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Thiol redox requirements and substrate specificities of recombinant cytochrome *c* assembly systems II and III☆

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ABSTRACT

The reconstitution of biosynthetic pathways from heterologous hosts can help define the minimal genetic requirements for pathway function and facilitate detailed mechanistic studies. Each of the three pathways for the assembly of cytochrome *c* in nature (called systems I, II, and III) has been shown to function recombinantly in *Escherichia coli*, covalently attaching heme to the cysteine residues of a CXXCH motif of a *c*-type cytochrome. However, recombinant systems I (CcmABCDEFGH) and II (CcsBA) function in the *E. coli* periplasm, while recombinant system III (CCHL) attaches heme to its cognate receptor in the cytoplasm of *E. coli*, which makes direct comparisons between the three systems difficult. Here we show that the human CCHL (with a secretion signal) attaches heme to the human cytochrome *c* (with a signal sequence) in the *E. coli* periplasm, which is bioenergetically (*p*-side) analogous to the mitochondrial intermembrane space. The human CCHL is specific for the human cytochrome *c*, whereas recombinant system II can attach heme to multiple non-cognate *c*-type cytochromes (possessing the CXXCH motif.) We also show that the recombinant periplasmic systems II and III use components of the natural *E. coli* periplasmic DsbC/DsbD thiol-reduction pathway. This article is part of a Special Issue entitled: Biogenesis/Assembly of Respiratory Enzyme Complexes.

1. Introduction

All *c*-type cytochromes function in the prokaryotic periplasm, the thylakoid lumen, or the mitochondrial intermembrane space. (See [1–4] for recent reviews on functions and assembly of *c*-type cytochromes). Typical *c*-type cytochromes have two thioether bonds that are formed between two cysteines and the two vinyl groups of heme (at a CXXCH motif, where the histidine acts as an axial ligand to the iron). This covalent linkage prevents heme loss and indirect evidence shows that most *c*-type cytochromes are degraded without heme attachment [5, 6]. The assembly of a *c*-type cytochrome requires that the heme and the apocytochrome are already transported to the periplasm or mitochondrial intermembrane space prior to attachment. Additionally, *in vitro* the heme iron and the two cysteines must be reduced for attachment, this attachment occurring spontaneously at the alpha carbons of the vinyl groups [7, 8].

Three systems have been described for cytochrome *c* assembly (Fig. 1). Many Gram negative bacteria (including *Escherichia coli*), archaea, and plant mitochondria use the system I pathway encoded by *ccm* (cytochrome c maturation) genes (Fig. 1A). System I is the most studied pathway and it involves apocytochrome *c* thiol-reduction by a specific periplasmic thioredoxin protein called CcmG (HelX) [9–11], which is itself reduced by the transmembrane DsbD (or the related CcdA) [12–14]. Other proteins are involved in heme export (CcmABCD), heme chaperoning (CcmE), apocytochrome trafficking and thiol redox (CcmH), and heme reduction (CcmF) prior to heme attachment to apocytochrome *c* by CcmF/H complex (recently reviewed in [1, 2]). Although the molecular mechanisms underlying apocytochrome *c* recognition and heme attachment by CcmF/H are poorly understood, many studies are in agreement that system I can assemble a wide variety of *c*-type cytochromes, with only the CXXCH as the recognition determinant for attachment (*e.g.*[15–21]).

System II is present in some Gram negative bacteria (*e.g.* Helicobacter, Wolinella), Gram positives *(e.g.* Mycobacterium, Bacillus), cyanobacteria, and the chloroplast (Fig. 1B). Genetic analyses of bacteria with system II have shown that a specific periplasmic thioredoxin (CcsX) [22], and again the generic DsbD (or CcdA) protein [22–26] are required. System II also involves export of heme, recognition of the apocytochrome *c*, and heme attachment, but these functions are carried out by the CcsBA integral membrane protein [27]. This was established by using a recombinant *E. coli* deleted for all its *ccm* genes, which was able to synthesize periplasmic cytochrome c_4

Abbreviations: CCHL, cytochrome *c* heme lyase; 6x:His, hexahistidine; B-PER, bacterial protein extraction reagent; DEAE, diethylaminoethyl; cyt, cytochrome; MBP, maltose binding protein; DTT, dithiothreitol; GST, glutathione transferase.

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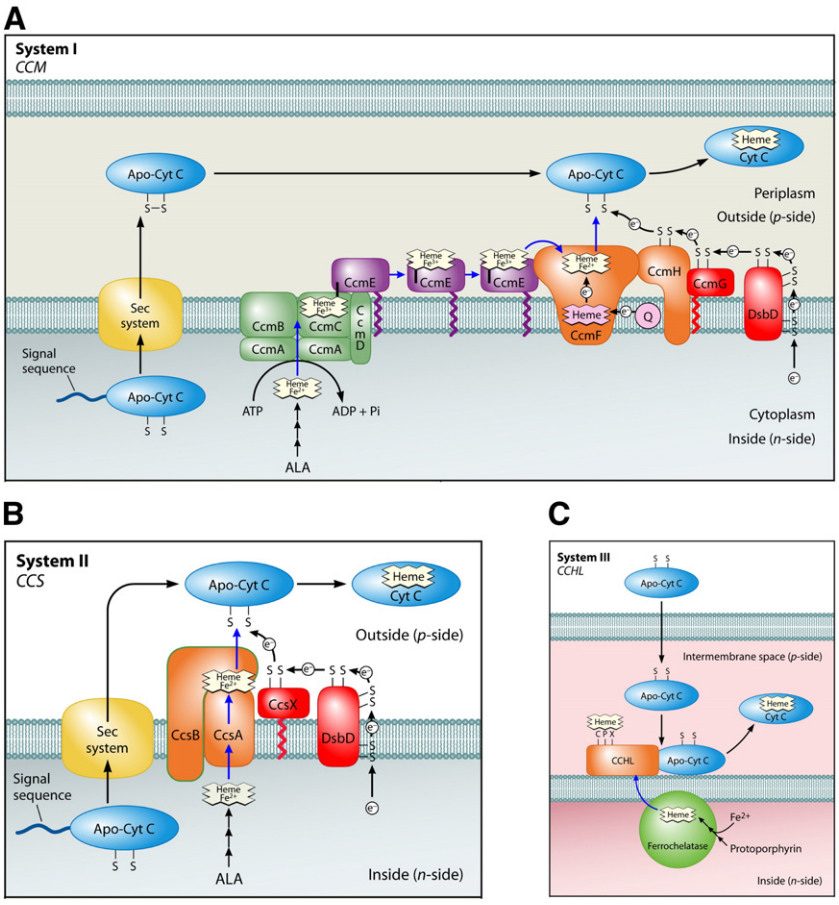

Fig. 1. Schematic models of the three *c*-type cytochrome assembly pathways (Systems I, II, and III). The cartoons are reproduced in modified form from Kranz et al. [1].

when only the Helicobacter fused *ccsBA* gene was expressed [27–29]. Although most organisms with system II have separate *ccsB* and *ccsA* genes, a few organisms have naturally fused *ccsBA* genes. Further studies on this recombinant CcsBA have indeed shown that the protein acts as a heme exporter and cytochrome *c* synthetase [27]. However, questions remain for the recombinant CcsBA as to what comprises the substrate recognition determinants and how the apocytochrome *c* is reduced (e.g. whether Dsb proteins are involved) [28, 30]. Here we address these questions.

System III appears to be the simplest, comprised of an enzyme first discovered by yeast genetic analyses [31–33], referred to as cytochrome *c* heme lyase (CCHL) (Fig. 1C). This enzyme has also been referred to as holocytochrome *c* synthase (HCCS), cytochrome *c* synthase, or cytochrome *c* synthetase *e.g.*[34–36]. Here we use CCHL to refer to the system III enzyme. Despite evidence that it is a soluble protein in the mitochondrial intermembrane space, and its involvement in some rare human genetic defects [34–36], it is a poorly understood enzyme [37]. The evidence that it represents the sole cytochrome *c* synthetase comes in part from reconstitution of its activity in the *E. coli* cytoplasm, using its cognate cytochrome *c* (e.g. [15, 38–40]). Interestingly, although yeast has two separate CCHLs, one for cytochrome *c*[31] and a second related enzyme for cytochrome c_1 (CC_1HL) [33], humans have a single CCHL [3, 34]. The human CCHL appears to recognize both cytochromes *c* and c_1. Moreover, the yeast CCHL can be over-expressed or easily selected to recognize both cytochromes [3, 41]. Recently, the recombinant cytoplasmic CCHL from yeast was used to begin to investigate apocytochrome *c* recognition determinants (i.e. residues or regions in cytochrome *c* required for heme attachment by CCHL) [39, 40, 42]. One of the caveats to studying cytoplasmic synthesis of *c*-type cytochromes is that no direct comparison to the periplasmic recombinants (systems I or II) can be made with respect to apocytochrome *c* folding and/or suitability for heme attachment. Here, we test whether a periplasmic recombinant human CCHL with a periplasmic human cytochrome *c* is functional (to allow such comparisons). We also address what endogenous periplasmic thiol reduction proteins are necessary.

2. Materials and methods

2.1. Bacterial growth conditions

E. coli were grown aerobically in Luria–Bertani (LB) media (Difco) at 37 °C with shaking at 300 rpm. Antibiotic concentrations are as follows: 50 μg mL^{-1} carbenicillin (carb), 20 μg mL^{-1} chloramphenicol (cm) and 100 μg mL^{-1} kanamycin (km). Expression of pRGK333 (CcmABCDEFGH; system I) [28], pRGK368 (CcsBA; system II) [29], pRGK399 (pMALp2x:CCHL; system III), and pRGK400 (pET226xHis:CCHL; system III) was induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and expression of pRGK332 (Cyt c_4) [28], pRGK389 (Cyt c_2; pBADc_2:6xHis), pRGK390 (human CycS; pBADCycS:6xHis), and pRGK401 (human CycS:*R. capsulatus* Cyt c_2 chimera; pBADCycS-c_2:6xHis) was induced by 0.2% arabinose.

2.2. Plasmid construction

The human CCHL was amplified from a cDNA clone (Origene) and cloned in frame into the EcoRI and HindIII sites of pMalp2x (New England Biolabs) to create pRGK399, a periplasmically targeted maltose binding protein (MBP) fusion (see Table 1 for strains and plasmids, Table S1 for oligonucleotide sequences). An N-terminal hexahistidine-tagged periplasmically targeted (*pelB* signal) version of the

human CCHL (pRGK400) was created by amplification from the above cDNA clone and in-frame insertion into the NcoI and HindIII sites of pET22b (EMD Biosciences). The cytochrome c_2 gene *cycA*, with its natural signal sequence, was amplified from *R. capsulatus* DNA and cloned into the KpnI and PstI sites of pRGK330 [28] to create pRGK389. The human cytochrome *c* gene *cycS* was amplified from cDNA clone MGC12367 (ATCC) and cloned into NotI and KpnI sites of pRGK331 [28] that was amplified to use the cytochrome c_4 signal sequence to create pRGK390. The human cytochrome *c* gene, containing a deletion of the DNA corresponding to amino acids 4–22, along with the surrounding plasmid was amplified from pRGK390 with phosphorylated oligonucleotides, containing the *R. capsulatus* Cyt c_2 DNA corresponding to amino acids 2–20, and blunt end ligated to create pRGK401.

2.3. Knockout strain construction

E. coli strains with knockout mutations in *dsb* genes were obtained from the Keio strain collection [43, 44]. The knockout mutations were transduced into RK103 (Δ*ccm*) [28] by P1 phage transduction [45]. Briefly, P1 phage were grown first in LB media, 5 mM $CaCl_2$, 0.2% glucose, with a *dsb* mutant strain as the donor strain. Cells were killed by addition of chloroform to the growth media and the phage were isolated. Dilutions of isolated phage (100 μL) were then incubated with 100 μL RK103 cell culture ($O.D._{600} = 0.8$–1.0) in 5 mM $CaCl_2$ and 100 mM $MgSO_4$ for 30 minutes at 37 °C. Physical interaction between the phage and cells was disrupted by addition of 66.7 mM sodium citrate pH 5.5 then the culture was added to 1 mL LB broth and incubated for 1 hour at 37 °C with shaking at 300 rpm. Transductants were selected by growth on LB-Kan plates and verified by PCR.

2.4. Protein purification

Hexahistidine-tagged proteins were purified with Talon affinity resin (Clonetech) from whole cell lysates. *E. coli* cultures of RK103 (Δ*ccm*) containing pRGK399 (pMalp2x:CCHL) or pRGK333 (system I) and pRGK390 (pBADCycS:6xHis) or RK112 (BL21(DE3) Δ*ccm*) containing pRGK400 (pET226xHis:CCHL) were inoculated (to 1%) with fresh overnight culture in LB medium supplemented with the appropriate antibiotics. One liter cultures were grown to an OD_{600} of 0.8–1.0 and protein over-expression was induced with IPTG and arabinose or IPTG alone for 4 hours (system I matured) or 12–14 hours (system III matured). Following induction the cells were harvested at 6000*g* for 10 min and the cell pellet was resuspended in 3.5 mL, per gram of cell pellet, of 1×Talon buffer (50 mM sodium phosphate buffer, 300 mM NaCl, pH 7.0) that was supplemented with 0.1 mg mL^{-1} egg white lysozyme (Sigma-Aldrich) and 1 mM phenylmethanesulfonyl fluoride (PMSF; Sigma-Aldrich). Following gentle agitation on ice (20 min), the cell suspensions were sonicated (on ice) for 2×3 min using a Branson 250 sonicator (50% duty, 70% output), and cellular debris was removed by centrifugation (10,000*g*, 15 min, 4 °C). The clarified lysate (Load) was passed by gravity flow over Talon resin, washed with 10 column volumes of 1× Talon buffer (Wash 1), five column volumes of 1× Talon buffer containing 5 mM imidazole (Wash 2), and eluted with five column volumes of 1× Talon buffer containing 150 mM imidazole (Elution). To ensure that all hexahistidine-tagged protein was eluted from the resin, the cobalt ions were stripped with five column volumes of 1× Talon buffer containing 0.2 M EDTA (Strip). The elution fraction was concentrated approximately 10-fold with an Amicon 10 K spin filter (ThermoFisher Scientific). The human CycS:6xHis was further purified (>95%) by passage over DEAE Sepharose Fast Flow (GE Healthcare). The 1× Talon buffer (containing 150 mM imidazole) was exchanged for a Tris based buffer (20 mM Tris, 25 mM NaCl, pH 8.0; loading buffer) and the final volume was brought to 4 mL. The buffer-exchanged Talon purified CycS:6xHis was loaded onto a 2.5 mL packed bed volume DEAE column, the flow through was collected along with another 2×column volumes of loading buffer (total 9 mL), and the column was washed with successive 2×column volumes of loading buffer containing 0.1 M, 0.3 M, 0.5 M, and 1 M NaCl, respectively. Using these conditions (i.e. loading buffer at pH 8.0) the human CycS:6xHis did not bind with the DEAE resin and thus, was present in the flow through. For mass spectrometry analysis the DEAE purified CycS:6xHis (matured by both system III and system I) was dialyzed against sterile distilled de-ionized H_20 and concentrated as above.

2.5. E. coli cellular protein fractionation

Isolation of periplasmic proteins was by a modification of the method of Feissner et al. [28]. *E. coli* cultures (100 mL) of RK103 (Δ*ccm*) or RK112 (BL21(DE3) Δ*ccm*) containing a system plasmid, pRGK333 (pGEXCcmABCDEFGH), pRGK399 (pMALp2x:CCHL) and a reporter plasmid, pRGK400 (pET226xHis:CCHL) and pRGK390 (pBADCycS:6xHis), pRGK389 (pBADc_2:6xHis) or pRGK401 (pBAD-CycS-c_2:6xHis chimera) were grown to an OD_{600} of 0.6 to 0.8, IPTG (1 mM) and arabinose (0.2%, when required) were added for protein over-expression, and induction proceeded for 12–14 hours. Cells were harvested at 6,000 *g* for 10 min at 4 °C and washed once at

Table 1
Description of strains and plasmids used in this study.

Strain or plasmid	Description and purpose	Reference
RK103	Δ*ccm E. coli* strain deleted for all *ccm* genes	[28]
RK106	Δ*ccm*Δ*dsbA E. coli* strain Generated by P1 phage transduction using strains JW3832 and RK103	This study
RK107	Δ*ccm*Δ*dsbB E. coli* strain Generated by P1 phage transduction using strains JW5182 and RK103	This study
RK108	Δ*ccm*Δ*dsbC E. coli* strain Generated by P1 phage transduction using strains JW2861 and RK103	This study
RK109	Δ*ccm*Δ*dsbD E. coli* strain Generated by P1 phage transduction using strains JW5734 and RK103	This study
RK110	Δ*ccm*Δ*dsbG E. coli* strain Generated by P1 phage transduction using strains JW0597 and RK103	This study
RK112	Δ*ccm* BL21(DE3) *E. coli* stain Generated by P1 transduction with RK103	This study
TC127034	cDNA clone from Origene-plasmid template for construction of pRGK390 and pRGK391	Origene
MGC12367	*E. coli* strain from ATCC with plasmid PCR template for construction of pRGK392	ATCC
pRGK333	Expression of GST-tagged CcmABCDEFGH (system I) from *E. coli*	[28]
pRGK368	Expression of GST-tagged CcsBA (fused system II) from *H. hepaticus*	[29]
pRGK330	pBAD24 based, chloramphenicol resistant plasmid for arabinose inducible expression	[28]
pRGK399	Periplasmic expression MBP-tagged human cytochrome *c* heme lyase (CCHL; system III)	This study
pRGK400	Periplasmic (*pelB* signal) of N-terminal 6x: His-tagged human CCHL	This study
pRGK331	Expression of cytochrome c_4 from *B. pertussis* fused to alkaline phosphatase. PCR template for construction of pRGK392	[28]
pRGK332	Expression of cytochrome c_4 reporter from *B. pertussis*	[28]
pRGK389	Expression of cytochrome c_2 reporter from *R. capsulatus*	This study
pRGK390	Periplasmic (cyt c_4 signal) expression of human cytochrome *c* CycS reporter	This study
pRGK401	Periplasmic (cyt c_4 signal) expression of chimeric human CycS and *R. capsulatus* cytochrome c_2 reporter	This study

room temperature with 10 mM Tris pH8.0. The washed cell pellet was resuspended in 10 mL of 100 mM Tris containing 20% (w/v) sucrose, warmed to 37 °C, 0.1 mg mL^{-1} lysozyme was added, and the suspension was gently rocked at 37 °C for 15 min. Periplasmic proteins were released from the spheroplasts by treatment with EDTA (10 mM final concentration) at 37 °C and separated by centrifugation at 12,000*g* for 10 min. The supernatant was saved as the periplasmic protein fraction. The spheroplasted cell pellet was resuspended in phosphate buffered saline (10 mM phosphate, 137 mM NaCl, 3 mM KCl, pH7.4), sonicated on ice for 3×20 sec (50% duty, 15% output on a Branson Model 250 sonicator), and the lysate was cleared via centrifugation at 12,000 *g* for 15 min. The membranes were separated from the cytoplasm by ultracentrifugation at 100,000*g* for 1 hour and solubilized in PBS containing 1% n-Dodecyl-β-D-maltoside. Crude protein extraction for cytochrome *c* assembly activity assays was accomplished with B-PER (Bacterial Protein Extraction Reagent; Pierce-Thermo Scientific). Briefly, 5-mL LB cultures, supplemented with the appropriate antibiotics, of a given strain of *E. coli* Δ*ccm* (or Δ*ccm* with single *dsbA*, *dsbB*, *dsbC*, *dsbD*, or *dsbG* deletions) containing the indicated system plasmid (pRGK333, pRGK368, pRGK399, or pRGK400) and/or the indicated cytochrome *c* over-expression plasmid (pRGK332, pRGK389, pRGK390, or pRGK401) were inoculated with 200 μl of fresh overnight culture and grown for 2.5 hours shaking at 37 °C. Protein over-expression was induced with IPTG and arabinose for 4 hours (system I matured cytochrome *c*) or 12–14 hours (system II and III matured cytochrome *c*). For experiments containing DTT, a final concentration of 3 mM DTT was added to the 5-mL cultures at the time of IPTG induction, incubation proceeded for 30 min, and then the cytochrome *c* reporters were induced with arabinose for the times given above. The cells were pelleted at 4,000 *g* for 10 min and resuspended in 200 μl B-PER.

2.6. Heme stains and Western blots

Total protein concentration was determined by BCA assay (Pierce Thermo Scientific) or A_{280} with a Nanodrop Spectrophotomer (Thermo Scientific). Protein fractions were subjected to SDS-PAGE, transferred to a Hybond C nitrocellulose membrane (GE Healthcare) and heme stains were conducted as described [46] using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce-Thermo Scientific). Western blots were performed with anti-His antibody (1:5000; Santa Cruz Biotechnology) with protein A peroxidase as the secondary label and chemiluminescent signal was developed with Super-Signal West Femto. Both heme stains and Western blots were visualized with an LAS-1000plus Luminescent Image Analyzer CCD camera system (Fujifilm/GE Healthcare).

2.7. Other methods

Reduced (sodium dithionite; Sigma-Aldrich) and as purified (air oxidized) cytochrome *c* UV–vis absorption spectra were recorded on a UV2101PC scanning spectrophotometer (Shimadzu). ESI-MS was performed by the Proteomics and Mass Spectrometry Facility at the Donald Danforth Plant Science Center on DEAE purified human CycS:6xHis.

3. Results and discussion

3.1. Expression of a functional CCHL (i.e. cytochrome c synthase) in E. coli

The human gene encoding CCHL was cloned in-frame to the *E. coli* gene (*malE*) encoding the periplasmic maltose binding protein (MBP) (Fig. 2A). Note that MBP contains its natural signal sequence for export to the periplasm, and such fusion proteins can be purified in one step on amylose-agarose columns. *E. coli* RK103 (Δ*ccm*) with this plasmid as well as RK103 with pMal-p2x (*malE*-only plasmid without CCHL) were induced with IPTG, periplasmic fractions were separated by SDS-PAGE, and stained with Coomassie Blue (Fig. 3). Both preparations show a major polypeptide the size of MBP (42KD). It is often the case that fusion proteins are proteolysed at the fusion junction resulting in MBP. With pMalp2X, a larger polypeptide (~53 kDa) that represents the fusion of lacZ' from the blue/white screening vector is also present (labeled as MBP:lacZ' in Fig. 3). This polypeptide is absent from preparations of the MBP:CCHL, as expected, and two new polypeptides are observed. One of these is likely the full length MBP:CCHL, migrating at approximately 83 kDa, the other is a truncated product of 59 kDa (labeled MBP:CCHL*).

To determine if the human CCHL that contains the MalE periplasmic signal sequence is functional in *E. coli*, the human cytochrome *c* gene (*cycS*) was engineered into an arabinose-inducible pBAD plasmid (Fig. 2C) which is compatible with pMalp2x. A periplasmic signal sequence from *Bordetella pertussis* cytochrome c_4 was engineered in frame to the N-terminus of CycS, and cloned downstream of the arabinose-inducible promoter. We have previously shown that the cytochrome c_4 signal is efficiently processed in the periplasm of *E. coli* Δ*ccm* expressing recombinant system I and II [28]. For affinity purification a hexahistidine tag was engineered onto the C-terminus of CycS. Expression and heme attachment to this periplasmically targeted CycS was initially tested using the *E. coli* system I pathway, all eight *ccm* genes expressed from pRGK333 in *E. coli* RK103 (Δ*ccm*). The human cytochrome *c* was expressed and assembled in the periplasm, as is evidenced by the heme stain of polypeptides separated by SDS PAGE (Supp Fig. 1), which suggests that CycS:6xHis with the c_4 signal is properly transported and folded in the periplasm. These results are in agreement with system I cytochrome c maturation requiring apocytochrome export to the periplasm for heme insertion and folding [47]. [Note that because the heme is covalently attached, even upon SDS PAGE the heme remains with the denatured polypeptide and is detected by staining for heme.] We next tested strain RK103 (*E. coli* Δ*ccm*) containing pRGK399 (pMalp2x:CCHL) and pRGK390 (pBADCycS:6xHis) for holoCycS:6xHis maturation. A culture containing the periplasmically targeted MBP:CCHL and CycS:6xHis was induced and the hexahistidine tagged CycS was purified over a cobalt column (Fig. 4A). Although the imidazole-eluted fraction showed multiple polypeptides (Fig. 4A, lane 7), one major polypeptide at 13 kDa stained intensely for heme (Fig. 4B, lane 7). Subsequent purification of the imidazole-eluted fraction over a DEAE column yielded (>95%) pure CycS (Fig. 4C, lane 3). This polypeptide (13 kDa) stained for heme (Fig. 4D), and, upon UV–vis spectroscopy, the pure protein showed the characteristic alpha absorption peak at 550 nm (Fig. 5A). The purified preparation was analyzed by ESI-MS to establish whether the signal sequence was cleaved and to confirm covalent heme attachment (Fig. 5B). All masses are given as ± 1 Dalton. Three cleavage products were detected of the masses indicated in Fig. 5B (see "1, 2, 3") and are predicted with an attached heme of 616 Daltons. (Note that the relative percentage of each holo-CycS, as determined from mass spectrometry peak profiles, are given to the right of the mass.) Even though each of the three predicted polypeptides (Fig. 5B, see "1, 2, 3") was found to be cleaved at a potential *E. coli* signal peptidase recognition site [48–50] we cannot rule out some nonspecific proteases are also responsible for this cleavage (in periplasm or cytoplasm). No polypeptides without heme were detected, which is consistent with the premise that the apocytochrome polypeptide is often degraded if heme is not attached (e.g. see [1, 4]). CycS matured by system I was also purified, as given above, and showed identical absorption maxima as system III matured CycS and a single polypeptide at 13329 Da was detected via ESI-MS (data not shown). These results, *e.g.* the presence of full-length MBP:CCHL in unpurified periplasmic shock fractions (Fig. 3 lane 3) and holoCycS that has the same mass and spectral characteristics as system I matured, suggest that at least some of the holoCycS is matured in the *E. coli* periplasm.

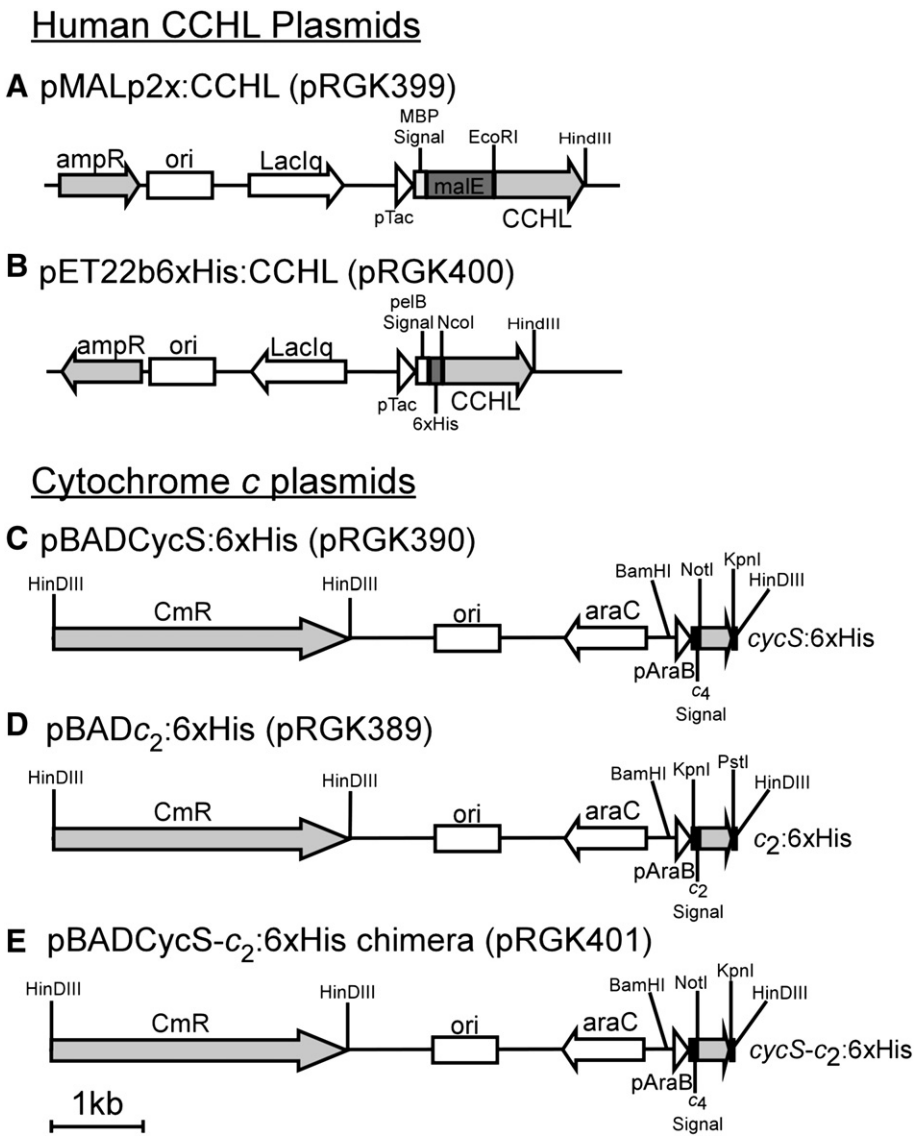

Fig. 2. Recombinant plasmids with the human system III (A and B) and indicated *c*-type cytochromes (C, D, and E).

This is the first report that CCHL may be functional in the *E. coli* periplasm, thus we wanted to confirm the potential periplasmic synthesis using a different secretion signal for CCHL (from *pelB*), and a different affinity tag for purification, an N-terminal hexahistidine tag rather than a MBP fusion (see Fig. 2B). We chose the an N-terminal hexahistidine tag because previous work with cytoplasmically expressed yeast CCHL suggested the N-terminal 6xHis tag was more soluble and was over-expressed to higher levels than the non-hexahistidine tagged version [15, 38]. The 6x:His-tagged CCHL, over-expressed from pRGK400 (pET226xHis:CCHL), could be partially purified over a cobalt column (Fig. 6A), and various polypeptides reacted with hexahistidine antisera upon Western blotting (Fig. 6B). From the Western blot, full-length CCHL of approximately 30 kDa was present in the load fraction (Fig. 6B, lane 2) and purified fraction (lane 6), which suggests that at least some of the CCHL is properly folded and stable. Two other immunoreactive polypeptides were observed in the pure fraction (Fig. 6B, lane 6), yet it is clear that in the load (lane 2) full-length CCHL is the major polypeptide. Thus, some of the CCHL is proteolysed during purification. When this plasmid construct is transformed into *E. coli* BL21(DE3) Δ*ccm* (RK112) that also possesses the *cycS* gene (with signal; pBADCycS:6xHis), human cytochrome *c* is synthesized, as detected by heme staining of periplasmic shock proteins (Fig. 6C, lane 1) and no holocytochrome is detected (lane 2) in the absence of CCHL (pET226xHis:CCHL). We conclude that CCHL can fold and properly function with its cognate cytochrome *c* in the bacterial periplasm. An important implication of these results

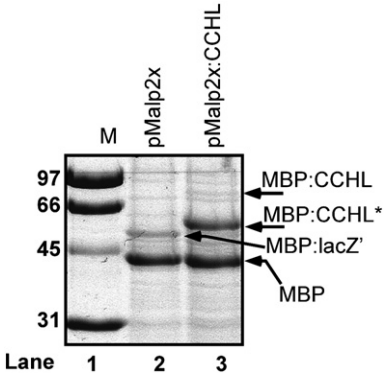

Fig. 3. Human cytochrome *c* heme lyase expressed as a MBP:CCHL fusion protein in the *E. coli* periplasm. Coomassie Blue stained SDS-PAGE profile of periplasmic shock proteins from IPTG-induced RK103 (Δ*ccm*) expressing pMalp2x (lane 2) and pMalp2X:CCHL (lane 3). Protein molecular weight standards are shown (labeled M) in lane 1 with indicated molecular masses (left).

is that some reduced heme is available in the periplasm for cytochrome *c* assembly by CCHL. Possibly this is a ramification of the peripheral association of CCHL [51] with the outer leaflet of the cytoplasmic membrane, where the amphipathic and still reduced heme may bind directly to CCHL for subsequent attachment. We have previously speculated that non-specific "flippases" may flip heme from inner leaflet to outer leaflet in a manner similar to phospholipid "flipping", as a mechanism to "export" heme for CCHL [1] (see Fig. 1C). Nevertheless, the levels of human cytochrome *c* matured by periplasmic CCHL are very low when compared to system I (see below). Since a previous study indicated that cytoplasmically matured yeast cytochrome *c* (by cytoplasmic CCHL) was up to two-fold more than a recombinant system I [15], we estimate that in our study at least ten- to one hundred- fold lower levels are periplasmically attached (compared to system I, thus to cytoplasmic CCHL). This may be a ramification of lower levels of available reduced heme, or to the secretory or folding requirements in the periplasm.

3.2. Apocytochrome c recognition requirements of systems II and III

Many studies have been carried out on the substrate recognition of system I, with the conclusion that naturally only the CXXCH appears to be necessary [16–20, 52]. However the substrate recognition requirements for system II are not as well studied. A recent report using our *Helicobacter pylori ccsBA* recombinant approach indicated that multiple *c*-type cytochromes were matured [21], suggesting that system II requires only the CXXCH motif for recognition. To further examine recognition determinants of system II, three heterologous cytochromes *c* were expressed (Fig. 2C, D, and pBADCytc_4:6xHis [28]) in *E. coli* strain RK103 (Δ*ccm*) in the presence or absence of the *H. hepaticus* CcsBA system II fusion protein. Heme stains of B-PER-isolated fractions were conducted to detect assembled cytochromes *c*. In the absence of CcsBA, no cytochrome *c* is assembled (Fig. 7A, lanes 1, 3 and 5). However, when CcsBA is present, all three periplasmic cytochromes *c* contain covalently attached heme (Fig. 7A, lanes 2, 4 and 6), demonstrating that system II is capable of maturing diverse cytochromes *c*. These include the diheme cytochrome c_4 from *B. pertussis* (system II), cytochrome c_2 from *R. capsulatus* (system I), and the human (system III) cytochrome *c*. Our results are in agreement with the study by Goddard et al. who showed that CcsBA from *H. pylori* matures monoheme cytochromes from the system I organisms *Paracoccus denitrificans* and *Hydrogenobacter thermophilus*[21]. We concur that the feature of apocytochromes *c* recognized by CcsBA is the CXXCH motif. Note that rare "alternative" CcsBA synthetases have been shown to recognize motifs other than CXXCH, such as CXXCK and CX15CH [53, 54], but that here we have studied the more common system II CcsBA.

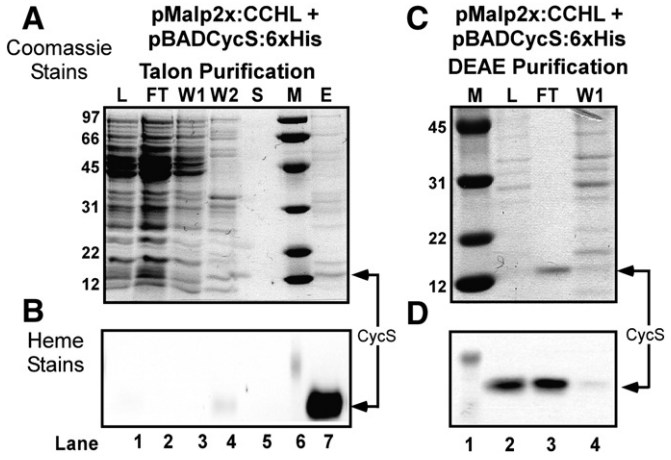

Fig. 4. Purification of hexahistidine tagged human cytochrome *c* (CycS) produced in IPTG- and arabinose-induced RK103 (Δ*ccm*) by pMALp2X:CCHL (MBP:CCHL; pRGK 399). SDS-PAGE of protein purification fractions stained with (A and C) Coomassie Blue and (B and D) heme stain. Hexahistidine-tagged Talon purification fractions (panel A) are indicated above each lane, where lane 1 (L; crude extract load), lane 2 (FT; Flow Through), lane 3 (Wash 1), lane 4 (Wash 2), lane 5 (EDTA strip), and lane 7 (imidazole elution). Note: The imidazole elution was performed before the EDTA strip. DEAE purification fractions are indicated above panel C where lane 2 (Talon elution as load), lane 3 (flow through), and lane 4 (0.3 M NaCl wash). Protein molecular weight standards are shown (labeled M) in lane 6 (A and B) and lane 1 (C and D) with the masses (kDa) shown on the left of A and C. The position of CycS is shown in the flow through with an arrow on the right.

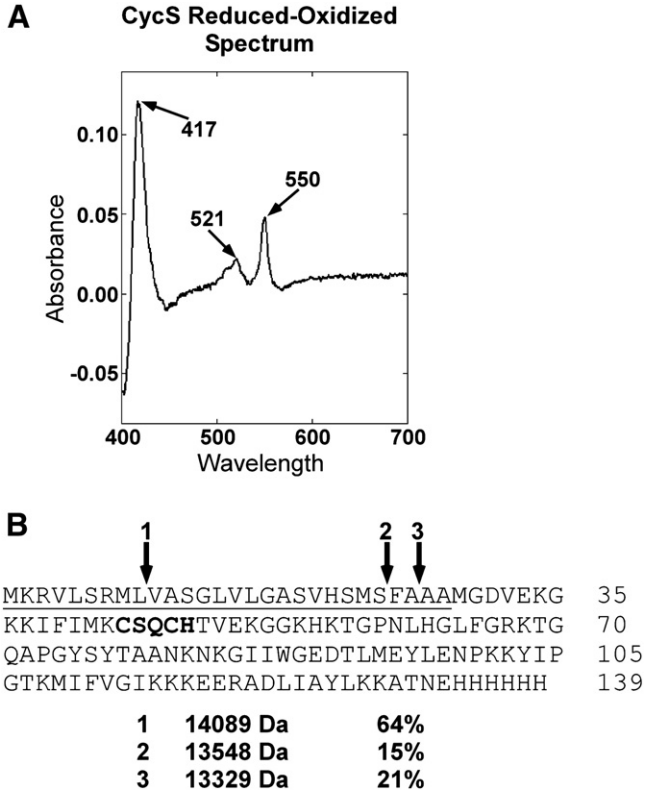

Fig. 5. Reduced minus oxidized UV-vis absorption spectra and ESI mass spectrometry analysis of purified human cytochrome *c* matured by MBP:CCHL. (A) UV-vis absorption spectra of human CycS expressed from RK103 (Δ*ccm*) containing pRGK399 (pMalp2x:CCHL) and pRGK390 (pBADCycS:6xHis). The absorption maxima are denoted with arrows. (B) Amino acid sequence of the cytochrome c_4 signal:CycS. The signal sequence is underlined, the CXXCH heme binding motif is bolded and the cleavage sites, as determined by ESI-MS, are denoted with arrows. The molecular masses (Da) and relative percentages of each cleaved product, as deduced from the mass spectrometry peak profile, are shown below the amino acid sequence.

It has been largely accepted that the mitochondrial CCHLs may have more stringent recognition requirements than the bacterial systems (e.g. more than CXXCH) [31, 33]. Recombinant studies of CCHLs in the *E. coli* cytoplasm [15, 38–40] and in yeast mitochondria [3, 41, 42] point to expanded recognition requirements (i.e. more than simply CXXCH or XXCH) [55]. Recently, studies using the cytoplasmic recombinant CCHL have started to define exact residues in apocytochrome *c* that are important for the cognate CCHL recognition [39, 40]. To address possible folding defects (rather than recognition defects), the same recombinant *c*-type cytochromes were assembled by system I in *E. coli*[39]. However these controls (system I) require the engineering of a signal sequence, thus not allowing exact comparison of substrates (or folding environments, periplasm versus cytoplasm). One of the uses of the recombinant system III (CCHL) described here is to facilitate direct comparisons of identical substrates in the same cellular compartment (periplasm). We test this approach by focusing on the human cytochrome *c* and the structurally related soluble monoheme cytochrome c_2[56–58] from *R.*

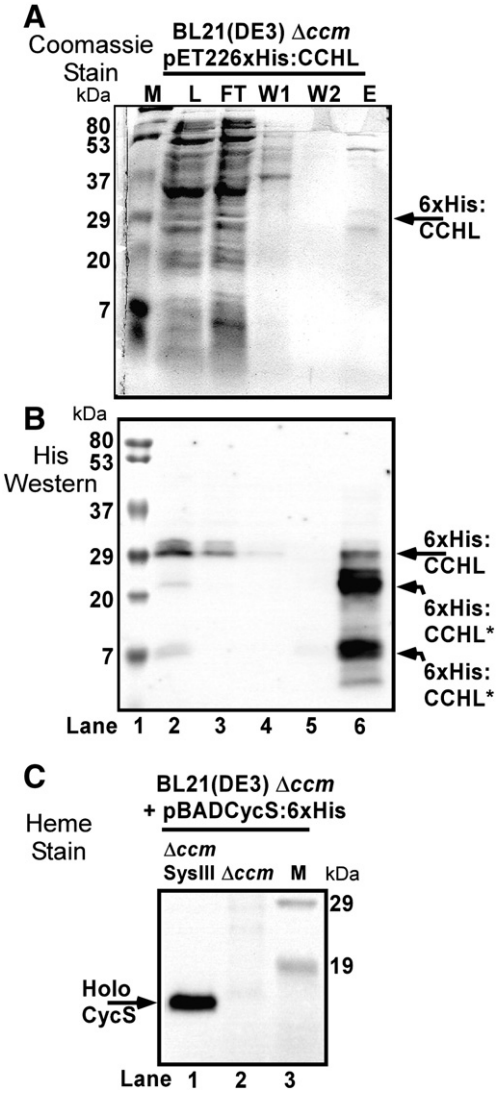

Fig. 6. Hexahistidine-tagged human CCHL (with a periplasmic signal) attaches heme to human cytochrome *c*. Cobalt (Talon) resin purification of IPTG-induced 6xHis:CCHL (pET226xHis:CCHL; pRGK400) expressed in RK112 (BL21(DE3) Δ*ccm*). Purification fractions run on SDS-PAGE and detected with (A) Coomassie Blue stain and (B) 6xHis antisera by Western blot. Protein fractions are given above each lane, where lane 2 (L; crude extract load), lane 3 (flow through), lane 4 (wash 1), lane 5 (wash 2), lane 6 (imidazole elution). (C) Heme stain of periplasmic shock proteins from IPTG- and arabinose-induced RK112 containing pRGK400 and pRGK390 (lane 1; labeled SysIII Δ*ccm*) or pRGK390 only (lane 2; labeled Δ*ccm*). Protein molecular weight standards are shown (labeled M) in lane 1 (A and B; masses on left) and lane 3 (C; masses on right). The position of 6xHis:CCHL and its proteolytic products (A and B) and of holo-CycS:6xHis (C) are denoted with arrows.

capsulatus. Three substrates were compared: the human cytochrome *c*, the *R. capsulatus* cytochrome c_2, and a chimeric molecule in which the human cytochrome *c* had a 19 residue region (encompassing the CXXCH) substituted with the analogous region of the *R. capsulatus* cytochrome c_2 (Fig. 7B). System I recognized and assembled all three substrates (Fig. 7C, lanes 1–3), which further confirms that system I can mature a wide variety of *c*-type cytochromes. However, the human CCHL recognized only its cognate human cytochrome *c* (Fig. 7C, lane 4) and not the cytochrome c_2 (lane 5) or chimera (lane 6). Because the expression level of system III was lower than that of system I, as noted previously with recombinant system II [28, 29], we concentrated by six-fold the recombinant periplasmic shock proteins and still no holocytochrome c_2 or chimera are detected (data not shown). We conclude that CXXCH is not sufficient for CCHL recognition, and that other residues within this region (i.e. of the 19 residues of the human cytochrome *c* defined in Fig. 7B) are important for recognition by human CCHL. This conclusion is consistent with previous cytoplasmic studies in *E. coli* that suggested the N-terminus (up to and including the CXXCH) of the apocytochrome *c* are important and sufficient for yeast CCHL activity (e.g. [40, 47]). Further analysis will be necessary to elucidate the exact residues necessary for recognition.

3.3. The periplasmic thiol redox requirements of recombinant system II and III

When results on the first functional recombinant system II were published [28], a commentary on the achievement [30] suggested that one significant question was what periplasmic thiol oxidation and reduction pathway(s) from *E. coli* were used. For secreted (and membrane) proteins the *E. coli* Dsb system is one of the best understood pathways for disulfide bond formation [59–61]. DsbA and DsbB represent the major pathway for oxidation of disulfides in the periplasm. For thiol reduction, the integral membrane protein DsbD transfers reducing equivalents from the cytoplasm to periplasmic thioredoxin-type proteins such as DsbC, or to CcmG (HelX) in the case of the natural system I in *E. coli* (see Fig. 1A). (Note that deletion of the *ccm* operon, necessary for studies on the recombinant system II and III, removes *ccmG* also). In order to determine which Dsb proteins, if any, are required for recombinant systems, deletions of selected *dsb* genes were constructed in a Δ*ccm* background. Using the recombinant system plasmids and selected cytochrome *c* expression plasmids, the attachment of heme was investigated in each

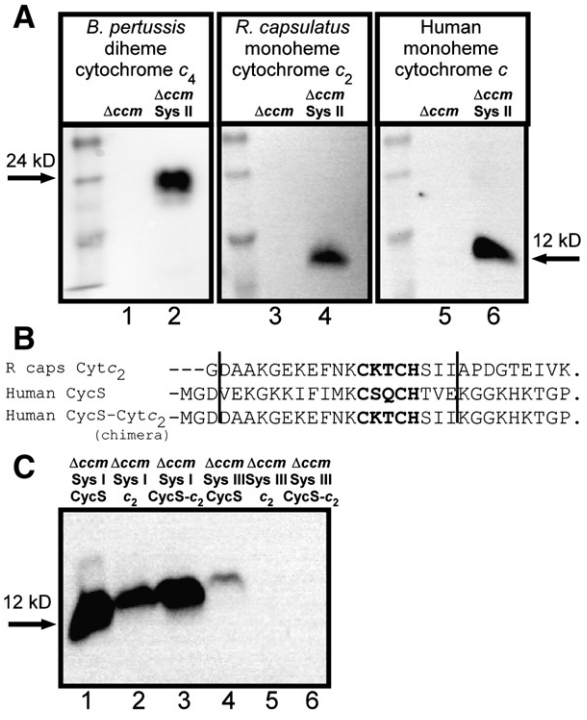

Fig. 7. Maturation of various *c*-type cytochromes by recombinant CcsBA (system II) and CCHL (system III). (A) Representative heme stains (three trials) of arabinose-induced, soluble B-PER extracts (40 μg) from RK103 (Δ*ccm*; lanes 1, 3, and 5) or RK103 containing pRGK368 (Sys II; CcsBA) and pRGK332 (lane 2; pBADCytc_4:6xHis), pRGK389 (lane 4; pBADCytc_2:6xHis), or pRGK390 (lane 6; pBADCycS:6xHis). (B) Amino acid sequence alignment of the N-termini of *R. capsulatus* cytochrome c_2, the human Cyc S, and the chimeric human CycS:c_2. The vertical lines denote the 19 amino acid region that was "swapped" in the chimera. (C) Heme stain of IPTG- and arabinose-induced periplasmic shock proteins (approx. 100 μg) from RK103 containing pRGK333 (SysI; system I) and pRGK390 (lane 1; pBADCycS:6xHis), pRGK389 (lane 2; pBADCytc_2:6xHis), pRGK401 (lane 3; CycS-c_2; pBADCycS-c_2:6xHis chimera) or pRGK399 (SysI; pMALp2xCCHL) containing pRGK390 (lane 4), pRGK389 (lane 5), pRGK401 (lane 6). The sizes of the respective holocytochromes *c* are denoted with arrows.

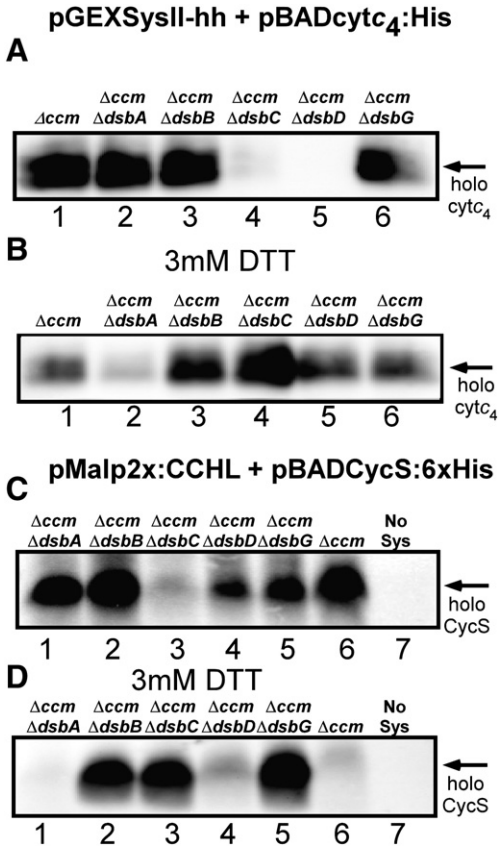

Fig. 8. Assembly of *c*-type cytochromes by CcsBA and CCHL in *E. coli* strains deleted of the indicated genes. Representative heme stains (based on three trials) of soluble B-PER extracts (40 μg) of IPTG- and arabinose-induced *ccm* or *ccm dsb* knockout(s) (indicated above each lane), expressing pRGK368 (CcsBA) and pRGK332 (pBADCytc_4:6xHis; A and B) or pRGK399 (pMalp2x:CCHL) and pRGK390 (pBADCycS:6xHis; C and D). Panels C and D lane 7 (No Sys) represent IPTG- and arabinose-induced *E. coli ccm* containing only pRGK390. B-PER extracts of induced *E. coli* strains where exogenous 3 mM DTT was included during growth and induction are shown in B and D. The positions of holoCytc_4 and holoCycS are denoted with arrows.

strain (Fig. 8). For system II, the *dsbD* and *dsbC* mutants produced little to no cytochrome c_4 (Fig. 8A), and these defects were largely corrected by adding exogenous DTT (Fig. 8B). Neither the *dsbA* nor *dsbB* mutant showed defects in assembly, suggesting that formation of a disulfide is unnecessary under these growth conditions. Various studies have suggested that sometimes DsbA and/or DsbB are necessary for the natural system I-mediated synthesis, and sometimes they are dispensable [13, 14, 62]. Such variation could be due to aerobic versus anaerobic growth conditions, but we have not studied this further. The requirement for DsbC and DsbD suggests that some of the apocytochrome c_4 (CXXC) may undergo oxidation (e.g. oxygen-mediated), and DsbD-/DsbC-mediated thiol reduction is necessary. DsbC encodes a periplasmic thioredoxin-like protein, thus we propose that it functions in the reduction of apocytochrome *c* directly, and is re-reduced by DsbD. In the case of recombinant system III (CCHL) and its cognate human cytochrome *c* (Fig. 8C, D), DsbC is partially required (holoCycS is reduced by 80%), while the *dsbD* mutant showed only 30% reduction (when averaged over three trials) in cytochrome *c* assembly. It is possible that as the human cytochrome *c* is exported it is more rapidly used by CCHL, thus its two cysteines are reduced and ready for heme attachment to CXXCH. (Alternatively, some of the holoCycS may be matured by unsecreted, cytoplasmically located CCHL.) In both systems II and III, DTT inhibits cytochrome *c* production in the absence of DsbA (and in Δ*ccm*). This result could be due to the osmofragility of *dsbA*$^-$ strains, thus a pleiotrophic effect [63]. We conclude that under the aerobic conditions used, both recombinant systems II and III require some thiol reduction and this periplasmic reduction is mediated by the natural *E. coli* DsbC and/or DsbD proteins. For system III, the lack of a strong DsbD requirement suggests that DsbC may be acting in a DsbD-independent manner, thus DsbC can obtain reductant from other periplasmic proteins directly or from reduced DsbA, as shown previously [63, 64]. In addition to the thiol reduction requirement, DsbC might protect the apocytochrome *c* cysteines from sulfenylation, which was recently reported as a function of DsbC [65]. The need for DsbC in system III underscores the conclusion that heme attachment can occur in the periplasm by this recombinant CCHL system, albeit at much reduced levels when compared to system I (or to cytoplasmic CCHL). The absolute dependence of recombinant system II on DsbC and DsbD (and correction by exogenous DTT), addresses how recombinant, periplasmic *c*-type cytochromes in *E. coli* can be reduced using various recombinant assembly systems (e.g. see [28, 30, 64].

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbabio.2011.09.008.

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