

c-Type Cytochrome Biogenesis Can Occur via a Natural Ccm System Lacking CcmH, CcmG, and the Heme-binding Histidine of CcmE^{*[S]}

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The Ccm cytochrome *c* maturation System I catalyzes covalent attachment of heme to apocytochromes *c* in many bacterial species and some mitochondria. A covalent, but transient, bond between heme and a conserved histidine in CcmE along with an interaction between CcmH and the apocytochrome have been previously indicated as core aspects of the Ccm system. Here, we show that in the Ccm system from *Desulfovibrio desulfuricans*, no CcmH is required, and the holo-CcmE covalent bond occurs via a cysteine residue. These observations call for reconsideration of the accepted models of System I-mediated *c*-type cytochrome biogenesis.

c-Type cytochromes are generally characterized by the covalent attachment of heme to the polypeptide via two thioether bonds formed by reaction of the vinyl groups of heme with the sulfur atoms of two thiols in a CXXCH motif in the protein. This post-translational modification reaction is, surprisingly, catalyzed by several different systems in different cell types and organelles (1–3). Of these, the cytochrome *c* maturation system (CcmABCDEFGH (4), also called System I) functions in the periplasm of many species of bacteria and in mitochondria of some eukaryotes, including plants (5, 6).

A large number of investigations of the *Escherichia coli* system have implicated the CcmABC proteins in ATP-dependent handling of heme via covalent attachment to CcmE (e.g. Refs. 7 and 8). CcmD appears to be a single helix protein involved in the interaction of heme with CcmE (9, 10). CcmE has been identified as a heme chaperone, covalently binding heme via an apparently conserved histidine residue (His¹³⁰ in *E. coli*) before transfer of the heme to apocytochromes (11, 12); to date, this particular heme-histidine bond has only been identified in CcmE (12, 13). Covalently bound heme on CcmE is transferred to the apocytochrome in a process that is believed to involve CcmF and CcmH (14). *E. coli* CcmH contains two distinct domains that exist as separate proteins in some organisms. The

protein called CcmH in most organisms comprises the N-terminal CXXC-containing globular domain of the *E. coli* protein. The C-terminal tetratricopeptide repeat-containing domain of *E. coli* CcmH is analogous to CcmI in other species of bacteria (15). *In vitro* studies have suggested that peptides carrying the CXXCH motif can interact with the CXXC region of bacterial and plant CcmH (16–18), and two-hybrid analysis indicated that plant CcmH interacts with apocytochrome *c* (18). CcmG is believed to transfer reductant from the transmembrane DsbD protein to CcmH and/or the apocytochrome (19), and it has been recently proposed, at least for *Rhodobacter capsulatus*, that CcmG can also interact with the apocytochrome *c* in a “holdase” role (20). The *E. coli* Ccm system is inactive in the absence of CcmG (21).

Recent sequence analysis (22) revealed an apparently novel CcmE in a number of archaeal and bacterial species, including two sulfate-reducing bacteria which are rich in, and highly dependent upon *c*-type cytochromes (e.g. Ref. 23). This CcmE contains a cysteine residue in place of the histidine that, in other CcmE proteins, forms a covalent bond to heme that is currently thought to be an essential intermediate in the cytochrome *c* maturation pathway (12). In addition, the putative *ccm* operons from *Desulfovibrio desulfuricans* and *Desulfovibrio vulgaris* lack *ccmH* and *ccmG*, although a candidate CcmG is encoded elsewhere on the genomes. A *ccmI* homologue was originally missed in the *ccm* operon (22) but was subsequently suggested to be encoded at the 3' end (2). These *in silico* findings clearly raise important questions about the function of the Ccm proteins. Genetic manipulation of sulfate-reducing bacteria is not straightforward, and *c*-type cytochromes are important for growth of such organisms. Therefore, we have used *E. coli* as an heterologous expression system to study *c*-type cytochrome biogenesis by the *D. desulfuricans* Ccm system.

Here we report maturation of *c*-type cytochrome when the *ccmEFABCDI* genes from *D. desulfuricans* are expressed in an *E. coli* strain lacking all endogenous *ccm* genes. We have termed this variant Ccm machinery “System I*.” This outcome calls for recognition that some current views concerning the function of core Ccm components require revision and illustrates how the acquisition of ever-growing numbers of bacterial genome sequences can generate unexpected challenges to accepted paradigms.

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[S] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Tables S1 and S2 and Fig. S1.

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EXPERIMENTAL PROCEDURES

Strains and Plasmids—*E. coli* strain EC06 (4) contains a chromosomal deletion of the *ccm* operon and was used to examine holocytochrome formation in the presence of each biogenesis system and its mutants. *E. coli* strain DH5 α (Invitrogen) was used for routine molecular biology. PCR used KOD polymerase from *Thermococcus kodakaraensis* (Novagen). All oligonucleotides (Sigma-Genosys) used in this study are listed in supplemental Table S1, and all plasmids are listed in supplemental Table S2. All DNA constructs were sequenced before use.

The *E. coli ccmABCDEFGH* operon (System I) was expressed from pEC86 (24). To create a comparable plasmid lacking any biogenesis system, inverse PCR was performed on pEC86 using AG234 and AG235, and the product self-ligated. This removed the entire *ccm* operon, and the plasmid created is AD377 (no biogenesis system). To create a suitable plasmid for expression of other biogenesis systems, a *Xho*I site was introduced immediately after the ATG start codon of *ccmA* in pEC86 via QuikChange mutagenesis using WC1 and WC2. The resultant construct is pEC86x in which the entire *ccm* operon can be excised by digestion with *Xho*I and *Stu*I. The *ccm* operon (*ccmEFABCDI*) was amplified from *D. desulfuricans* genomic DNA using AG81 and AG82 and cloned into the unique *Xho*I and *Stu*I sites in pEC86x to generate pDD86. The same methodology was utilized to clone the truncated operons *ccmEFABCD* (using AG81 and AG83 resulting in plasmid AD457) and *ccmEFABC* (AG81 and AG286 resulting in plasmid AD153). *Paracoccus denitrificans* cytochrome *c*₅₅₀ was expressed from the isopropyl-1-thio- β -D-galactopyranoside (IPTG)⁴-inducible promoter of pKPD1 (25), a derivative of pKK223-3. Similarly, a CXXCH variant of *Trypanosoma brucei* cytochrome *c* was expressed from IPTG-inducible pKK223-Tbctc_{peri}CXXCH as described previously (26). The CXXCK variant of *P. denitrificans* cytochrome *c*₅₅₀ has been described previously (27).

To generate mutants in *D. desulfuricans* CcmE, the HindIII fragment containing the *ccmE* open reading frame was excised from pDD86 and cloned into HindIII-digested pTZ19R. Inverse PCR was performed on this construct using AG267 and AG266 (C127H), AG285 (C127M), or AG284 (C127A). The resulting construct was sequenced before the HindIII fragment was reintroduced into pDD86 to create the appropriate mutant plasmids, which are indicated in supplemental Table S2.

To generate a construct for expressing *D. desulfuricans* membrane-bound CcmE, PCR was performed on *D. desulfuricans* genomic DNA using oligonucleotides JS3 and JS4. The product was cloned into the *Nde*I and *Eco*RI sites of pISC2 to create pDDCcmE. To generate soluble *D. desulfuricans* CcmE, PCRs were performed using AG298 and, sequentially, AG295, AG296, and AG297. The template for the first PCR was pDD86, and subsequent templates were the purified products of the previous PCR. The final product was cloned into the *Eco*RI and HindIII sites of pKK223-3 to create pDDCcmE^{sol}. The C127H

mutant in this construct was generated via inverse PCR on pDDCcmE^{sol} using AG266 and AG267.

To generate a construct for expressing soluble *E. coli* CcmE, inverse PCR was performed on pEC415 (12) using FR1 and FR2 to remove the His₆ tag. The resultant plasmid is pECCcmE^{sol}. A H130C mutation was created in this construct by QuikChange mutagenesis using oligonucleotides FR3 and FR4. This plasmid is pECcmE^{sol}H130C. To generate the mutation changing the ¹³⁰HDENY motif of *E. coli* CcmE to CPSKY, QuikChange mutagenesis was performed on pEC86 using JS1 and JS2 to create pEC86^{CPSKY}.

In each case the plasmid bearing the biogenesis system confers resistance to chloramphenicol, and expression of the biogenesis system is constitutive. The plasmid bearing the cytochrome is IPTG-inducible and confers resistance to carbenicillin. pISC2-derived plasmids are arabinose inducible.

Routine cell growth was conducted using LB (Luria-Bertani) media supplemented with antibiotics where appropriate. Growth on solid media used liquid growth medium supplemented with 1.5% bacteriological agar. For preparation of periplasmic fractions, single colonies containing appropriate plasmids were picked into 500 ml of 2 \times TY medium (16 g liter⁻¹ peptone, 10 g liter⁻¹ yeast extract, 5 g liter⁻¹ NaCl) supplemented with 1 mM IPTG or 0.1% (w/v) arabinose and appropriate antibiotics in 2 liter flasks. Cultures were grown at 37 °C with shaking at 200 rpm for 20–24 h before harvesting. Carbenicillin was used at 100 μ g ml⁻¹, and chloramphenicol was at 34 μ g ml⁻¹.

Analysis of Cytochrome Production—Periplasmic extractions were performed as described previously (28). Where appropriate, these fractions were analyzed by SDS-PAGE (Invitrogen pre-cast 10% Bis-Tris gels) followed by heme staining (29) which detects heme covalently bound to protein. Samples were normalized for wet cell weight, and 5–10 μ g of protein were loaded per lane. See-Blue Plus 2 (Invitrogen) prestained protein marker was used. UV-visible spectroscopy was performed using a PerkinElmer Life Sciences Lambda 2 UV-visible spectrophotometer; samples were reduced by the addition of a few grains of disodium dithionite (Sigma). The extinction coefficient for reduced *P. denitrificans* cytochrome *c*₅₅₀ at 415 nm is 140 mM⁻¹ cm⁻¹. Pyridine hemeochrome spectra were obtained according to the method of Bartsch (30).

Analysis of Covalent Heme Binding by CcmE—Membrane fractions were prepared from cultures containing the appropriate biogenesis system plasmid. Cultures were grown as described above for preparation of periplasmic fractions, and membranes were extracted as described previously (31). 30 μ g of total membrane protein was analyzed by SDS-PAGE followed by heme staining (29).

Purification of Soluble *D. desulfuricans* CcmE—*E. coli* EC06 cells containing pDDCcmE^{sol} were grown in 2 \times TY medium containing 1 mM IPTG and carbenicillin for 20 h. Cells were harvested at 5000 \times g for 20 min at 4 °C and resuspended in Tris-HCl buffer (50 mM Tris, 150 mM NaCl, pH 7.5) containing a protease inhibitor mixture. Periplasmic extraction was carried out using β -Polymyxin (final concentration of 3 mg ml⁻¹) followed by incubation at 37 °C for 1 h. The spheroplasts were pelleted by centrifugation at 10,000 \times g for 40 min at 4 °C.

⁴ The abbreviations used are: IPTG, isopropyl-1-thio- β -D-galactopyranoside; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

A Variant System I for *c*-Type Cytochrome Biogenesis

Proteins in the resulting periplasm (supernatant) were purified using a Streptactin (IBA) containing column equilibrated with the above Tris buffer and eluted with the same buffer containing 2.5 mM desthiobiotin. Protein concentration determination was carried out using the bicinchoninic acid (BCA) assay (Pierce).

Western Blot Analysis—Western blotting was carried out following SDS-PAGE by first transferring onto nitrocellulose (Hybond C-Extra, Amersham Biosciences). Blocking was achieved by incubation with 3% bovine serum albumin in Tris-buffered saline (50 mM Tris, 120 mM NaCl, 0.1% Tween 20, pH 7.5). Antibodies were used in the following dilutions: alkaline phosphatase conjugated anti-Strep2 (1:5,000, IBA); anti-CcmE from rabbit (1:1,000, from Prof. Linda Thony-Meyer (12)); alkaline phosphatase conjugated anti-rabbit (1:30,000, Sigma). Development was by a colorimetric reaction using a SigmaFast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablet dissolved in 10 ml of ultrapure water. The reaction was stopped by decanting off the developing solution and washing the membrane with ultrapure water.

Proteomics Analysis—Samples were digested with sequencing-grade trypsin (porcine, Promega), and analysis was done using liquid chromatography-tandem mass spectrometry on a Thermo LTQ Orbitrap mass spectrometer coupled to a Dionex Ultimate 3000 nano-high performance liquid chromatography

system or by MALDI-TOF/mass spectrometry on an Ultraflex instrument (Bruker).

RESULTS

Expression of *D. desulfuricans* *ccmEFABCDI* Restores *c*-type Cytochrome Biogenesis in *E. coli* Δ *ccm*—Initial analysis of the *ccm* operon from *D. desulfuricans* (22) revealed the presence of homologues of *ccmEFABCD*. However, we subsequently identified a predicted 22-kDa tetratricopeptide repeat-containing protein at the 3' end of the operon in which the predicted start codon overlaps the 3' end of *ccmD* (Fig. 1) (2). This is a potential homologue of *ccmI*. Therefore, we cloned the operon, including *ccmI*, into a modified version of pEC86, a vector routinely used to express the *E. coli* Ccm proteins, to create pDD86. The predicted *ccm* operon from *D. desulfuricans* lacks homologues of *ccmH* (*E. coli* N-CcmH) and *ccmG*; pDD86 allows us to assess if the predicted *ccmEFABCDI* genes are sufficient to support *c*-type cytochrome maturation.

The *E. coli* strain EC06 lacks the endogenous *ccm* system and, thus, is unable to make *c*-type cytochromes (4). Here we have used *P. denitrificans* cytochrome *c*₅₅₀ expressed from pKPD1 as an exogenous *c*-type cytochrome to assess the ability of the *D. desulfuricans* Ccm system to restore *c*-type cytochrome biogenesis in *E. coli* EC06 cells. To allow comparison of the various systems, the amount of cytochrome produced is expressed as absorbance/g of wet cell pellet where the absorbance is calculated as the absorbance difference between the base line of the spectra at 650 nm and the maximal absorbance of the Soret peak of reduced cytochrome *c*₅₅₀ (415 nm). Co-expression of plasmids AD377 (derived from pEC86 and containing no biogenesis genes (32)) and pKPD1 did not result in production of any detectable *c*-type cytochrome. *E. coli* EC06 cells containing pEC86 (*E. coli* *ccmABCDEF*) and pKPD1 produced 1.03 absorbance units/g, equivalent to 3.3

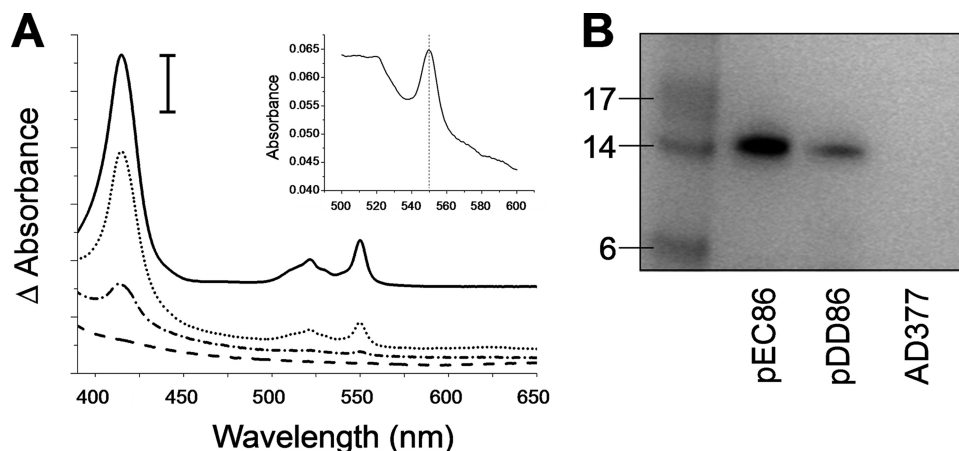
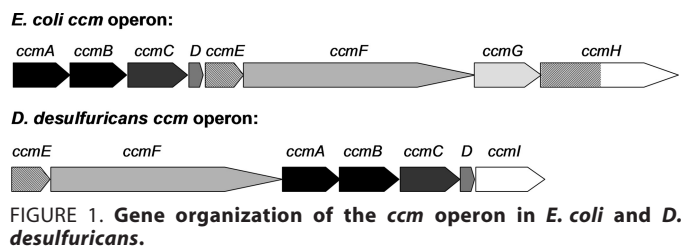


FIGURE 2. Production of *c*-type cytochromes by various biogenesis systems. A, shown are visible absorption spectra reflecting the formation of *P. denitrificans* cytochrome *c*₅₅₀ in *E. coli* catalyzed by *E. coli* CcmABCDEF (solid line), *D. desulfuricans* CcmEFABCDI (dotted line), *D. desulfuricans* CcmEFABCD (alternating dashed/dotted line), and as a negative control, no biogenesis system (dashed line). The vertical scale bar represents 0.04 absorbance units. The spectra are vertically offset for clarity. Samples were reduced by the addition of a few grains of disodium dithionite. The periplasmic fraction from cells expressing pEC86 was diluted 20-fold before analysis. The absorbance maxima are at 415, 521.5, and 550 nm. The inset shows the reduced pyridine hemochrome spectrum of cytochrome *c*₅₅₀ produced by *D. desulfuricans* CcmEFABCDI. The vertical line indicates 550 nm. B, detection of *c*-type cytochromes via SDS-PAGE analysis of periplasmic fractions from cells expressing *P. denitrificans* cytochrome *c*₅₅₀ and the indicated biogenesis system and subsequent heme-staining of the gel. The left-most lane contains See-Blue Plus 2 protein markers of the indicated molecular mass (kDa).

mg of cytochrome *c*₅₅₀/g wet cell pellet. Cells containing pDD86 (*D. desulfuricans* *ccmEFABCDI*) and pKPD1 produced 0.045 absorbance units/g (0.15 mg cytochrome *c*₅₅₀/g wet cell pellet), corresponding to ~5% of the level produced by pEC86. Fig. 2A shows the visible absorption spectra of the cytochrome *c*₅₅₀ produced by cells containing pDD86; it is indistinguishable from material made using the *E. coli* Ccm system or in *P. denitrificans* itself (25). Qualitatively similar results to those in this paragraph were obtained when pKPD1 was replaced by a plasmid encoding a CXXCH variant of *T. brucei* mitochondrial cytochrome *c* (26), indicating that the *D. desulfuricans* *ccm* system is active toward apocytochromes *c* from different sources. No detectable cytochrome was produced by either of the biogenesis

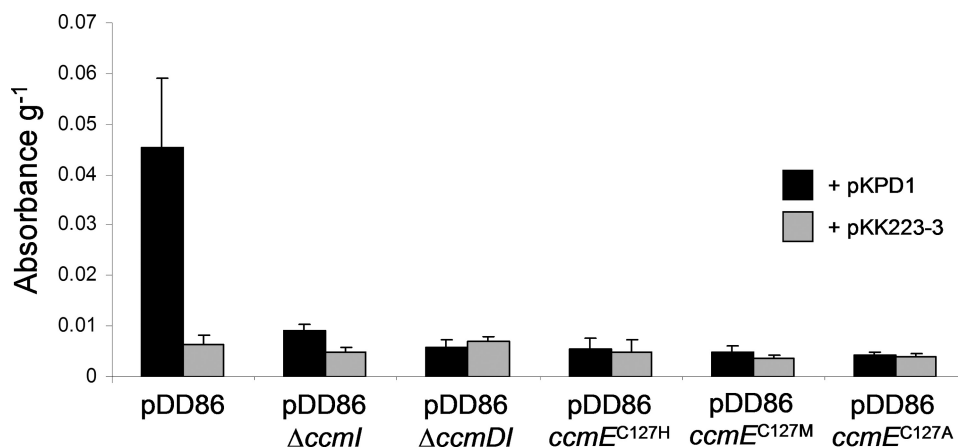


FIGURE 3. Production of *c*-type cytochromes by mutant *D. desulfuricans* Ccm systems. Quantitation of cytochrome c_{550} production by the indicated biogenesis system (data shown are means of six separate determinations \pm S.D. and are expressed as absorbance per gram of wet cell pellet). Details of quantitation are provided under "Results". For comparison, *E. coli* EC06 cells containing pEC86 and pKPD1 produced 1.03 absorbance units/g. The "basal" level of absorbance observed in all strains is not due to *c*-type cytochrome production, as can be deduced from the dashed spectrum in Fig. 2A that was obtained from cell material from which *c*-type cytochromes were completely absent.

systems when pKPD1 was replaced by pKK223-3, a plasmid lacking the cytochrome gene.

To define the lower limits of detection of *P. denitrificans* cytochrome c_{550} , we diluted the periplasmic extract from cells containing both pEC86 and pKPD1 (supplemental Fig. S1). The results indicate that UV-visible spectroscopy is slightly more sensitive than heme staining but that both detection methods have very low thresholds. UV-visible spectroscopy can detect *P. denitrificans* cytochrome c_{550} via the Soret peak at concentrations down to 3×10^{-8} M, and heme-staining can detect $\sim 1 \times 10^{-9}$ mol of cytochrome c_{550} .

To ensure covalent attachment of heme to apocytochrome c_{550} had occurred, periplasmic fractions from cells expressing pKPD1 together with *E. coli* ccmABCDEF, *D. desulfuricans* ccmEFABCDI, or AD377 (no biogenesis system) were subjected to SDS-PAGE analysis and subsequent heme staining (Fig. 2B). The presence of a band at ~ 14 kDa, corresponding to the mass of holocytochrome c_{550} , indicated covalent attachment of heme. To determine whether the cytochrome c_{550} produced by the *D. desulfuricans* system had heme correctly bound, we examined the reduced pyridine hemochrome spectrum. This was characteristic of a *c*-type cytochrome in which heme is bound via two thioether bonds to the protein, with the maximal absorbance of the α -band at 550 nm (Fig. 2A, inset). A variant of cytochrome c_{550} that contained the CWSCK motif (rather than the endogenous CKACH motif), found as a heme attachment site in some NrfA nitrite reductases, was not a substrate for the *D. desulfuricans* Ccm system, paralleling observations with the *E. coli* system (27).

The Requirement for CcmG, CcmI, and CcmD for Cytochrome *c* Biogenesis by the *D. desulfuricans* Ccm System—In addition to lacking a potential homologue of the N-terminal region of *E. coli* CcmH, the ccm operon in *D. desulfuricans* also lacks a homologue of ccmG (Fig. 1). It is clear from the evidence presented above that this protein is not required for *c*-type cytochrome biogenesis by the *D. desulfuricans* proteins when expressed in *E. coli* EC06. The endogenous ccmG is absent from EC06.

In *E. coli*, CcmH appears to be a fusion of two distinct domains, of which only the N-terminal domain is essential for cytochrome *c* biogenesis (33, 34). The CcmI protein encoded within the *D. desulfuricans* operon is homologous to the C-terminal domain of *E. coli* CcmH. Therefore, we created a plasmid containing the *D. desulfuricans* ccm operon but lacking ccmI (ccmEFABCD). Co-expression of this operon and pKPD1 resulted in production of cytochrome c_{550} at a level $\sim 20\%$ (0.0092 absorbance units/g, equivalent to 0.03 mg of cytochrome c_{550} /g of wet cell pellet) that observed in cells containing the ccmEFABCDI operon (Fig. 3). The spectrum did not show any detectable

difference from cytochrome c_{550} produced in the presence of CcmI (Fig. 2A). No cytochrome was observed in the absence of pKPD1. This suggests that, although the presence of *D. desulfuricans* CcmI significantly enhances cytochrome *c* production, it is not essential for this process. Therefore, the *D. desulfuricans* ccmEFABCD genes alone are sufficient for *c*-type cytochrome biogenesis.

Because of the small size and poor conservation of CcmD proteins, we determined whether the putative ccmD homologue in the *D. desulfuricans* operon was essential for cytochrome *c* biogenesis. We created a plasmid containing the *D. desulfuricans* ccm operon but lacking ccmD and ccmI (ccmEFABC). Co-expression of this operon and pKPD1 or pKK223-3 did not result in detectable production of cytochrome c_{550} (Fig. 3), suggesting that CcmD is essential for *c*-type cytochrome biogenesis, as it is in *E. coli* (9).

Exogenous Reductant Does Not Increase Cytochrome c_{550} Production—Mutations in *E. coli* CcmG, CcmH, and DsbD (which supplies reductant to CcmG) can be complemented by provision of exogenous reductant, notably 2-mercaptoethane sulfonic acid, in the growth media (33, 35, 36). However, the addition of 5 mM 2-mercaptoethane sulfonic acid or 1 mM cysteine had no effect on the yield of *c*-type cytochrome produced by the *D. desulfuricans* Ccm system when operating in *E. coli* EC06.

Desulfovibrio CcmE Covalently Binds Heme—*E. coli* CcmE has been demonstrated to covalently bind heme via His¹³⁰ (12). However, *D. desulfuricans* CcmE contains a cysteine at the corresponding position (Cys¹²⁷; Fig. 4A). To establish whether *D. desulfuricans* CcmE can covalently bind heme, membrane fractions were prepared from *E. coli* EC06 cells expressing the *D. desulfuricans* ccmEFABCDI, ccmEFABCD, and ccmEFABC operons and also those expressing no biogenesis system. The ccmEFABCD operon was included to provide a defect (a lack of CcmI) downstream of CcmE in the cytochrome biogenesis pathway in anticipation of maximizing, by analogy with the work of Reid *et al.* (37) with *E. coli*, the proportion of CcmE that

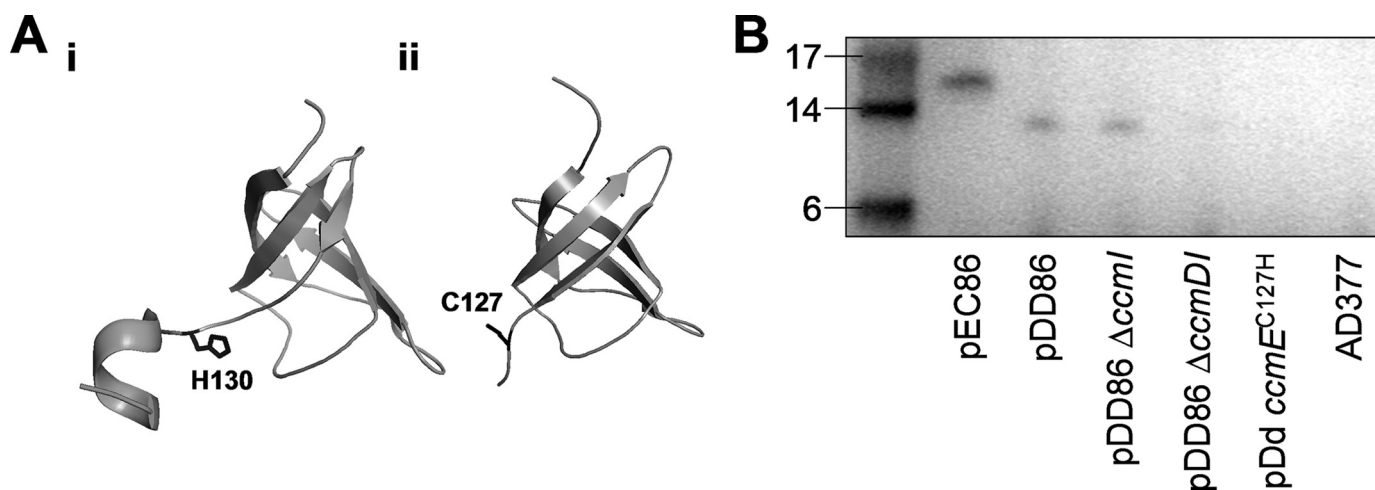


FIGURE 4. **Attachment of heme to CcmE.** A, shown are NMR structures of *E. coli* apoCcmE (PDB entry 1LIZ) (i) and *D. vulgaris* apoCcmE (PDB entry 2KCT) (ii). Structures were rendered in PyMOL. B, membrane fractions from the indicated strains were prepared and analyzed via SDS-PAGE and subsequent heme-staining for the presence of a covalent complex between *E. coli* or *D. desulfuricans* CcmE and heme. The left-most lane contains See-Blue Plus 2 protein markers of the indicated molecular mass (kDa).

may have covalently bound heme. In each case the genes for exogenous *c*-type cytochromes were absent. Membrane extracts from cells containing pEC86 encoding the entire *E. coli* *ccm* operon were included as a positive control (Fig. 4B). Equal protein loading for each extract was confirmed by examination of an equivalent Coomassie Blue-stained gel.

As reported previously (12), a heme-staining band was observed at ~16 kDa from cells expressing pEC86 after separation of the membrane fraction by SDS-PAGE (Fig. 4B, second lane). This is consistent with the presence of the *E. coli* holo-CcmE covalent complex (molecular weight, ~18 kDa). This band was not observed in the absence of a biogenesis system (AD377; Fig. 4B, seventh lane). In membrane extracts from *E. coli* EC06 cells expressing the *D. desulfuricans* *ccmEFABCDI* operon, a heme staining band was observed at ~14 kDa (Fig. 4B, third lane), consistent with the presence of holo-CcmE; *D. desulfuricans* CcmE has a predicted molecular weight of 15,114 Da, and heme has a mass of 616.5 Da. In both cases the holo-CcmE complexes migrate at a lower molecular weight than predicted; this is not unusual for membrane proteins. We conducted mass spectrometry of trypsin-digested proteins in a gel slice cut from an equivalent Coomassie-stained gel containing the region corresponding to the heme-staining band believed to be *D. desulfuricans* holo-CcmE. This revealed the presence of several fragments of *D. desulfuricans* CcmE (17.5% total sequence coverage). No *c*-type cytochromes were detected in the peptide fragments from the gel slice.

In membranes from cells expressing the *D. desulfuricans* *ccmEFABCD* operon (i.e. lacking CcmI) a heme-staining band of the same mass and approximate intensity as that present in the case of *ccmEFABCDI* was observed (Fig. 4B, fourth lane). This suggests that the absence of CcmI does not affect the accumulation of holo-CcmE. Membranes from cells expressing the *ccmEFABC* operon had a scarcely detectable amount of holo-CcmE (Fig. 4B, fifth lane), implicating CcmD in transfer of heme to CcmE. Kranz and co-workers recently reported that loss of *ccmD* from the *E. coli* system resulted in formation of less holo-CcmE (9).

Cys¹²⁷ of D. desulfuricans CcmE Is Essential for Heme Binding and Cytochrome c Biogenesis—*E. coli* CcmE covalently binds heme via His¹³⁰, and although the H130C variant can covalently bind heme, the heme cannot subsequently be transferred to apocytochrome *c* (38, 39). The equivalent residue in *D. desulfuricans* CcmE, Cys¹²⁷, was mutagenized to histidine, methionine, and alanine. Histidine was chosen as the analogous residue to that in *E. coli* CcmE. Methionine is a common iron ligand to which heme can be non-covalently bound. In each case *E. coli* EC06 cells expressing the resultant *ccmE^{mut}FABCDI* operon and pKPD1 were unable to synthesize cytochrome *c*₅₅₀ at a level detectable either by heme staining or spectroscopically (Fig. 3). This demonstrates the importance of cysteine at this position in *D. desulfuricans* CcmE and that this residue plays an integral role in *c*-type cytochrome biogenesis. It was also tested whether CcmE^{C127H} could covalently bind heme. SDS-PAGE analysis of membranes prepared from cells expressing the *ccmE^{C127H}FABCDI* operon did not result in any heme-staining bands (Fig. 4B, sixth lane). Thus, covalent attachment of heme to *D. desulfuricans* CcmE requires and is most likely directly via Cys¹²⁷.

The D. desulfuricans Ccm System Attaches Heme Covalently to Cys¹²⁷ of Soluble D. desulfuricans CcmE—Because of limitations of expression and subsequent analysis of the membrane-anchored *D. desulfuricans* CcmE, we generated a construct encoding the soluble portion of CcmE (lacking the N-terminal transmembrane helix) preceded by a signal peptide and a Streptavidin II tag, termed CcmE^{sol}. Expression of this construct in the presence of pDD86 (*D. desulfuricans* *ccmEFABCDI*) and subsequent UV-visible spectroscopic analysis of the protein purified by affinity chromatography and reduced using dithionite revealed an absorption maximum at 419 nm (Fig. 5A). Pyridine hemochrome analysis showed an α -band at 551.5 nm (Fig. 5A, inset), a value similar to that reported for *E. coli* holo-CcmE (39). It is notable that single-cysteine *c*-type cytochromes generally show maxima at 552–553 nm in pyridine hemochrome analysis (e.g. Ref. 26). This difference may reflect the nature of the cysteine-heme bond formed in *D. desulfuricans* holo-CcmE.

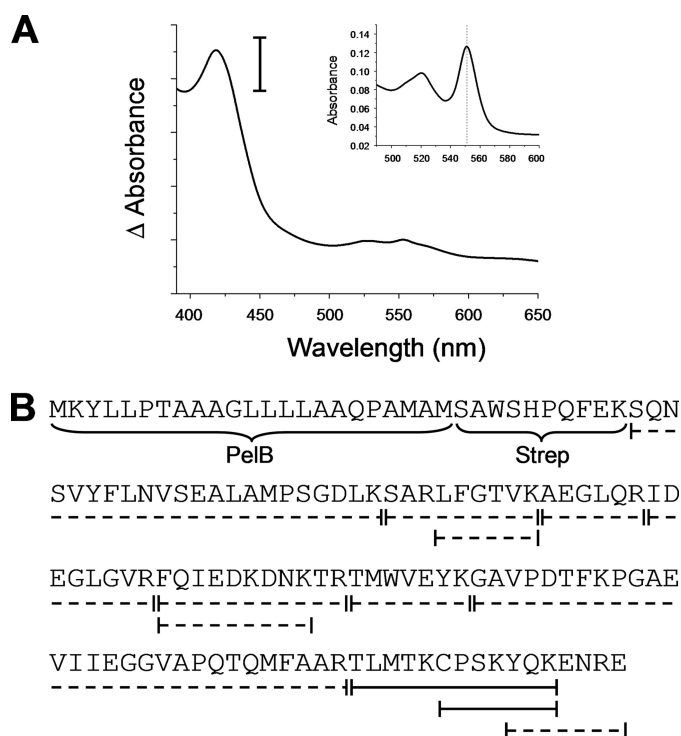


FIGURE 5. Analysis of *D. desulfuricans* holo-CcmE^{sol}. A, shown is the visible absorption spectrum reflecting the formation of *D. desulfuricans* holo-CcmE^{sol} by *D. desulfuricans* CcmEFABCDI expressed in *E. coli*. The absorption maximum is at 419 nm. The sample was reduced by the addition of a few grains of disodium dithionite. The vertical scale bar represents 0.2 absorption units. The inset shows the reduced pyridine hemochrome spectrum of *D. desulfuricans* holo-CcmE^{sol}. The vertical line indicates 551.5 nm. B, shown is a schematic representation of peptide fragments determined by MALDI-TOF mass spectrometry after digestion of purified *D. desulfuricans* holo-CcmE^{sol} with trypsin. PelB represents the signal peptide, and Strep indicates the position of the Streptavidin II affinity tag. Dotted lines represent peptides with the expected molecular mass detected by mass spectrometry. Solid lines represent peptides with an additional mass of 616 Da, indicating covalent attachment of heme to the peptide.

The purified *D. desulfuricans* CcmE^{sol} protein was subjected to trypsin digestion followed by mass spectrometry. This revealed a series of peptide fragments encompassing the entire *D. desulfuricans* CcmE sequence (Fig. 5B). Two peptides corresponding to the C-terminal region of *D. desulfuricans* CcmE were of particular note. The first of these (CPSKYQK) had a mass 616 Da greater than would be expected for the peptide alone, indicating covalent attachment of otherwise-unmodified heme. A second peptide (YQKENRE) was detected that did not have the additional mass, indicating attachment of the heme to Cys¹²⁷. Attachment of heme to Pro¹²⁸, Ser¹²⁹, or Lys¹³⁰, the only other possibilities, appears highly unlikely. This is consistent with data above in which no heme binding to membrane-bound *D. desulfuricans* CcmE was observed upon mutagenesis of Cys¹²⁷.

***D. desulfuricans* CcmE and *E. coli* CcmE Are Not Functionally Interchangeable**—Expression of membrane-anchored *D. desulfuricans* CcmE (pDDCcmE) in *E. coli* EC65 (Δ ccmE (12)) did not restore c-type cytochrome biogenesis of an exogenous c-type cytochrome (data not shown). This indicates that *D. desulfuricans* CcmE cannot be functionally incorporated into the *E. coli* Ccm system. Additionally, expression of the *E. coli* ccm operon in which the ¹³⁰H DENY CcmE heme-bind-

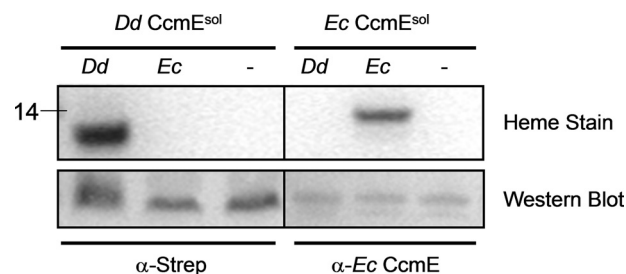


FIGURE 6. Specificity of holo-CcmE^{sol} formation. *D. desulfuricans* CcmE^{sol} or *E. coli* CcmE^{sol} were co-expressed with the *D. desulfuricans* ccmEFABCDI operon (Dd), the *E. coli* ccmABCDEFGH operon (Ec), or no biogenesis system (—). Top panel, formation of holo-CcmE^{sol} was monitored by the detection of heme-staining bands of appropriate molecular masses (weight indicated in kDa). Bottom panel, expression of protein was detected by Western blotting whole cell extracts using an anti-streptavidin antibody for *D. desulfuricans* CcmE^{sol} and an anti-*E. coli* CcmE antibody and periplasmic extracts for *E. coli* CcmE^{sol}.

ing motif was replaced with CPSKY (the equivalent residues in *D. desulfuricans* CcmE; pEC86^{CPSKY}) was unable to restore endogenous c-type cytochrome biogenesis in *E. coli* EC06 (Δ ccmABCDEFGH; data not shown).

To investigate if the *E. coli* Ccm system was capable of attaching heme to *D. desulfuricans* CcmE^{sol}, we coexpressed pEC86 (*E. coli* ccmABCDEFGH) and *D. desulfuricans* CcmE^{sol}. There was no detectable heme attachment to *D. desulfuricans* CcmE^{sol} by the *E. coli* Ccm system (Fig. 6). In the complementary experiment, *D. desulfuricans* CcmEFABCDI was unable to attach heme detectably to *E. coli* CcmE^{sol}. The two biogenesis systems were able to attach heme covalently to their own respective soluble CcmE proteins (as indicated by the heme-staining bands in Fig. 6), which could be detected with appropriate antibodies under all conditions.

DISCUSSION

Reported here are several striking results that indicate a need for reappraisal of the functions of at least some of the Ccm proteins. Firstly, the identification of a functional, natural, CcmE variant with cysteine (Cys¹²⁷) replacing the hitherto invariant and essential histidine residue, which for the *E. coli* protein (and a plant CcmE (40)), forms a covalent bond to heme. The replacement of Cys¹²⁷ in *D. desulfuricans* CcmE by histidine or alanine resulted in loss of any detectable cytochrome c assembly. The failure of CcmE^{C127A} to support activity parallels the result found in the H130A variant of the *E. coli* protein (12), but the inactivity of C127H shows that the CcmE of *D. desulfuricans* is a variant adapted to employ cysteine at a crucial site in the protein. It is striking that the *E. coli* protein is non-functional when its histidine is replaced by cysteine (H130C (38)).

An important question is whether *D. desulfuricans* CcmE forms a covalent bond to heme. It is clear from our data, in which heme-staining of SDS-PAGE gels (Fig. 4) is supported by spectroscopy and mass spectrometry detecting two heme-containing peptides (Fig. 5), that such a bond is formed. The covalent bond was not detected when Cys¹²⁷ was mutated to histidine. The conclusion that it is Cys¹²⁷ which covalently binds heme is confirmed by analysis of peptide fragments (Fig. 5). In the case of *E. coli* CcmE, His¹³⁰ has been reported through structural analysis of a tryptic peptide as being joined to the

heme via a novel bond between the N^{δ1} of the histidine and the β-carbon of a vinyl group of the heme (13). Hence, both the *E. coli* and *D. desulfuricans* Ccm systems involve a covalent attachment of heme to CcmE.

The structure of *E. coli* apoCcmE has been determined by NMR (Fig. 4A, panel i (41)). Briefly, the N-terminal domain forms a β-barrel of six β-sheets, and His¹³⁰ is surface-exposed near a hydrophobic platform to which heme is proposed to bind. The structure of *D. vulgaris* CcmE, which is highly homologous to the *D. desulfuricans* protein, has been determined by NMR (PDB accession number 2KCT; Fig. 4A, panel ii). Unfortunately, the available structure only encompasses residues 44–128, lacking the N-terminal transmembrane helix but also terminating at the proline of the CPSKY motif (where the cysteine is Cys¹²⁷), thereby excluding the conserved tyrosine that is a heme-iron ligand in *E. coli* CcmE (42, 43). Nevertheless, the β-barrel structure bears a striking similarity to that of the *E. coli* protein despite relatively low sequence identity. Cys¹²⁷ also appears to be surface-exposed, consistent with its role in heme binding. Extensive future work will be required to identify any compensating differences in the Ccm proteins of *D. desulfuricans* compared with *E. coli* that permit the use of an essential cysteine rather than histidine in CcmE.

There is no detectable CcmH analogue encoded on the genome of *D. desulfuricans*. The *D. desulfuricans* *ccm* operon confers cytochrome *c* maturation capability on *E. coli* lacking its endogenous *ccm* system, demonstrating that this variant System I can function in the absence of CcmH. This contrasts with the situation in *E. coli* itself where the N-terminal region of its CcmH protein is essential for *c*-type cytochrome synthesis (33). Analysis of the *D. desulfuricans* Ccm proteins did not reveal the presence of additional CXXC motifs or extra domains compared with their *E. coli* counterparts. Thus, it appears that the reductive role of CcmH is absent and not simply incorporated into another of the *D. desulfuricans* Ccm components. However, an atypical reductant-transfer motif cannot be excluded. For example, CcmF from *D. desulfuricans* and *D. vulgaris* contains several cysteines, although these are not conserved in other System I*-containing organisms. Genomes of some organisms encoding CcmE proteins with cysteine in place of the histidine do contain CcmH homologues (22). Therefore, it would seem that such CcmE proteins can function in the presence of CcmH but that, in contrast to *E. coli*, CcmH is not essential for *c*-type cytochrome biogenesis in such a system.

In the present experiments *c*-type cytochromes have been produced in *E. coli* without a CcmG protein. This also contrasts with the situation in *E. coli* where CcmG has been shown to be an essential member of the endogenous Ccm system (21). However, and similarly, it has been shown that expression of *c*-type cytochrome biogenesis System II in a Δ*ccm* *E. coli* strain leads to cytochrome *c* production without CcmG (or ResA, the analogous protein from System II) (32, 44). Normally, CcmG transfers reductant from DsbD to the rest of cytochrome *c* assembly apparatus and/or the apocytochrome. Presumably instances of continued cytochrome *c* synthesis in the absence of CcmG can be explained on the basis that either reductant from DsbD is not required or that there are other pathways catalyzing reductant transfer from DsbD to the apparatus.

The addition of exogenous reductant has previously been reported to complement for a disruption in DsbD (35) and site-directed mutants of the active cysteines in CcmG (36). However, the addition of either cysteine or 2-mercaptoethane sulfonic acid had no significant effect on cytochrome *c*₅₅₀ production in *E. coli* EC06 strains expressing pDD86 and pKPD1. Thus, the lower yield of *c*-type cytochrome produced by the *D. desulfuricans* system in *E. coli*, relative to that produced by cells containing pEC86, must be attributed to other factors. Among these is a possible lower heterologous expression of *D. desulfuricans* Ccm proteins compared with the homologous expression of *E. coli* counterparts. Also, it is possible that other factors in the highly reducing environments in which sulfate-reducing bacteria grow can compensate for the variations in the Ccm system seen in these organisms that depend more extensively on the function of *c*-type cytochromes than does *E. coli*.

The results presented here imply that CcmF is the irreducible core of heme attachment via the Ccm system and that CcmG and CcmH may be ancillary, but dispensable, components. We can find only one copy of *ccmF* on the genomes of *Desulfovibrio* species despite the fact that these organisms all contain NrfA nitrite reductase which has an active site heme-attachment motif CXXCK (45), known in other organisms to require dedicated variant forms of CcmF for heme attachment (46). A further variant form of CcmF has also been implicated in *Shewanella* for the attachment of heme to a CX₁₅CH motif (47). Thus, it may be that the CcmF of *Desulfovibrio* differs from its counterparts in *E. coli* in that it can participate in heme attachment to both CXXCH and CXXCK. However, we originally noted that *D. desulfuricans* contains a gene known as *resC* or *ccsA* (22). This encodes part of the System II *c*-type cytochrome assembly apparatus found in various species of bacteria and thylakoids. Homologues of ResC are implicated in substrate recognition of, and heme attachment to, CXXCK and CX₁₅CH in *Wolinella succinogenes* (48). We have identified a strong correlation between the presence of ResC alongside the *Desulfovibrio*-type System I* and the presence of a NrfA protein bearing the CXXCK motif. This raises the important possibility that ResC can operate in concert with some parts of System I to attach heme to CXXCK in sulfate-reducing bacteria. Testing this hypothesis is not trivial, although we note that *D. desulfuricans* CcmEFABCDI cannot mature a CXXCK variant of *P. denitrificans* cytochrome *c*₅₅₀ when expressed in *E. coli*. This supports involvement of ResC in maturation of CXXCK-containing cytochromes in *D. desulfuricans*. Clearly this is another unexpected outcome of the present work, which calls for a new perspective on *c*-type cytochrome assembly pathways.

Crucially, this work raises questions about the role of the histidine-heme bond in CcmE from many organisms. There is strong evidence that a covalent bond between heme and CcmE is a competent intermediate in *c*-type cytochrome assembly (12), so it is remarkable that the novel heme-histidine chemistry can be substituted by heme-cysteine chemistry. The observation that the histidine-heme bond in CcmE is not essential along with the completely unexpected alterations observed within *D. desulfuricans* System I* challenge the current paradigm *E. coli* system as a universal model. Overall, our data suggest that only CcmF and CcmE are at the heart of heme attach-

ment to apocytochromes *c* and demonstrates a greater degree of variation within System I-mediated cytochrome *c* biogenesis than previously anticipated.

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c-Type Cytochrome Biogenesis Can Occur via a Natural Ccm System Lacking CcmH, CcmG, and the Heme-binding Histidine of CcmE

Alan D. Goddard, Julie M. Stevens, Feng Rao, Despoina A. I. Mavridou, Weelee Chan, David J. Richardson, James W. A. Allen and Stuart J. Ferguson

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c-Type cytochrome biogenesis can occur via a natural Ccm system lacking CcmH, CcmG, and the heme-binding histidine of CcmE.

Alan D. Goddard, Julie M. Stevens, Feng Rao, Despoina A. I. Mavridou, Weelee Chan, David J. Richardson, James W. A. Allen, and Stuart J. Ferguson

PAGE 22885:

In the left hand column, eight lines from the top, the sentence should read as follows: UV-visible spectroscopy can detect *P. denitrificans* cytochrome c_{550} via the Soret peak at concentrations down to 3×10^{-8} M, and heme staining can detect $\sim 1 \times 10^{-12}$ mol of cytochrome c_{550} .

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DOI 10.1074/jbc.A110.153692

Extended binding site on fibronectin for the functional upstream domain of protein F1 of *Streptococcus pyogenes*.

Lisa M. Maurer, Bianca R. Tomasini-Johansson, Wenjiang Ma, Douglas S. Annis, Nathan L. Eickstaedt, Martin G. Ensenberger, Kenneth A. Satyshur, and Deane F. Mosher

PAGE 41091:

The experiment shown in Fig. 3A was done with 18.6 μ M (not 8.95 μ M) fibronectin, and the experiment on 70K shown in Fig. 3B was done at 40 °C (not 25 °C). We have added the thermodynamic parameters to Table 1 that correspond to Fig. 3A and added a third row to Table 1 that has the parameters for binding to 70K at 25 °C. The conclusions are unchanged.

Interaction	T	[FUD]	[Fn]/[70K]	ΔH	ΔS	ΔG	K_d	n
	°C	μ M	μ M	kcal mol ⁻¹	cal mol ⁻¹	kcal mol ⁻¹	nM	
FUD-FN	25	204	18.6	-34.6	-82.9	-9.9	58	1.4
FUD-70K	25	15.8	1.40	-46.1	-117	-11.2	5.2	1.1
FUD-70K	40	17.9	1.70	-113	-324	-11.6	8.8	1.1

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Two neuronal nicotinic acetylcholine receptors, $\alpha 4\beta 4$ and $\alpha 7$, show differential agonist binding modes.

Nyssa L. Puskar, Xinan Xiu, Henry A. Lester, and Dennis A. Dougherty

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Stabilization of HIV-1 envelope in the CD4-bound conformation through specific cross-linking of a CD4 mimetic.

Grégoire Martin, Brian Burke, Robert Thaï, Antu K. Dey, Olivier Combes, Oscar H. P. Ramos, Bernadette Heyd, Anthony R. Geonnotti, David C. Montefiori, Elaine Kan, Ying Lian, Yide Sun, Toufik Abache, Jeffrey B. Ulmer, Hocine Madaoui, Raphaël Guérois, Susan W. Barnett, Indresh K. Srivastava, Pascal Kessler, and Loïc Martin

Dr. Oscar H. P. Ramos was inadvertently left off the author list. The affiliation for Dr. Ramos is the Commissariat à l'Energie Atomique, iBiTecS, Service d'Ingénierie Moléculaire des Protéines, F-91191 Gif-sur-Yvette, France. The correct author list is shown above.

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Identification of a gene essential for the first committed step in the biosynthesis of bacteriochlorophyll c.

Zhenfeng Liu and Donald A. Bryant

The locus tag for the *bciC* gene of *Candidatus Chloracidobacterium thermophilum* was indicated as Cabther_B0031 throughout the text and in Fig. 3. The correct locus tag for this *bciC* gene is Cabther_B0081.

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