve. Error bars correspond to the standard deviation calculated from three independent experiments.

DISCUSSION

Previous studies have identified Cyc2p, a pyridine nucleotide-dependent flavoprotein, as a redox component controlling the maturation of mitochondrial *c*-type cytochromes (Bernard *et al.* 2003, 2005). Genetic analyses revealed that Cyc2p is not absolutely required for *c*-type cytochrome maturation but becomes essential when the cytochrome c_1 heme-binding site is modified from CAACH to CAPCH, an indication that the C*XX*CH motif could be the relevant target of Cyc2p action *in vivo* (Bernard *et al.* 2005). Our initial interpretation of this observation was that the A-to-P mutation altered the redox chemistry of the cysteinyl thiols so that the apocytochrome c_1 CAPCH heme-binding site exists largely in an oxidized form (*i.e.*, the cysteines form an intramolecular disulfide bond) and is no longer able to participate in the heme attachment without Cyc2p intervention. It is now well recognized that the nature of the residues between the cysteines at a C*XX*C redox motif influences the propensity of this motif to be in the oxidized (disulfide) or reduced form (dithiol). Examples come from the documented changes in midpoint potential caused by mutations of the residues between the two cysteines at the redox active site of thioredoxin (Krause *et al.* 1991; Lundstrom *et al.* 1992; Mossner *et al.* 1999), protein disulfide isomerase (Chivers *et al.* 1996) and DsbA oxido-reductase (Grauschopf *et al.*

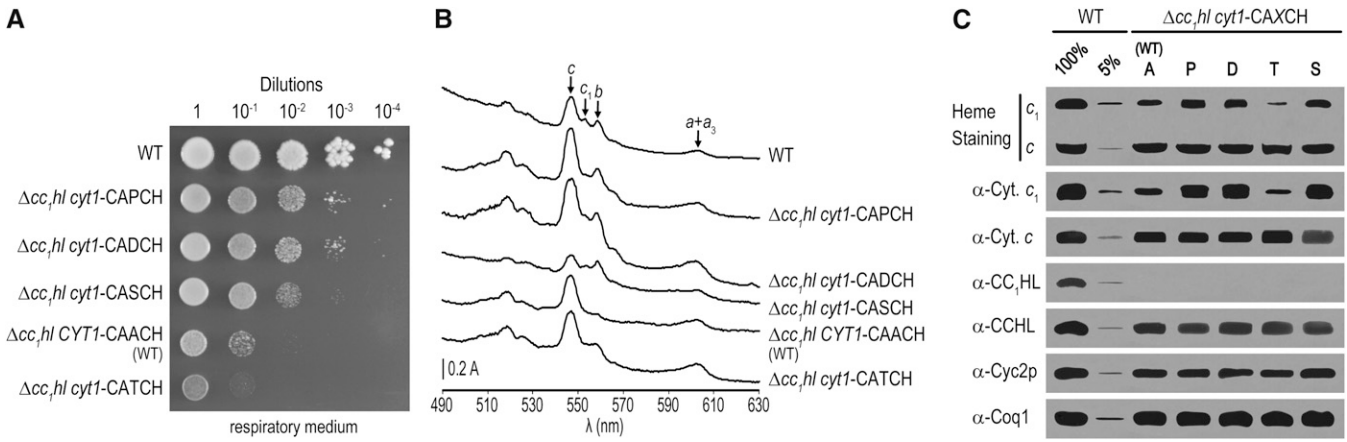

FIGURE 5.—Impact of the mutations at the cytochrome c_1 heme-binding motif in the absence of CC_1HL. (A) Respiratory growth of the Δ*cc*$_1$*hl* mutants. Serial dilutions of wild-type (WT) (W303-1A), Δ*cc*$_1$*hl cyt1*-CAPCH (UV34), Δ*cc*$_1$*hl, cyt1*-CADCH (UV48), Δ*cc*$_1$*hl cyt1*-CASCH (YDM4-1B), Δ*cc*$_1$*hl* carrying the wild-type allele CAACH (SMY1), and Δ*cc*$_1$*hl cyt1*-CATCH (YDM1-1B) strains were grown on ethanol/glycerol as respiratory substrates as mentioned in Figure 2A. In a *cc*$_1$*hl*-null context, the *cyt1* mutants carrying the P, D (BERNARD *et al.* 2003), or S (this work) mutations at the heme-binding motif are restored for respiratory growth when compared to a Δ*cc*$_1$*hl*. Δ*cc*$_1$*hl cyt1*-CATCH, -CAHCH, and -CALCH all display the same respiratory growth (not shown). (B) Cytochrome absorbance spectra of the Δ*cc*$_1$*hl* mutants. The absorption spectra of cytochromes *c*, c_1, *b*, and $a + a_3$ were detected as previously described (see Figure 1B). (C) Heme stain and immunodetection of mitochondrial *c*-type cytochromes and assembly proteins in the Δ*cc*$_1$*hl* mutants. Heme staining and protein immunodetection were performed as indicated in MATERIALS AND METHODS. Coq1 immunodetection was used as a loading control.

1995). To better understand why Cyc2p is required for the assembly of cytochrome c_1 with a CAPCH heme-binding site, we isolated secondary mutations that enable Cyc2p-independent assembly of cytochrome c_1. All the bypass mutations that we isolated change the proline at the cytochrome c_1 heme-binding motif to A (wild-type), H, L, S, or T residues and restored a wild-type level of holocytochrome c_1 assembly (Figure 2). As stated above, we had originally interpreted this result in the context of a change in thiol chemistry at the heme-binding site and postulated that cytochrome c_1 with a CAHCH, CALCH, CASCH, or CATCH is able to be assembled because the presence of a H, L, S, and T residue instead of P results in a shift of the equilibrium of the C*XX*CH heme-binding site toward the reduced form (*i.e.*, dithiol) that is competent for the heme ligation reaction. Measurements of midpoint potentials reveal that there is no significant difference between wild-type, CAPCH, and CATCH cytochromes c_1, an indication that, at redox equilibrium, the thermodynamic stability of the disulfide bond at the heme-binding site is not influenced by the P and T mutations (Figure 4). Hence it is unlikely that the Cyc2p-dependent assembly of CAPCH cytochrome c_1 can be explained by a change in the equilibrium dithiol–disulfide ratio of the apocytochrome c_1 heme-binding site. However, because the midpoint potential reflects only the redox state at thermodynamic equilibrium (SEVIER and KAISER 2002), it is conceivable that the P residue has altered the reactivity of cysteines in the C*XX*CH motif so that Cyc2p activity is required for the heme attachment to apocytochrome c_1.

We had previously shown that apocytochrome c_1 is a substrate of both CC_1HL and CCHL (BERNARD *et al.* 2003). While we demonstrate in this work that Cyc2p is not required for the CC_1HL-dependent assembly of apocytochrome c_1, its activity is absolutely essential for the CCHL-dependent assembly of apocytochrome c_1 (BERNARD *et al.* 2003). In the Δ*cyc2 cyt1*-CAPCH mutant, the holocytochrome c_1-deficient phenotype can be explained by the fact that both CCHL- and CC_1HL-dependent assembly pathways for apocytochrome c_1 are inactive. Whereas the absence of Cyc2p can account for the lack of cytochrome c_1 assembly via the CCHL pathway, we also envision that CC_1HL-dependent assembly of cytochrome c_1 is abolished in the Δ*cyc2 cyt1*-CAPCH mutant due to the inability of CC_1HL to interact with CAPCH apocytochrome c_1. This is supported by the fact that the level of assembled CAPCH holocytochrome c_1 is not influenced by the presence or the absence of CC_1HL (Figures 3 and 5), an indication that CAPCH holocytochrome c_1 assembly is dependent only on CCHL. Because the A-to-P modification in cytochrome c_1 was recovered in a search for mutations that suppress the absence of CC_1HL (BERNARD *et al.* 2003), it is conceivable that the P residue enhances the activity of CCHL toward apocytochrome c_1 while simultaneously preventing the CC_1HL from acting on its cognate substrate. The finding that the H, L, S, and T mutations (Figure 2) and CC_1HL overexpression (Figure 1) suppress the cytochrome c_1 defect in a Δ*cyc2 cyt1*-CAPCH context can be explained by a restoration of the CC_1HL-dependent assembly of cytochrome c_1, presumably via enhanced interaction between CC_1HL and its cognate apocytochrome c_1 substrate.

The observation that the Δ*cyc2 cyt1*-CAPCH mutant also displays a cytochrome *c* deficiency suggests that

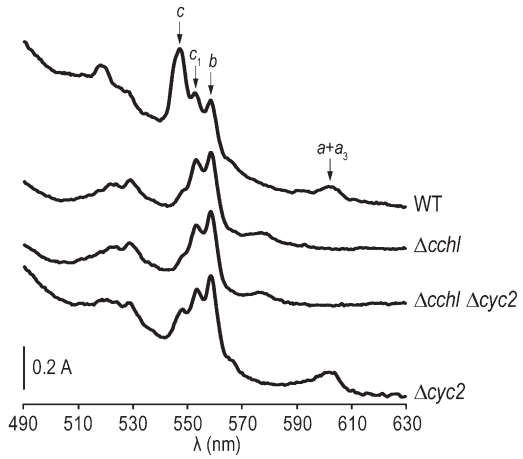

FIGURE 6.—Cyc2p is not required for the CC_1HL-dependent assembly of cytochrome c_1. Whole-cell absorption spectra of wild type (WT) (W303-1A), Δ*cchl* (SMY4), Δ*cchl* Δ*cyc2* (YCT2-14C), and Δ*cyc2* (YPH1) strains were recorded as mentioned in Figure 1B. Note the absence of spectrally detectable cytochromes $a + a_3$ in the Δ*cchl* and Δ*cchl* Δ*cyc2* mutants. This phenotype is not a specific trait of the *cchl*-null mutants but results from the loss of cytochrome oxidase assembly as a secondary effect due to the absence of holocytochrome *c* (SHERMAN *et al.* 1965; PEARCE and SHERMAN 1995).

holocytochrome *c* assembly is also blocked as a result of an A-to-P mutation in the heme-binding site of cytochrome c_1. As apocytochrome *c* can be acted upon only by CCHL, we postulate that CCHL activity toward apocytochrome *c* is inactivated in the Δ*cyc2 cyt1*-CAPCH strain. Two possible scenarios can be envisioned to account for this phenotype. In one model, CCHL can no longer act on apocytochrome *c* because CAPCH apocytochrome c_1 is trapped in a complex with the lyase. However, we view this model as unlikely on the basis of our observation that overexpression of CCHL in the Δ*cyc2 cyt1*-CAPCH strain does not relieve the block in holocytochrome *c* assembly (not shown). The observation that the apoform of the molecule is not immunodetected in the Δ*cyc2 cyt1*-CAPCH strain suggests that it has undergone proteolytic degradation, which does not appear consistent with a model in which apocytochrome c_1 is trapped in a stable CCHL complex. Instead, we favor an alternative scenario in which, in the absence of Cyc2p, CAPCH apocytochrome c_1 is engaged in a transient interaction with CCHL resulting in a deactivated enzyme that cannot assemble apocytochrome *c*. Note that the Δ*cyc2* Δ*cc*$_1$*hl* strain also displays a dual deficiency in cytochromes *c* and c_1, similarly to the Δ*cyc2 cyt1*-CAPCH mutant (BERNARD *et al.* 2005). Hence, we postulate that this deactivation is Cyc2p-dependent and takes places in situations where apocytochrome c_1 maturation is dependent only on CCHL (*i.e.*, when the apocytochrome c_1 heme-binding site carries the A-to-P mutation or in the absence of CC_1HL).

The proposal that the H, L, S, and T residues restore the CC_1HL-dependent assembly of cytochrome c_1 further underscores the critical role of this residue in the maturation process. Interestingly, while the S residue at this position enhances CCHL-dependent assembly of cytochrome c_1, the presence of H, L, or T further decreases the intrinsic activity of CCHL toward apocytochrome c_1 (Figure 5). This suggests that the identity of the second intervening amino acid in the C*XX*CH motif is crucial in modulating the CCHL-dependent assembly of cytochrome c_1. The fact that CAPCH apocytochrome c_1 has become dependent on CCHL alone for its assembly indicates that the P residue is essential in determining the CCHL- *vs.* CC_1HL-dependent assembly of cytochrome c_1. It is likely that the nature of the residue is a determinant for enzyme–substrate interaction, but it is also possible that CCHL- or CC_1HL-catalyzed assembly requires a specific residue at this position for the enzymatic reaction.

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