SUPPORTING INFORMATION

Construction of strains and plasmids. All oligonucleotide primer sequences, plasmids, and strains are given in Table S1. To create RK113, we deleted the endogeneous *ccm* operon in *E. coli* BW25113 by P1 transduction. Briefly, P1 phage were grown first in LB media containing 5 mM $CaCl_2$ and 0.2 % glucose (wt/vol) with the donor strain (RK103 (*1*)). Donor cells were killed by addition of chloroform and phage were isolated. Dilutions of isolated phage (100 µL) were incubated with 100 µL of the recipient strain (BW25113) in LB containing 5 mM $CaCl_2$ and 100 mM $MgSO_4$ at 37^{o}C for 30 min. Physical interaction between the phage and cells was disrupted by addition of 66.7 mM sodium citrate (Fisher), pH 5.5, and the culture was added to 1 mL LB broth and incubated for one hour at 37^{o}C while shaking at 300 rpm. Transductants were selected by growth on LB + Kan, and cells were purified from P1 phage by repeated streaking on LB + Kan plates containing sodium citrate. Deletion of the *ccm* operon in RK113 was confirmed by genomic PCR. To create RK111, we used the pKNOCK system (*2*). Briefly, the *cyt* c_4:His6 gene along with the *araC* gene were cloned from pRGK332 (*1*) into pKNOCK-Gm (*2*). The *araC* gene was included to serve as the site for homologous recombination with the chromosome. Cloning was done in *E. coli* S17-1/λ*pir*, and the plasmid was conjugally transferred to *E. coli* RK103 (*1*). Putative exconjugants were selected for resistance to 10 ug ml^{-1} gentamicin, and correct integration of *cyt* c_4:His was verified by genomic PCR.

E. coli strains TB1 and HB101 were used as host strains for cloning. pRGK333 (*1*) containing the full system I operon (*ccmABCDEFGH*) was the template for all PCR amplifications unless otherwise indicated. A plasmid containing *ccmABCDE* (Δ*ccmFGH*) was constructed by PCR amplification of *ccmABCDE* with Ccm_N-term and Del_CcmF_Left, the product of which was digested, and ligated into BamHI and EcoRI digested pGEX-4T-1 (GE

Healthcare) to make pRGK402. To generate the His173Ala, His261Ala, and His303Ala mutants, short PCR products were amplified using Del_CcmE_Right and CcmF_His173Ala_Rev, CcmF_His261Ala_Rev, or CcmF_His303Ala_Rev. These products were gel purified and used in a second amplification with CcmH_XhoI_Rev using pRGK386 (*3*) as template to generate *ccmF*(His173Ala):His6*GH*, *ccmF*(His261Ala):His6*GH*, and *ccmF*(His303Ala):His6*GH* (His6 refers to a hexahistidine tag at the carboxy terminus of CcmF). The final, full-length products were ligated into NdeI and XhoI digested pRGK402 to generate pRGK403, pRGK404, and pRGK405, respectively. A plasmid containing *ccmABCDEGH* (Δ*ccmF*) was constructed by PCR amplification of *ccmGH* with Del_CcmF_Right and Ccm_C-term primers, the product of which was digested and ligated into EcoRI and NdeI digested pRGK402 to make pRGK406. For the His491Ala mutant, a short PCR product was generated by amplifying with CcmF_His491Ala_Fwd and CcmF_ 6xHis_NdeI_Rev. This product was gel purified and then used in a second amplification with Del_CcmE_Right to generate *ccmF* (His491Ala):His6. The final, full-length product was ligated into the single NdeI site of pRGK406 to generate pRGK407.

To insert *ccmF*:His6, *ccmF*(His261Ala):His6, or *ccmF*(His491Ala):His6 into pRGK330 (*1*) for arabinose-inducible expression in the absence of other *ccm* genes, the corresponding *ccmF* gene was amplified from pRGK386, pRGK404, or pRGK407, respectively, with CcmF_NcoI_Fwd and CcmF_6xHis_PstI_Rev, digested, and ligated into NcoI and PstI digested pRGK330 to make pRGK408, pRGK409, and pRGK410. His261Gly, Cys, Tyr, and Met were generated by amplifying with the appropriate reverse mismatch primer and CcmF_NcoI_Fwd to generate short PCR products. These products were gel purified and used in a second amplification with CcmF_6xHis_PstI_Rev. His491Gly, Cys, Tyr and Arg were generated by

amplifying with the appropriate forward mismatch primer and CcmF_6xHis_PstI_Rev to generate short PCR products. These products were gel purified and used in a second amplification with CcmF_NcoI_Fwd. The final, full-length products for all site-directed mutants at His261 and His491 described above were digested with NcoI and PstI and inserted into pRGK330 to generate pRGK411-418. Each of the final constructs was sequenced to confirm the mutation.

The *ccmF-3* gene from *Shewanella oneidensis* (SO_0478) was PCR amplified from genomic DNA, digested, and ligated into the NcoI and PstI sites in pRGK330 to generate pRGK420. The *ccmF* gene from *Roseobacter denitrificans* (RD1_3223) was amplified from genomic DNA, digested, and ligated into the EcoRI and KpnI sites of pRGK330 to make pRGK424. *Roseobacter denitrificans* genomic DNA was provided generously by the Blankenship lab. The *ccmF-2* gene from *E. coli* (b4074) was amplified from genomic DNA, digested, and ligated into the NcoI and XbaI sites of pRGK330 to make pRGK421. The *ccmF* genes from *Shewanella oneidensis* (SO-0266), *Desulfovibrio vulgaris* (DVU_1050), and *Thermus thermophilus* (TTHA1404) were each amplified from genomic DNA with the appropriate primers and cloned into pCR-Blunt II-TOPO (Invitrogen). From pCR-Blunt II-TOPO, the *ccmF* gene from *Shewanella oneidensis* was ligated into the NheI and XhoI sites of pRGK330 to make pRGK419, the *ccmF* gene from *Desulfovibrio vulgaris* was ligated into the NcoI and XbaI sites to make pRGK423, and the *ccmF* gene from *Thermus thermophilus* was ligated into the KpnI and NheI sites to make pRGK422. *Desulfovibrio vulgaris* subsp. *vulgaris* ATCC 29579 and *Thermus thermophilus* ATCC 27634 strains were obtained from ATCC and cultured according to ATCC recommendations, and genomic DNA was prepared using the Puregene System Cell and Tissue DNA Isolation Kit (Gentra Systems).

Table S1. Oligonucleotides, plasmids, and strains used in this study

Primer	Sequence (5'-3')	Plasmid Constructed
Del_CcmF_Left	CCGAATTCTGGCATCATATGGCTGGGTCCTTAT	pRGK402
Ccm_N-term	TTGCAGATCTATGCTTGAAGCCAGAG	pRGK402
Del_CcmE_Right	GACCCAGCCATATGATGCCAGAAATTGGTAACG	pRGK403-405, 407
CcmF_His173Ala_Rev	GCAGAGGCGGAGCGAAGATGAGCCCCGG	pRGK403
CcmF_His261Ala_Rev	CCGCCAGTGACGCCATCAGCGCAGTCC	pRGK404
CcmF_His303Ala_Rev	CAGACGCAAACGCGGCTACAGATACCAGCACGCC	pRGK405
CcmH_XhoI_Rev	GCGCTCGAGTTTTTATTCACTCTCCTGCGG	pRGK403-405
Del_CcmF_Right	GGCCATATGAAGCGCAAAGTATTGTTAATTCCG	pRGK406
Ccm_C-term	CGGAATTCTTTTTATTTACTCTCCTGC	pRGK406
CcmF_His491Ala_Fwd	GTGGCGGCTGCCCTTGGGCTG	pRGK407
CcmF_6xHis_NdeI_Rev	GATCATATGTCAGTGGTGGTGGTGGTGGTGTACGGCCTCCGGCGCAG	pRGK407
CcmF_6xHis_PstI_Rev	GATCTGCAGTCAGTGGTGGTGGTGGTGGTGTACGGCCTCCGGCGCAG	pRGK408-418
CcmF_NcoI_Fwd	CGCCATGGTGCCAGAAATTGGTAACGGAC	pRGK408-418
CcmF_His261Gly	CGCCAGTGAGCCCATCAGCGC	pRGK411
CcmF_His261Cys	CGCCAGTGAGCACATCAGCGC	pRGK412
CcmF_His261Tyr	CGCCAGTGAATACATCAGCGC	pRGK413
CcmF_His261Met	CGCCAGTGACATCATCAGCGC	pRGK414
CcmF_His491Gly	GTGGCGGCTGGCCTTGGGCTG	pRGK415
CcmF_His491Cys	GTGGCGGCTTGCCTTGGGCTG	pRGK416
CcmF_His491Tyr	GTGGCGGCTTACCTTGGGCTG	pRGK417
CcmF_His491Arg	GTGGCGGCTCGCCTTGGGCTG	pRGK418
Shew_CcmF_Fwd	GGCCATGGCCCCAGAACTGGGACACTTTTCGCTG	pRGK419
Shew_CcmF_Rev	GGGGTCGACTTAGTGGTGGTGGTGGTGTTGAGCGGTAGCTAATTTCGC	pRGK419
Shew_CcmF3_Fwd	CGCCATGGTTCCAGAAATCGGGCA	pRGK420
Shew_CcmF3_Rev	ATCTGCAGTCAGTGGTGGTGGTGGTGTTGGGTTACCGTCTC	pRGK420
Ecoli_CcmF2_Fwd	GCACCATGGTGACCCCGTTGACGGCCTTC	pRGK421
Ecoli_CcmF2_Rev	GCTCTAGATTAGTGGTGGTGGTGGTGTTCATCGCGCTTCTTCCCCC	pRGK421
Thermus_CcmF_Fwd	ATGCTAGCAGGAGGAATTCACCATGACCCCGGCCCTTCTCGGCAAC	pRGK422
Thermus_CcmF_Rev	ACAAGCTTTCAGTGGTGGTGGTGGTGGTGCGCCGGGCTCACCCCCCTAG	pRGK422
Desulfo_CcmF_Fwd	AACCATGGCCCTTTCCGCCTACCTGCTTCTCGTAGCCTCGCTGCTGTTC	pRGK423
Desulfo_CcmF_Rev	AGGATCCTTAGTGGTGGTGGTGGTGGTGCGCCTCCGCTGTCTTCCGGC	pRGK423
Roseo_CcmF_Fwd	GCGAATTCACCATGATTACAGAACTCGGACA	pRGK424
Roseo_CcmF_Rev	ATGGTACCTCAGTGGTGGTGGTGGTGGTGTTCCGCAGGCACCGGTGCGGC	pRGK424

Plasmid	Description	Reference
pRGK330	pBAD CmR	(1)
pRGK332	pBAD cytochrome *c*4:His6	(1)
pRGK333	pGEX *ccmABCDEFGH*	(1)
pRGK386	pGEX *ccmABCDEF*:His6*GH*	(3)
pRGK402	pGEX *ccmABCDE*	This work
pRGK403	pGEX *ccmABCDEF*(His173Ala):His6*GH*	This work
pRGK404	pGEX *ccmABCDEF*(His261Ala):His6*GH*	This work
pRGK405	pGEX *ccmABCDEF*(His303Ala):His6*GH*	This work
pRGK406	pGEX *ccmABCDEGH*	This work
pRGK407	pGEX *ccmABCDEF*(His491Ala):His6*GH*	This work
pRGK408	pBAD *ccmF*:His6	This work
pRGK409	pBAD *ccmF*(His261Ala):His6	This work
pRGK410	pBAD *ccmF*(His491Ala):His6	This work
pRGK411	pBAD *ccmF*(His261Gly):His6	This work
pRGK412	pBAD *ccmF*(His261Cys):His6	This work
pRGK413	pBAD *ccmF*(His261Tyr):His6	This work
pRGK414	pBAD *ccmF*(His261Met):His6	This work
pRGK415	pBAD *ccmF(*His491Gly):His6	This work
pRGK416	pBAD *ccmF*(His491Cys):His6	This work
pRGK417	pBAD *ccmF*(His491Tyr):His6	This work
pRGK418	pBAD *ccmF*(His491Arg):His6	This work
pRGK419	pBAD *ccmF*:His6 (*S. oneidensis*)	This work
pRGK420	pBAD *ccmF-3*:His6 (*S. oneidensis*)	This work
pRGK421	pBAD *ccmF-2*:His6 (*E. coli*)	This work
pRGK422	pBAD *ccmF*:His6 (*T. thermophilus*)	This work
pRGK423	pBAD *ccmF*:His6 (*D. vulgaris*)	This work
pRGK424	pBAD *ccmF*:His6 (*R. denitrificans*)	This work

Strain	Description	Reference
RK103	MG1655 Δ*ccm*	(1)
RK111	MG1655 Δ*ccm cyt c*4:His6 chromosomal integrate	This work
RK113	BW25113 Δ*ccm* Δ*araBAD*	This work
ATCC 27634	*Thermus thermophilus*	This work
ATCC 29579	*Desulfovibrio vulgaris* subsp. *vulgaris*	This work

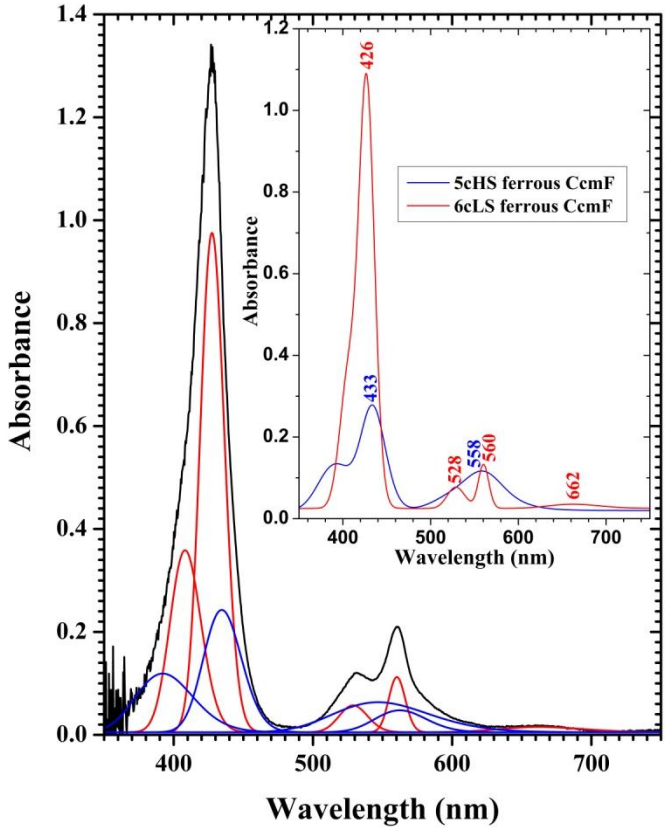

Figure S1. Peak fitting of the ferrous CcmF UV-visible absorbance spectrum. The fit peaks reveal speciation between 6cLS (red) and 5cHS (blue) hemes with a significant fraction being 5cHS. Assuming that the extinction coefficients at the HS and LS Soret maxima are roughly equal, the samples of ferrous CcmF(WT) used in this study comprise approximately 20 % 5cHS heme. Based on similarities among the UV-visible spectra and insensitivity of the ν_3 ratios to [DDM] between 0.48 % and 0.02 %, this speciation is essentially independent of DDM concentration.

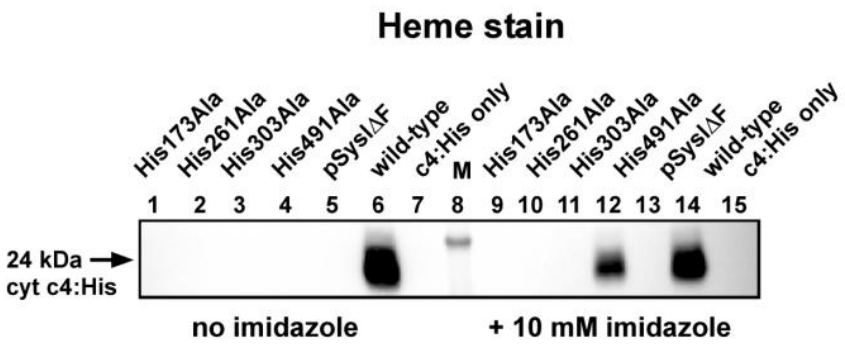

Figure S2. Representative heme stain showing cytochrome c_4:His6 assembly (i.e., heme attachment) by the indicated CcmF proteins in the presence or absence of 10 mM imidazole. Arrow indicates 24 kDa holocytochrome c_4:His6 matured by a functional system I. "pSysIΔF" denotes a system I deleted for *ccmF*; "c4:His only" denotes an absence of all *ccm* genes; "M" denotes molecular weight standards (shown is the 28 kDa standard). 100 µg of BPER-isolated proteins was loaded into each lane for SDS-PAGE prior to heme staining.

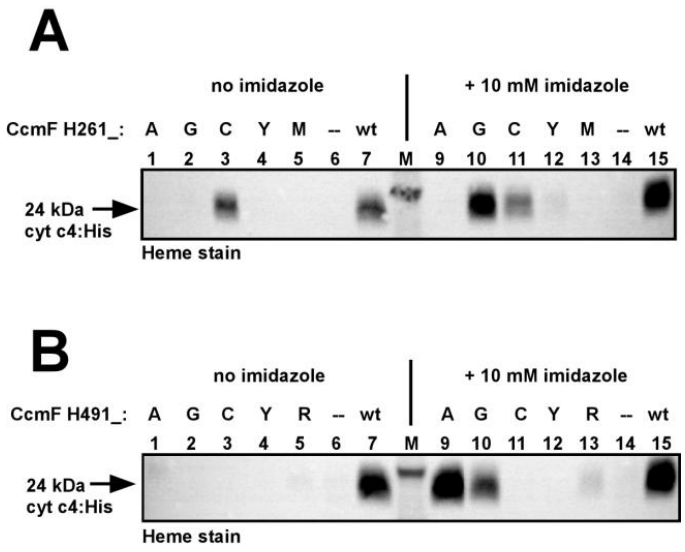

Figure S3. Representative heme stains showing cytochrome c_4:His6 assembly of mutants at His261 (A) and His491 (B) in the presence or absence of 10 mM imidazole (as described in Figure S1). Arrow indicates 24 kDa holocytochrome c_4:His6 matured by a functional system I. Substitutions: A, Ala; G, Gly; C, Cys; Y, Tyr; M, Met; R, Arg. The dash denotes the negative control condition (absence of CcmF) and "wt" denotes wild-type CcmF. "M" denotes molecular weight standards (shown is the 28 kDa standard). 100 µg of BPER-isolated proteins was loaded into each lane for SDS-PAGE prior to heme staining.

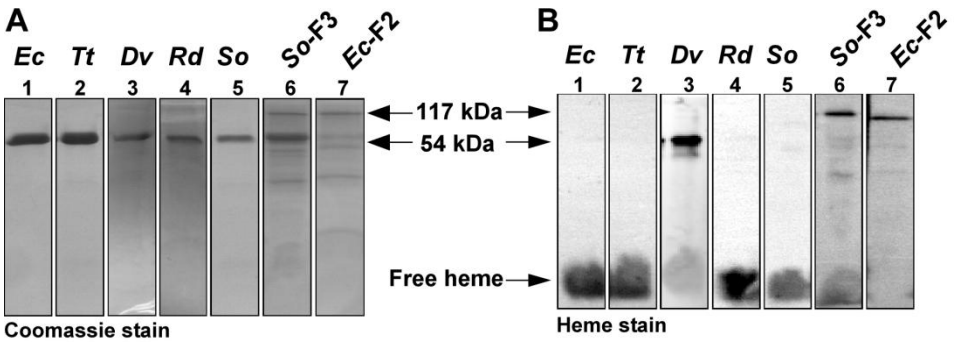

Figure S4. Coomassie stain (A) and corresponding heme stain (B) after SDS-PAGE of CcmF:His6 from *E. coli* (*Ec*), *Thermus thermophilus* (*Tt*; Deinococcus group), *Desulfovibrio vulgaris* (*Dv*), *Roseobacter denitrificans* (*Rd*), *Shewanella oneidensis* (*So*), *Shewanella oneidensis* CcmF-3 (*So*-F3), and *E. coli* CcmF-2 (*Ec*-F2). Arrows indicate full-length 54 kDa CcmF:His6 and free heme at the SDS-PAGE dye front. Note that some of the *So*-CcmF3 and *Ec*-CcmF2 aggregate at approximately 117 kDa, possibly a dimeric form, under the conditions of SDS-PAGE used. Additionally, note that for *Dv*, *So*-CcmF3 and *Ec*-F2, some heme is retained in the full length protein and/or in the higher molecular weight forms. For each of the proteins analyzed, heme was found to be non-covalent as determined by pyridine hemachromagen (data not shown). Thus, *Dv*, *So*-CcmF3 and *Ec*-F2 may bind heme in a partially SDS-resistant, although non-covalent, manner. 30 µg of purified hexahistidine-tagged protein was analyzed for each.

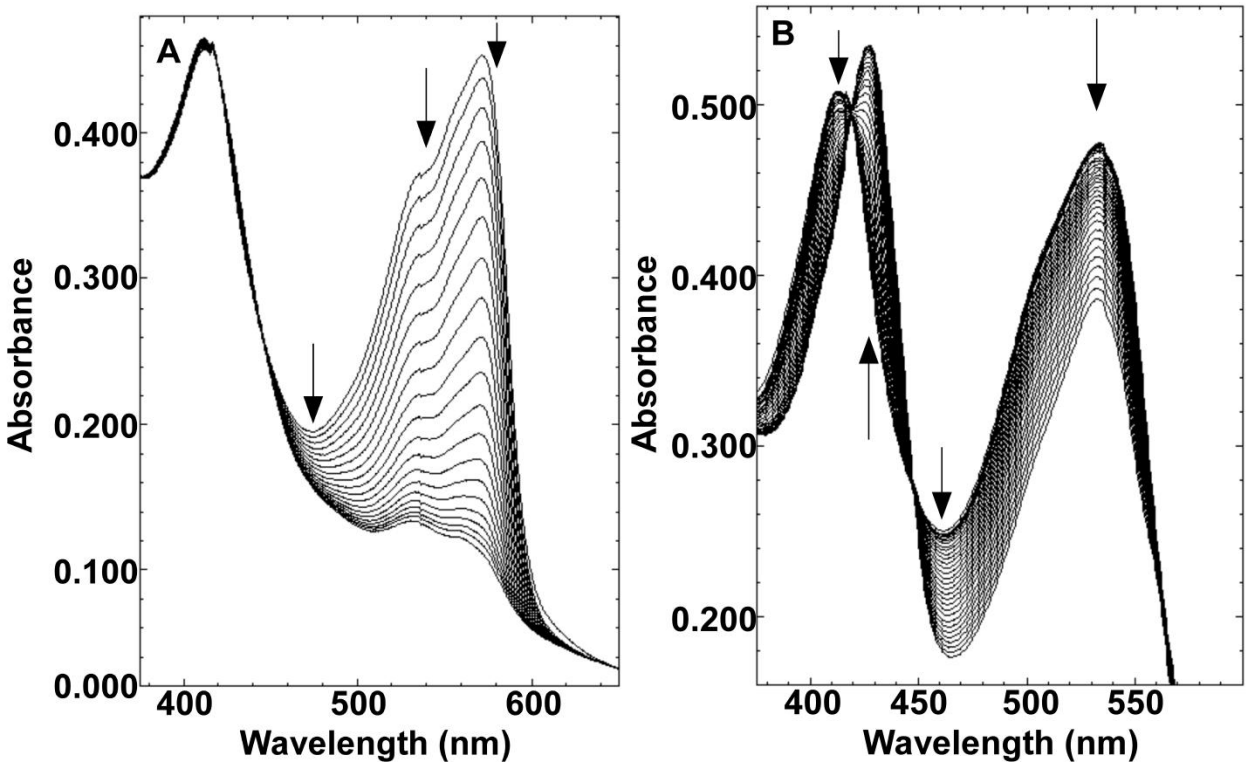

Figure S5. Redox titration of the CcmF *b*-heme with alternate redox dyes. Spectra collected during reduction of CcmF *b*-heme with resorufin (A) or safranin O (B). Arrows indicate the direction of changes in absorption during the course of the titration. The decreases in absorbance at 534 nm and 572 nm in (A) are due to reduction of resorufin. In (B), the decrease in absorbance at 412 nm and the increase in absorbance at 426 nm are indicative of reduction of the CcmF *b*-heme, and the decrease in absorbance at 532 nm is due to reduction of safranin O. Note that the dye resorufin (E_m = -50 mV) (*4*) is completely reduced before reduction of the CcmF *b*-heme, while the CcmF *b*-heme is completely reduced before reduction of safranin O (E_m = -280 mV) (*4*). This indicates that the relative midpoint potential of the CcmF *b*-heme is in between that of resorufin and safranin O. Conditions: 20 mM Tris-HCl, pH 7, 100 mM NaCl, 0.02 % DDM.

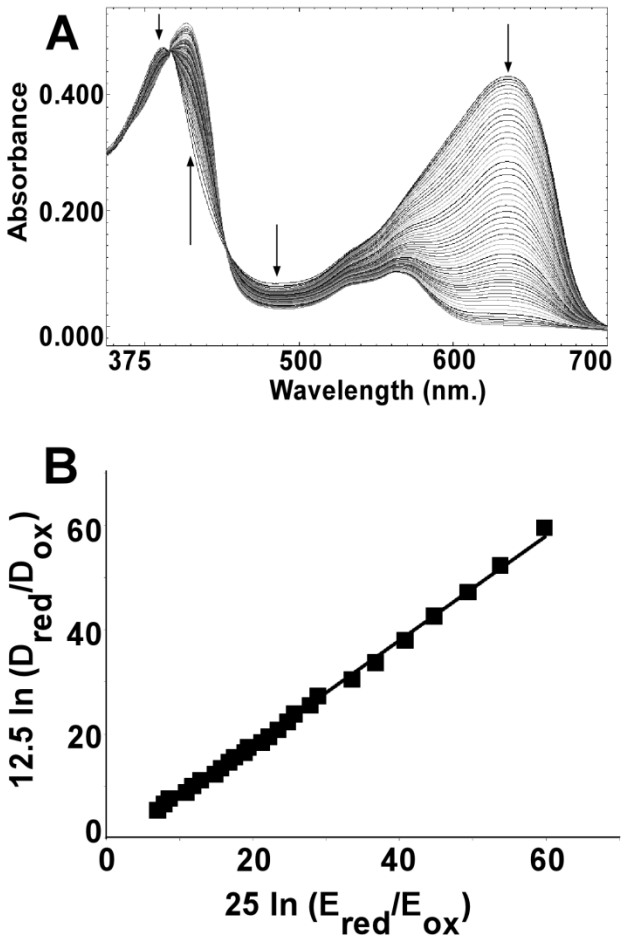

Figure S6. Redox titration of the CcmF *b*-heme in high DDM (1.2 %). Spectra collected during a reductive titration of CcmF *b*-heme with nile blue chloride (E_m = -116 mV) (*4*) (A) and the corresponding linear Nernst plot (B). Arrows in (A) indicate the direction of changes in absorption during the course of the titration. In (B), [25 mV ln (*b*-heme$_{red}$/*b*-heme$_{ox}$)] was used for the one-electron reduction of heme and [12.5 mV ln (dye$_{red}$/dye$_{ox}$)] was used for the two-electron reduction of dye, where *b*-heme$_{red}$/*b*-heme$_{ox}$ and dye$_{red}$/dye$_{ox}$ represent ratios of the molar concentrations of the reduced and oxidized forms of the *b*-heme and the dye, respectively. The reduction potential in 1.2 % DDM was substantially more positive (E_m = -110 ± 4 mV) than in 0.02 % DDM. Conditions: 20 mM Tris-HCl, pH 7, 100 mM NaCl, 1.2 % DDM.

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