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Engineering a prokaryotic apocytochrome *c* as an efficient substrate for *Saccharomyces cerevisiae* cytochrome *c* heme lyase

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Abstract

Cytochromes *c* are heme proteins that require multiple maturation components, such as heme lyases, for cofactor incorporation. *Saccharomyces cerevisiae* has two heme lyases that are specific for apocytochromes *c* (CCHL) or *c*₁ (CC₁HL). CCHL can covalently attach heme *b* groups to apocytochrome *c* substrates of eukaryotic but not prokaryotic origin. Besides their conserved Cys-Xxx-Xxx-Cys-His heme-binding motifs, the amino-terminal regions of apocytochrome *c* substrates appear to be important for CCHL function. In this study, we show for the first time that only two amino acid changes in the amino-terminal region of the non-CCHL substrate apocytochrome *c*₂ from *Rhodobacter capsulatus* are necessary and sufficient for efficient holocytochrome *c* formation by CCHL. This finding led us to propose a consensus sequence located at the amino-terminus of apocytochromes *c* and critical for substrate recognition and heme ligation by CCHL.

Keywords

Cytochrome *c* maturation; cytochrome *c* heme lyase; substrate specificity; apocytochrome *c*; heme attachment

1. Introduction

Cytochromes *c* are ubiquitous hemoproteins that act primarily as electron carriers between respiratory or photosynthetic energy transduction pathways, and are also involved in other

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cellular processes. They contain at least one heme *b* (protoporphyrin IX-Fe) cofactor bound covalently to the polypeptide chain via (usually) two thioether bonds between the vinyl groups of the porphyrin ring and the cysteine-sulfhydryl groups of a Cys-Xxx-Xxx-Cys-His heme-binding site [1–2]. Multiple distinct biogenesis machineries responsible for post-translational attachment of heme to apocytochromes *c* have been identified in various organisms and organelles [3–6]. One of these machineries (Ccm system III) is confined to mitochondria of fungi, metazoans and some protozoa. It includes one or two components with defined heme lyase activities towards apocytochrome *c* substrates, and a NAD(P)H-dependent heme reductase as an accessory factor [7–9]. Some eukaryotes like *Saccharomyces* (*S.*) *cerevisiae* contain two heme lyases with substrate specificities for either apocytochromes *c* (CCHL) or *c*₁ (CC₁HL) [10–11]. Other eukaryotes, including human and mouse, have only one heme lyase (also known as holocytochrome *c* synthetase or HCCS) with a broader substrate specificity, able to mature both cytochromes *c* and *c*₁ [11–13]. CCHLs, CC₁HLs and HCCSs show significant sequence similarity (~ 35% amino acid identity), and harbor one to four Cys-Pro-Val motifs [12,14], frequently found in proteins interacting with heme *b* groups [15], but shown to be not necessary for CCHL-dependent holocytochrome *c* formation [16].

Previous studies [17–32] have revealed that heme can be ligated to various apocytochromes *c* of eukaryotic origin in the cytoplasm of *Escherichia* (*E.*) *coli* hosts upon coproduction with *S. cerevisiae* CCHL, demonstrating that CCHL matures efficiently holocytochromes *c* in a redox-controlled heterologous environment (Fig. 1). However, holocyt *c* formation does not occur using apocyt *c* of prokaryotic origin, such as *Paracoccus* (*P.*) *denitrificans* cytochrome *c*₅₅₀ [30]. Yet, a chimeric apocytochrome *c* formed of *S. cerevisiae* iso-2-cyt *c* (Cyc7p) and *P. denitrificans* cytochrome *c*₅₅₀ can be ligated with heme by CCHL provided that at least the first 28 amino acids of Cyc7p, including its conserved heme binding site, are present [33]. Recently, data obtained with similar chimeric apocytochrome *c* constructs confirmed these findings [27,34], and established that the substrate recognition domain for CCHL is confined the amino-terminal region encompassing the heme binding motif of apocytochromes *c*. Thus, the determination of the amino acid composition of the amino-terminal portion of apocytochromes *c* is critical for the understanding of the mechanisms underlying substrate recognition and heme attachment by CCHL.

In this work, we first showed that, like *P. denitrificans* cytochrome *c*₅₅₀, the prokaryotic *Rhodobacter* (*R.*) *capsulatus* cytochrome *c*₂ (CycA) is produced in the cytoplasm of *E. coli* only as an apoform, both in the absence or presence of *S. cerevisiae* CCHL. Then, we carried out mutational analyses that stem from the comparison of the available amino-terminal sequences of mitochondrial cyts *c*, known to be matured by CCHL in the cytoplasm of *E. coli* (Fig. 1). We found that a Glu to Leu substitution at position 9, together with a Lys insertion at position 11 in *R. capsulatus* CycA are necessary and sufficient to render it an efficient CCHL substrate. These findings led us to propose a consensus sequence motif for apocytochrome *c* recognition and heme attachment by CCHL.

2. Materials and Methods

2.1 Cloning and Expression

Molecular biology techniques were performed according to Sambrook *et al* [35]. All constructs were confirmed by DNA sequencing. The bacterial strains and plasmids used in this work are described in Table 1. *R. capsulatus* cytochrome *c*₂ (CycA) mutants were constructed by site-directed mutagenesis using the QuickChange site directed mutagenesis kit (Stratagene, La Jolla, CA) and the plasmid pCS1726 as a template. This plasmid contains an in-frame Strep-tag II epitope (WSHPQFEK) sequence fused at the 5'-end of *cycA* encoding CycA without its native signal peptide [36]. *E. coli* strain EC06, carrying a deletion of all cytochrome *c* maturation genes *ccmABCDEFGH* [37], was first transformed with pCS315 harboring the gene encoding *S. cerevisiae* CCHL fused with a 6xHis-tag sequence at its 5'-end [33]. Then, the EC06 strain harboring pCS315 was transformed with compatible plasmids containing appropriate apocytochrome *c* mutants. Overnight cultures (1 ml) of the EC06/pCS1726+pCS315 transformants (as well as the other apocytochromes variants) in Luria–Bertani (LB) broth medium, supplemented with 100 µg/ml ampicillin and 50 µg/ml chloramphenicol were used to inoculate 10 mL of the same medium in 50 mL tubes, and grown at 30 °C with gentle shaking until an OD_{600nm} of 0.6. At this OD, cultures were induced by adding 1 mM IPTG for 4 h under the same growth conditions. Cells were then harvested by centrifugation and stored at –20 °C until further use.

2.2 Protein Analysis

E. coli cells were resuspended in BugBuster® Protein Extraction Reagent (Novagen®) supplemented with 300 mM NaCl and 1 mM PMSF. After 45 min incubation with gentle stirring at room temperature, insoluble material was removed by centrifugation at 16 000 × *g* for 15 min at 4 °C. Protein concentration of the clear supernatants was determined using the Bicinchoninic Acid kit (Sigma, Inc.) with bovine serum albumin as a standard. SDS-PAGE was performed using 15% acrylamide-bisacrylamide gels according to [38]. Samples were resuspended in 2× loading buffer without addition of any reducing agent and incubated at 42 °C for 10 min. For immunodetection of Strep-tag fusions to cytochromes *c*, gel-resolved proteins were electroblotted onto Immobilon-PVDF membranes (Millipore, Inc.), and probed with Strep•Tag® II Monoclonal antibodies (Novagen®). Horseradish peroxidase conjugated anti-mouse IgGs (GE Healthcare, Inc.) were used as secondary antibodies, and detection was performed using the SuperSignal West Pico Chemiluminescent Substrate® (Thermo Scientific, Inc.). For detection of proteins containing covalently attached heme, SDS-PAGE resolved proteins were electroblotted onto nitrocellulose membranes (Schleicher and Schüll), which were washed with 50 mM Tris-HCl (pH 8.0) and 150 mM NaCl, and the presence of heme-dependent peroxidase activity was monitored using the SuperSignal West Pico Chemiluminescent Substrate® [39].

Visible spectra were recorded at room temperature in a Cary 60 spectrophotometer (Agilent Technologies, Inc) between 400 and 600 nm. Reduction of the total protein extracts was carried out by adding a few grains of sodium dithionite prior to recording the spectra.

3. Results and Discussion

3.1 Amino acid residues at the amino-terminal portion of apocytochromes *c* critical for CCHL activity

Mitochondrial cytochromes *c* have been reported to be covalently attached with heme by *S. cerevisiae* CCHL upon coproduction in the cytoplasm of *E. coli* whereas cytochromes *c* of prokaryotic origin are not [17–32]. Yet, fusion proteins containing the amino-terminal portion including the Cys-Xxx-Xxx-Cys-His heme binding motif of mitochondrial and the carboxyl-terminal portion of prokaryotic cytochromes *c* can be covalently ligated with heme by CCHL. Thus, only the amino-terminal portions of mitochondrial cytochromes *c* are needed for CCHL-dependent holocytochrome *c* formation, suggesting that the amino acid sequences and/or structural properties of the other apocytochrome regions are not essential for heme ligation [27,33–34].

In this study, we used cytochrome *c*₂ (CycA) from *R. capsulatus* as a prokaryotic, non-CCHL apoprotein substrate model and showed that it is produced in the cytoplasm of *E. coli* only as an apoform, independent of the presence or absence of *S. cerevisiae* CCHL (Fig. 2B and 2C, lanes 4). Then, we compared the amino-termini of mitochondrial cytochromes *c* that are matured by CCHL in the same heterologous host compartment with those of prokaryotic or mutant mitochondrial cytochromes *c* that are not CCHL substrates (Fig. 1). As a first mutational step, we fused the amino-terminus of *S. cerevisiae* Cyc7p, including its heme binding site Cys14-Gln15-Gln16-Cys17-His18, with the carboxyl-terminal portion of *R. capsulatus* CycA, creating Cyc7p^{H18-S19}CycA (Fig. 2A). As expected, this fusion protein became competent for covalent heme ligation upon coproduction with CCHL (Fig. 2B and 2C, lanes 2). Next, we sought to define the amino acid residues in the amino-terminal portion of *R. capsulatus* CycA that are required for efficient heme ligation by CCHL.

Amino acid residues Cys14, Cys17, and His18 (horse cytochrome *c* numbering) are conserved in virtually all cytochromes *c*, with the sole exception of some *Euglenozoa* mitochondrial cytochromes *c* [40]. However, a *S. cerevisiae* iso-1-cytochrome *c* (Cyc1p) variant with Cys14 to Ser substitution can be ligated with heme by CCHL, while a variant of the same cytochrome *c* with a Cys17 to Ser replacement cannot [31]. The fact that CCHL does not require Cys14 for proficient heme attachment to apocytochrome substrates, together with its mitochondrial location, suggest a plausible link between the CCHL (System III) and the proposed Euglenozoan Ccm system V, which ligates heme *b* groups to mitochondrial cytochromes *c* with a single Cys heme binding sequence (Xxx-Xxx-Xxx-Cys-His) [4]. However, this fact clearly distinguishes the substrate specificity of CCHL from that of Ccm system I (found in bacteria, archaea, and plant mitochondria), which requires a Cys-Xxx-Xxx-Cys-His heme binding motif for covalent and stereo-specific heme incorporation into cytochromes *c* [3,41]. Like Cys17, His18 is also essential for heme attachment (Fig. 1). It acts as a proximal axial ligand for the heme-iron and is invariantly present in all cytochromes *c* [1]. The heme binding motif of horse cytochrome *c* (*Equus caballus* CYCS) can be changed to Cys-Xxx-Xxx-Xxx-Cys-His (but not to Cys-Xxx-Xxx-Xxx-Xxx-Cys-His), and still remains a competent CCHL substrate, albeit at a lower efficiency [28]. A

similar spacing flexibility between the Cys residues in the heme binding site sequence of apocytochrome *c* substrates has also been observed for the Ccm system I [42–43].

Besides the conserved Cys-Xxx-Xxx-Cys-His heme binding site, mitochondrial cytochromes *c* have in general conserved Gly1, Lys5, Gly6, Phe10 and Gln16, as well as a positively charged residue at position 13 (Lys or Arg) and an hydrophobic residue at position 9 (Iso or Leu) [44]. Mutation of Gly6 and Phe10 to Ala in the horse cytochrome *c* or the yeast Cyc1p eliminates CCHL-dependent holocytochrome *c* production [27–28], underlining their critical role for CCHL activity. In the contrary, mutation of Gln16 in Cyc7p [32] or Gln15 in horse cytochrome *c* [24] to Cys does not hinder the ability of CCHL to mature the corresponding cytochrome *c* variants. Similar flexibility for equivalent amino acid residues within the heme binding site has also been observed for Ccm system I and its apocytochromes *c* substrates [42]. To confirm this plasticity at positions 15 and 16, we constructed a fusion protein (Cyc7p^{R13-C14}CycA) with the amino-terminal part of Cyc7p up to the residue Arg13, and the carboxyl-terminal portion of CycA, including its heme binding site Cys14-Lys15-Thr16-Cys17-His18 (Figure 2A). Indeed, this fusion protein was covalently ligated with heme upon coproduction with CCHL in *E. coli* (data not shown). The same results were also obtained with a fusion protein containing the amino-terminal portion of Cyc1p and the C-terminal portion of *P. denitrificans* cytochrome *c*₅₅₀ including the latter heme binding sequence (Cys14-Lys15-Ala16-Cys17-His18) [27].

Change of Gly1, Lys5, or Arg13 to Ala in Cyc1p, Lys5 to Ala in horse cytochrome *c*, and Arg13 to Cys in Cyc7p do not affect production of the respective cytochrome *c* variants by CCHL [27,32]. When we constructed a fusion protein, ^{K5}Cyc7p^{H18-S19}CycA, composed of a truncated amino terminus of Cyc7p from Lys5 to His18 (deletion of the amino acid residues from Ala-9 to Lys4) and the carboxyl terminal portion of CycA (Fig. 2A), we observed that it was ligated with heme upon coproduction with CCHL in *E. coli* (Fig. 2B and 2C, lanes 3). Therefore, our data confirm that fusion proteins containing the amino-terminal portion of mitochondrial cytochromes *c* (starting with Lys5 up to the heme binding site Cys14-Xxx-Xxx-Cys17-His18) and the carboxyl-terminal portion of prokaryotic cytochromes *c* can be efficient CCHL substrates. Overall, these observations suggest that the amino acid sequence and/or structural determinants required for heme ligation by CCHL reside between the positions 5 to 18 of the mitochondrial cytochromes *c*.

3.2 Mutational analysis of the positions necessary for holocytochrome *c* formation by CCHL

Besides the conserved amino acid residues described above, the sequence alignment shown in Fig. 1 highlights that all mitochondrial cytochromes *c* have a stretch of three amino acid residues between Phe10 and Cys14. In contrast, the non-CCHL substrates *P. denitrificans* cytochrome *c*₅₅₀ and *R. capsulatus* cytochrome *c*₂ (CycA) have at position 9 a negatively charged Glu instead of a hydrophobic residue, and only two amino acid residues between the conserved Phe10 and the first Cys at the heme binding site.

In order to assess the role(s) of these residues on heme ligation by CCHL, we constructed four mutants using the non-CCHL substrate CycA (Fig. 2A). We tested whether any of the mutant proteins is produced (immunodetection of fused Strep-tag epitope, Fig. 2C), and

covalently attached with heme (chemiluminescence based detection of covalently attached heme in the presence of SDS, Fig. 2B) by a functional His-tagged *S. cerevisiae* CCHL in the cytoplasm of *E. coli*. First, the Glu residue at position 9 of CycA was replaced by the hydrophobic amino acid Leu, as in Cyc1p and Cyc7p (Fig. 1), originating the CycA-E9L variant. This variant was not matured upon coproduction with CCHL in *E. coli*, like the wild-type CycA (Fig. 2A, as well as Fig. 2B and 2C, lanes 4 and 8). Next, a Lys residue was inserted at position 11, producing the variant CycA-insK11 with a stretch of three amino acids between the conserved Phe10 and Cys14 as in mitochondrial cytochromes *c*, e.g., Cyc1p and Cyc7p. In the presence of CCHL, this variant yielded holocytochrome *c* although at a low efficiency (Fig. 2A, as well as Fig. 2B and 2C, lanes 5). Finally, two additional derivatives of CycA-insK11 were constructed to further probe the efficiency of CycA as a substrate for CCHL. In one construct, Asn12 was replaced by a Thr (as in Cyc1p and Cyc7p; other mitochondrial cytochromes *c* contain a Gln or Met at this position) to yield CycA-insK11+N12T (Fig. 2A). In the other construct, Glu9 was substituted with a Leu (like CycA-E9L) yielding CycA-insK11+E9L (Fig. 2A). In the presence of CCHL, the holocytochrome *c* form of CycA-insK11+N12T decreased to a quasi-undetectable level (Fig. 2B and Fig. 2C, lanes 7), but remarkably, with CycA-insK11+E9L the amount of holocytochrome *c* increased dramatically to that seen with Cyc7p, or with the fusion proteins (Fig. 2B and Fig. 2C, lanes 1, 2, 3, and 6). Covalent heme attachment by CCHL to CycA-insK11+E9L was further confirmed by visible spectroscopy using CycA as a control (Fig. 3). The spectrum of the dithionite-reduced total protein extract revealed a Soret band centered at 417 nm, as well as β - and α -bands at 520 and 550nm, respectively, typical for holocytochromes *c*. A α -band located at 550 nm is indicative of the successful formation of two thioether bonds between the vinyl groups of the heme tetrapyrrole ring and the two thiol groups of the Cys residues at the Cys-Lys-Thr-Cys-His heme binding site of *R. capsulatus* cytochrome *c*₂.

Thus, by inserting a Lys at position 11 and by substituting Glu9 with Leu, we were able to convert for the first time a non-CCHL substrate (*i. e.*, CycA) to a highly proficient CCHL substrate (*i. e.*, CycA-insK11+E9L) in the used heterologous *E. coli* system. Finally, based on our overall findings, we propose that the first 14 amino acid residues including the conserved heme binding motif at the amino-terminal portions of cytochromes *c* (Fig. 1) define the consensus sequence “Lys/Ala5-Gly6-Xxx-Xxx-Leu/Ile9-Phe10-Xxx-Xxx-Xxx-Cys14-Xxx-Xxx-Cys17-His18” that is crucial for apoprotein substrate recognition and heme attachment by *S. cerevisiae* CCHL. Our protein engineering approach provides a new rationale for defining the substrate requirements of various mitochondrial heme lyases that are specific for apocytochromes *c* or *c*₁, or both, and may help to elucidate the catalytic mechanism(s) of these poorly understood cytochrome *c* heme lyases.

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Abbreviations

Ccm	cytochrome <i>c</i> maturation
CCHL	cytochrome <i>c</i> heme lyase
CC₁HL	cytochrome <i>c</i> ₁ heme lyase
HCCS	holocytochrome <i>c</i> synthetase
Cyc1p	<i>Saccharomyces cerevisiae</i> iso-1-cytochrome <i>c</i>
Cyc7p	<i>Saccharomyces cerevisiae</i> iso-2-cytochrome <i>c</i>
CycA	<i>Rhodobacter capsulatus</i> cytochrome <i>c</i> ₂
IPTG	isopropylthiogalactoside
PMSF	phenylmethylsulfonyl fluoride

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Highlights

- We test the substrate specificity of *Saccharomyces cerevisiae* cytochrome *c* heme lyase (CCHL)
- CCHL can only ligate heme *b* groups to apocytochromes *c* of eukaryotic origin
- The amino-terminal region of eukaryotic apocytochromes *c* is critical for CCHL activity
- We convert a prokaryotic apocytochrome *c* into an efficient CCHL substrate with two mutations
- We define a consensus sequence for holocytochrome *c* formation by CCHL

Origin	Sequence					Reference
	1	6	10	14	18	
<i>H. sapiens</i> CYCS	GDVEKGGKKIFIMK	QSOCH				[17,18]
<i>M. musculus</i> , somatic, CYCS	GDVEKGGKKIFVOK	QAOCH				[19]
<i>M. musculus</i> , testis, CYCT	GDAEAGKKIFVOK	QAOCH				[19]
<i>T. californicus</i> CYC	GDIDKGGKKIFVOK	QTOCH				[20]
<i>E. caballus</i> CYCS	GDVEKGGKKIFVOK	QAOCH				[21,22]
	GDVEKGGKKIFVOK	QAOCH				[23]
	GDVEKGGKKIFVOK	QAOCH				[23]
	GDVEKGGKKIFVOK	QAOCH				[23]
	GDVEKGGKKIFVOK	QAOCH				[23]
	GDVEKGGKKIFVOK	QAOCH				[24]
	GDVEKGGKKIFVOK	QAOCH				[24]
	GDVEKGGKKIFVOK	QAOCH				[24]
	GDVEKGGKKIFVOK	QAOCH				[25]
	GDVEKGGKKIFVOK	QAOCH				[25]
	GDVEKGGKKIFVOK	QAOCH				[25]
	GDVEKGGKKIFVOK	QAOCH				[25]
	GDVEKGGKKIFVOK	QAOCH				[25]
	GDVEKGGKKIFVOK	QAOCH				[25]
	GDVEKGGKKIFVOK	QAOCH				[26]
	GDVEKGGKKIFVOK	QAOCH				[27]
	GDVEKGGKKIFVOK	QAOCH				[27]
	GDVEKGGKKIFVOK	QAOCH				[27]
	→ GDVEKGGKKIFVOK	QAOCH				[27]
	GDVEKGGKKIFVOK	QAOCH				[27]
	GDVEKGGKKIFVOK	QAOCH				[27]
	→ GDVEKGGKKIFVOK	QAOCH				[27,28]
<i>S. cerevisiae</i> Cyc1p	TEFKAGSAGKGGATLEKTR	LOCH				[29,30]
	TEFKAGSAGKGGATLEKTR	LOCH				[31]
	→ TEFKAGSAGKGGATLEKTR	LOCH				[31]
	GSAGKGGATLEKTR	LOCH				[26]
	GSAGKGGATLEKTR	LOCH				[26]
	GDVEKGGKKIFVOK	QAOCH				[26]
	TEFKAGSAGKGGATLEKTR	LOCH				[27]
	TEFKAGSAGKGGATLEKTR	LOCH				[27]
	→ TEFKAGSAGKGGATLEKTR	LOCH				[27]
	→ TEFKAGSAGKGGATLEKTR	LOCH				[27]
	TEFKAGSAGKGGATLEKTR	LOCH				[27]
<i>S. cerevisiae</i> Cyc7p	AKESTGFKPGSAGKGGATLEKTR	QOCH				[30]
	AKESTGFKPGSAGKGGATLEKTR	QOCH				[32]
	AKESTGFKPGSAGKGGATLEKTR	QOCH				[32]
	AKESTGFKPGSAGKGGATLEKTR	QOCH				[32]
	AKESTGFKPGSAGKGGATLEKTR	QOCH				[32]
	AKESTGFKPGSAGKGGATLEKTR	QOCH				[32]
<i>P. denitrificans</i> CycA	→ QDGDAAKGEKEF-NKOKACH					[30]
<i>R. capsulatus</i> CycA	→ GDAAKGEKEF-NKOKTCH					This work

Figure 1.

Sequence alignment of the amino-terminal portions of eukaryotic and prokaryotic cytochromes *c*. *H. sapiens* CYCS: *Homo sapiens* (or human) cytochrome *c*; *M. musculus*, somatic, CYCS: *Mus musculus* (or mouse) somatic cytochrome *c*; *M. musculus*, testis, CYCT: *Mus musculus* testis-specific cytochrome *c*; *T. californicus* CYC: *Tigriopus* (Tigerpod) *californicus* cytochrome *c*; *E. caballus* CYCS: *Equus caballus* (or horse) cytochrome *c*; *S. cerevisiae* Cyc1p and Cyc7p: *Saccharomyces cerevisiae* (or baker's yeast) iso-1-cytochrome *c* and iso-2-cytochrome *c*, respectively; *P. denitrificans* CycA: *Paracoccus denitrificans* cytochrome *c*₅₅₀; *R. capsulatus* CycA: *Rhodobacter capsulatus* cytochrome *c*₂. Absolutely conserved amino acid residues are highlighted in grey, and mutations in various cytochromes *c* tested experimentally for CCHL-dependent holo-cytochrome *c* formation are indicated in black. Cytochrome *c* derivatives that cannot be covalently ligated with heme by CCHL in the heterologous *E. coli* system are marked with an arrow in the left (→).

Figure 2.

R. capsulatus apocytochrome *c*₂ (CycA) fusion and mutant proteins tested for CCHL-dependent holocytochrome *c* formation in *E. coli*. Amino-termini sequences of different chimeric proteins formed of *S. cerevisiae* iso-2-cytochrome *c* (Cyc7p) and *R. capsulatus* CycA, and CycA mutants used are shown in panel A. Amino acid residues originating from Cyc7p and CycA are depicted in grey and black, respectively. Of the cytochromes *c* used only eight amino acids immediately following the Cys-Xxx-Xxx-Cys-His heme binding site are depicted to show the junction sequence of the chimera. In the fusion proteins amino acid residues flanking each portion are indicated. Absolutely conserved amino acids are boxed in grey, and mutations in the CycA derivatives are boxed in black. Panels B and C show the heme staining and Strep-tag detection data, respectively, for various cytochromes *c* coproduced with CCHL (lanes 1–8) or for CCHL produced alone (lane 9) in *E. coli* EC06 (Ccm) using 75 µg of total proteins in each case. Molecular markers (kDa) are shown on the left.

Figure 3. Visible spectra of the dithionite-reduced protein extracts (3.2 mg/mL) from *E. coli* coproducing *S. cerevisiae* CCHL with *R. capsulatus* CycA (dotted line) and CycA-insK11+E9L (solid line) variants.

Table 1

Strains and Plasmids used in this work

Strain/Plasmid	Relevant Characteristics	References
HB101	<i>E. coli</i> strain used for cloning	Stratagene
EC06	<i>E. coli ccmABCDEFGH::kan</i> strain used for gene expression	[37]
pCS308	= pACCyc3p: pLysS (Novagen®) derivative with T7 lysozyme gene replaced by expression cassette containing T7 promoter/ <i>lac</i> operator system and 6xHis-tag sequence fused <i>CYC3</i> from <i>S. cerevisiae</i> , Clm ^R ; CCHL	[30]
pCS315	pCS308 derivative with T7 promoter region replaced by <i>tac</i> promoter region of pMal-c2 (New England BioLabs®, Inc), Clm ^R	This work
pCS905	pET-3a (Novagen®) derivative with T7 promoter region replaced by <i>tac</i> promoter region of pMal-c2 (New England BioLabs®, Inc.), Amp ^R	[33]
pCS1303	pCS905 derivative containing a decahistidine sequence-fused <i>GFP</i> gene variant, Amp ^R	[45]
pCS703	= pSCyc7p: pET-3a (Novagen®) derivative containing Strep-tag II (IBA®) sequence-fused <i>CYC7</i> from <i>S. cerevisiae</i> , Amp ^R	[30]
pCS1000	Strep-tag II (IBA) sequence-fused <i>CYC7</i> from pCS703 cloned XbaI and BamHI into pCS1303, replacing 10xHis::GFP, Amp ^R ; Cyc7p	This work
pCS1011	pCS1000 derivative with <i>CYC7</i> encoding the amino acid sequence C-terminal of its CXXCH heme binding site replaced by <i>R. capsulatus cycA</i> to generate a fusion of Cyc7p and CycA joined at Cyc7p His 18, Amp ^R ; Cyc7p^{His18}-s¹⁹CycA	This work
pCS1736	pCS1000 derivative, like pCS1011 but Cyc7p and CycA are joined N-terminally of the CXXCH motif of CycA at Cys14, Amp ^R ; Cyc7p^{R13}-C14CycA	This work
pCS1769	pCS1011 derivative, deletion of the codons encoding thirteen amino-terminal amino acid residues (Ala-9 to Lys4) of the Cyc7p portion Amp ^R ; K5Cyc7p^{His18}-s¹⁹CycA	This work
pCS1302	pCS905 derivative, Strep-tag II (IBA) sequence-fused <i>GFP</i> gene variant expressed under control of <i>tac</i> promoter/ <i>lac</i> operator system, Amp ^R	[36]
pCS1726	pCS1302 derivative containing Strep-tag II (IBA) sequence-fused <i>cycA</i> (matured cyt <i>c</i> ₂ gene variant) from <i>R. capsulatus</i> CycA, Amp ^R ; CycA	[36]
pCS1730	pCS1726 derivative with a codon for a Lys inserted at position II, Amp ^R ; CycA-insK11	This work
pCS1760	pCS1726 derivative with the Glu9 codon exchanged to a Leu codon, Amp ^R ; CycA-E9L	This work
pCS1748	pCS1730 derivative with the Glu9 codon exchanged to a Leu codon, Amp ^R ; CycA-insK11+E9L	This work
pCS1754	pCS1730 derivative with the Asn12 codon exchanged to a Thr codon, Amp ^R ; CycA-insK11+N12T	This work