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A dedicated haem lyase is required for the maturation of a novel bacterial cytochrome *c* with unconventional covalent haem binding

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Summary

In bacterial *c*-type cytochromes, the haem cofactor is covalently attached via two cysteine residues organized in a haem *c*-binding motif. Here, a novel octahaem *c* protein, MccA, is described that contains only seven conventional haem *c*-binding motifs (CXXCH), in addition to several single cysteine residues and a conserved CH signature. Mass spectrometric analysis of purified MccA from *Wolinella succinogenes* suggests that two of the single cysteine residues are actually part of an unprecedented $CX_{15}CH$ sequence involved in haem *c* binding. Spectroscopic characterization of MccA identified an unusual high-potential haem *c* with a red-shifted absorption maximum, not unlike that of certain eukaryotic cytochromes *c* that exceptionally bind haem via only one thioether bridge. A haem lyase gene was found to be specifically required for the maturation of MccA in *W. succinogenes*. Equivalent haem lyase-encoding genes belonging to either the bacterial cytochrome *c* biogenesis system I or II are present in the vicinity of every known *mccA* gene suggesting a dedicated cytochrome *c* maturation pathway. The results necessitate reconsideration of computer-based prediction of putative haem *c*-binding motifs in bacterial proteomes.

Introduction

C-type cytochromes are a widespread class of proteins essential for the life of almost all organisms (Moore and Pettigrew, 1990; Scott and Mauk, 1995). They are characterized by the covalent attachment of haem (Fe-protoporphyrin IX) to a polypeptide chain via two thioether bonds obtained from the reaction of the thiol groups of two reduced cysteine residues with two haem *b* vinyl groups. The two cysteine residues are arranged in the amino acid sequence CXXCH (or more rarely CX_3CH, CX_4CH or CXXCK), the so-called haem *c*-binding motif. Mammalian and fungal mitochondrial cytochrome *c* is by far the best-known member of this protein class, but there is a wide range of other such proteins, often with several haems attached to one polypeptide chain and structurally unrelated to mitochondrial cytochromes (Mowat and Chapman, 2005). Cytochromes *c* typically function in electron transfer, but *c*-type cytochrome centres are also found in the active site of many enzymes. Bacterial *c*-type cytochromes are located either at the outside of the cytoplasmic membrane or, in case of Gram-negative bacteria, in the periplasmic compartment or on the surface of the outer membrane. It is still enigmatic why haem is covalently attached in *c*-type cytochromes and what advantages might result from this feature (Wood, 1983; 1991; Allen *et al*., 2003; 2005a; Stevens *et al*., 2004). Exceptionally, in the Eukaryotic domain of life, the cytochromes *c* and c_1 from mitochondria of *Euglena* species and trypanosomatids (various *Trypanosoma* and *Leishmania* species) contain only a single cysteine residue in the respective haem *c*-binding motif (AA/GQCH or FAPCH) (Allen *et al*., 2004). In contrast, cytochrome *c* biogenesis in Bacteria has been considered to be strictly dependent on the presence of two cysteine residues arranged in a $CX_{2\text{-}4}CH/K$ haem *c*-binding motif.

The stereospecific covalent attachment of haem to the reduced cysteine residue(s) of the apo-cytochrome haem *c*-binding motif is the last step in cytochrome *c* biogenesis and is catalysed by the enzyme cytochrome *c* haem lyase (CCHL). Remarkably, two distinct cytochrome *c* biogenesis systems were identified in bacteria that differ in their enzymic components (Thöny-Meyer, 1997; Kranz *et al*., 1998; Allen *et al*., 2003; Stevens *et al*., 2004). The systems are known as Ccm system (cytochrome *c* maturation, also known as system I) and Ccs system (cytochrome *c* synthesis, system II). The Ccm system usually consists of eight essential proteins (CcmA, -B, -C, -D, -E,

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-F, -G and -H) and has been predominantly characterized in *Escherichia coli*. The Ccs system involves at least four essential proteins (CcsA, CcsB, CcdA and ResA) and occurs, among others, in ε-proteobacteria. The CCHL enzymes in system I and II are most likely CcmF/-H and CcsB/-A respectively (Feissner *et al*., 2006). CcsA is a membrane-bound protein of about 250–350 amino acid residues (Goldman *et al*., 1998; Hamel *et al*., 2003). The *ccsA* gene is often located adjacent to *ccsB* encoding another essential membrane protein that possibly forms a complex with CcsA (Dreyfuss *et al*., 2003). Exceptionally, in genomes of ε-proteobacteria like *Wolinella succinogenes* and *Campylobacter* spp., the *ccsB* and *ccsA* genes are fused resulting in genes coding for membrane-bound proteins of about 900 residues (Hartshorne *et al*., 2006).

Haem attachment to a CXXCK haem *c*-binding motif requires a different CCHL enzyme to that used for the conventional CXXCH motif. The CXXCK motif is unique to cytochrome *c* nitrite reductase (NrfA) and binds the high-spin haem group of the catalytic centre that is axially ligated by the lysine residue of the motif (Einsle *et al*., 1999; Simon, 2002). The NrfE, -F and -G proteins that are homologous to Ccm proteins are specifically involved in covalent haem *c* attachment to the CXXCK motif, acting in an as yet unknown manner (Eaves *et al*., 1998). The NrfE, -F and -G proteins are therefore thought to make up a dedicated haem lyase for NrfA that evolved from the Ccm system. A functionally equivalent system has been characterized in system II of *W. succinogenes* where the NrfI protein, a CcsBA fusion protein, is the dedicated CCHL enzyme that recognizes the CXXCK motif of NrfA (Pisa *et al*., 2002). To date, it is not known how the eukaryotic A/FXXCH haem-binding motif is recognized but this process apparently also requires a dedicated maturation system (Allen *et al*., 2004). The genomes of several trypanosomatids (mentioned above) do not encode proteins of either bacterial cytochrome *c* biogenesis system and also do not comprise the commonly found mitochondrial haem lyase referred to as system III (Kranz *et al*., 1998). The latter enzyme, which is not similar to any system I or II component, is the only protein necessary to attach haem to apo-cytochromes *c* with CXXCH motifs in mitochondria of fungi, invertebrates and vertebrates.

We report here the purification and characterization of a novel bacterial multihaem cytochrome *c* from *W. succinogenes* with an unprecedented type of covalent haem attachment for a bacterial protein that involves the cysteines of a novel $CX_{15}CH$ sequence. Its maturation is dependent on a special CCHL that is presumably dedicated to the newly found haem *c*-binding sequence. The identification of MccA gives an example of how to retrieve as yet undiscovered *c*-type cytochrome genes from data banks and calls for careful re-inspection of genome sequence annotation routines.

Results and discussion

Discovery of a novel multihaem cytochrome c *(Mcc)*

In an approach to identify unconventional haem *c* binding sites in Bacteria, an open reading frame was discovered that encodes a conserved multihaem *c*-type cytochrome, MccA (Fig. 1). As of January 2007, 17 complete and one partial MccA primary structure were identified in bacterial genomes of ε-proteobacteria (*W. succinogenes*, *Campylobacter* spp., *Anaeromyxobacter dehalogenans*) and various *Shewanella* species (see Fig. S1 in *Supplementary material* for an alignment). All sequences (length between 645 and 709 residues) show at least 50% pair wise sequence identity. They include a typical Sec-dependent signal peptide and seven canonical haem *c*-binding motifs (CXXCH) within the N-terminal two-thirds of the sequence. In addition, there are four completely conserved single cysteine residues, one of which is arranged in a CH signature (consensus sequence A/N-K/G/S-G-C-H-S) that resembles the rare mono-cysteine haem *c*-binding motif of some eukaryotic mono-haem *c*-type cytochromes (see *Introduction*). It is also notable that the spacing between this cysteine and an upstream single cysteine is also conserved, raising the possibility of a $CX_{15\ or\ 17}CH$ haem-binding motif that far exceeds the maximal intercysteine spacing of four residues thus far reported.

Overproduction and purification of W. succinogenes *MccA*

The representative MccA protein from *W. succinogenes* DSMZ 1740 (data bank designation WS0379, Accession No. NP_906626) was purified in order to determine its number of covalently bound haem groups. *W. succinogenes* is a well-known model organism to study anaerobic respiration using various terminal electron acceptors like fumarate, nitrate, nitrite or polysulphide (Kröger *et al*., 2002; Simon, 2002; Baar *et al*., 2003). As the MccA protein has not been previously detected by haem staining in extracts of cells grown under any of these conditions, the *mccA* gene was expressed in mutant *W. succinogenes* P_{frd}-*mccA* upon replacement of the genuine promoter, supposed to be located immediately upstream of *mccA*, by the fumarate reductase promoter (Fig. 1, see *Experimental procedures* for details). Using this strategy the amino acid sequence of mature MccA remained unchanged. The MccA protein was detected in cell extracts of either fumarate- or nitrate-grown mutant cells by haem staining (Fig. 2, lanes 1 and 2). The amount of MccA was lower in nitrate-grown cells, which is similar to the pattern for the content of fumarate reductase under these conditions. The MccA protein was found to be located entirely in the soluble cell fraction as expected for

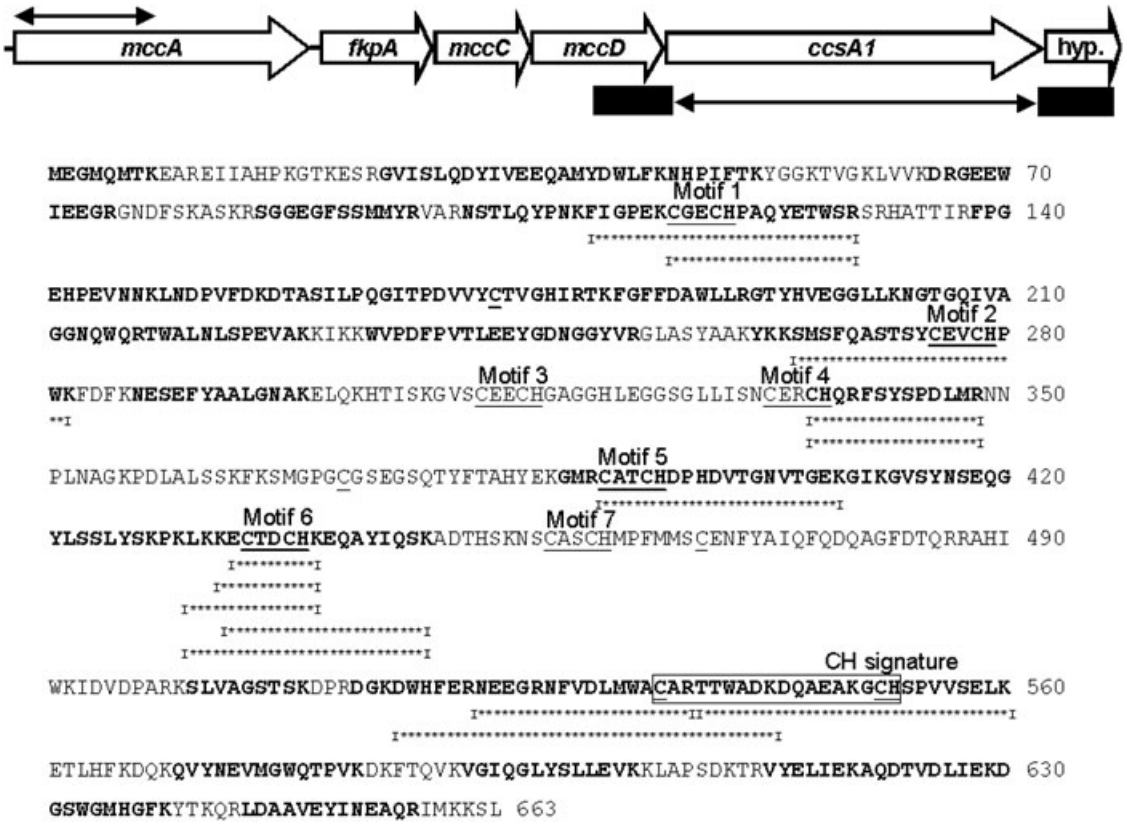

Fig. 1. The *mccA* gene of *W. succinogenes* and its predicted primary structure.
Top. Context of *W. succinogenes mccA* on the genome. The genes *mccC* and *mccD* are predicted to encode a soluble iron-sulphur protein (similar to NrfC) and a quinone-reactive membrane-bound protein (similar to NrfD) respectively (Hartshorne *et al*., 2006). These proteins might function in electron transport from the reduced quinone pool to periplasmic MccA. The *fkpA* gene predicts a peptidyl-prolyl *cis-trans* isomerase. The double-headed arrows refer to the construction of mutants as described in *Experimental procedures*. The arrow above *mccA* designates the region of homologous recombination between the genome and pMccA in order to overexpress the *mccA* gene under the control of P_{frd}. The arrow below *ccsA1* indicates the region replaced by the kanamycin resistance gene in the *ccsA1* deletion mutant. hyp., open reading frame encoding a hypothetical protein.
Bottom. Primary structure of mature *W. succinogenes* MccA. The seven CXXCH motifs, the CH signature and other cysteine residues are underlined. The $CX_{15}CH$ sequence is boxed. Residues that are part of tryptic fragments (with or without haem) detected by MALDI-MS are printed in bold (see Table S1 in *Supplementary material*). Haem-containing fragments detected unambiguously by tandem mass spectrometry are marked underneath the sequence.

a periplasmic *c*-type cytochrome (not shown). MccA was purified (about 15 mg of MccA was obtained per litre cell culture) and found to have an apparent size of 75 kDa as judged from SDS-PAGE (Figs 2 and 3). The N-terminus was experimentally determined as MEGMQ indicating that a signal peptide of 27 residues is cleaved during maturation (Fig. 1). The identity of the cleavage site was further proven by the detection of the N-terminal tryptic fragment (MEGMQMTK) by mass spectrometry (MS) (Table S1 in *Supplementary material*).

To determine the molecular weight of MccA in solution, samples of MccA at concentrations between 0.5 and 20 μM were subjected to sedimentation equilibrium analysis using analytical ultracentrifugation (Fig. 4). The molecular weight of the protein in solution ranged between 210 kDa at 0.5 μM and 233 kDa at 20 μM, consistent with a trimeric protein conformation. The absorbance coefficients and spectrometrically determined monomer mass (see below) was used to determine the dissociation constant of the trimeric MccA complex. A K_d value of 4.0×10^{-13} M was determined by fitting data sets from five MccA concentrations (0.5, 1, 2, 10 and 20 μM) on the basis of a model in which an 80 kDa monomer associates into a trimeric complex with a molecular weight of 240 kDa.

Spectroscopic analyses of MccA

The predicted mass of processed apo-MccA (663 residues) is 74 756, but masses of 79 683 (±72) and 79 766 (±72) were determined for the purified protein by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) using internal and external calibration respectively (Fig. S2 and Table S2 in *Supplementary*

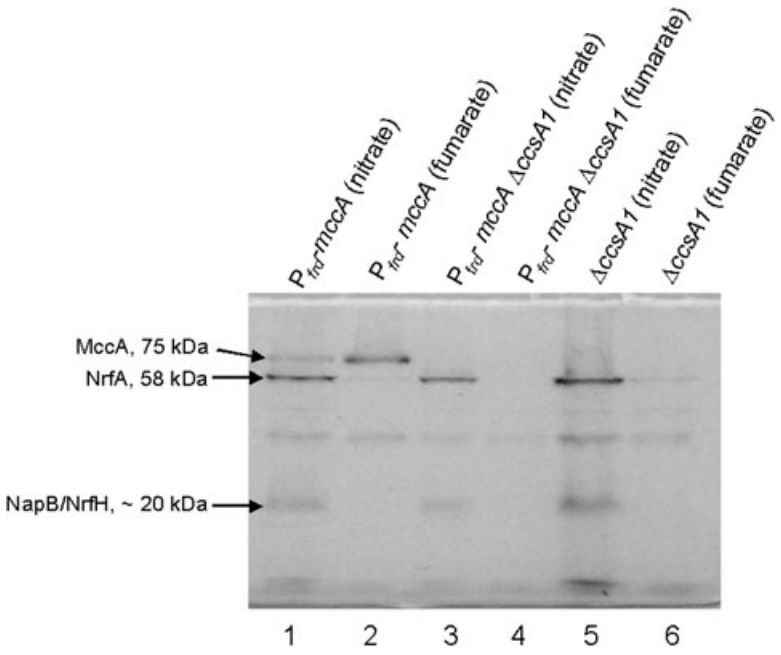

Fig. 2. Haem stain of cell extracts from different *W. succinogenes* mutants separated by SDS-PAGE. One hundred micrograms of protein from cells grown under fumarate- or nitrate-respiring conditions were applied. A haem stain pattern similar to lanes 5 and 6 was obtained when using wild-type cells (not shown). The detected *c*-type cytochromes beside MccA are the catalytic subunit of the cytochrome *c* nitrite reductase complex (NrfA), the small subunit of the cytochrome *c* nitrite reductase complex (NrfH) and the cytochrome *c* subunit of the periplasmic nitrate reductase (NapB). P_{frd}-*mccA*, MccA-overproducing strain *W. succinogenes* P_{frd}-*mccA*; Δ*ccsA1*, *W. succinogenes* deletion mutant lacking the *ccsA1* gene; P_{frd}-*mccA* Δ*ccsA1*, *W. succinogenes* mutant overproducing MccA in the absence of the *ccsA1* gene.

material). As one covalently bound haem group accounts for a mass of 616, the observed mass difference indicates the attachment of eight (calculated mass of 79 684) rather than the seven haems (79 068) expected from the seven canonical CXXCH motifs. This suggests that the eighth haem is covalently attached to the MccA polypeptide in a non-conventional manner.

To probe the co-ordination environment of the ferric haems in MccA, the air-oxidized haem protein was subjected to EPR spectroscopy (Fig. 5). The continuous wave 10 K perpendicular mode X-Band EPR spectrum is dominated by signals at g ~1.51, 2.22 and ~2.90. These g values are characteristic of rhombic trios (g_x, g_y, g_z) that arise from the paramagnetic Fe(III) ions of low-spin

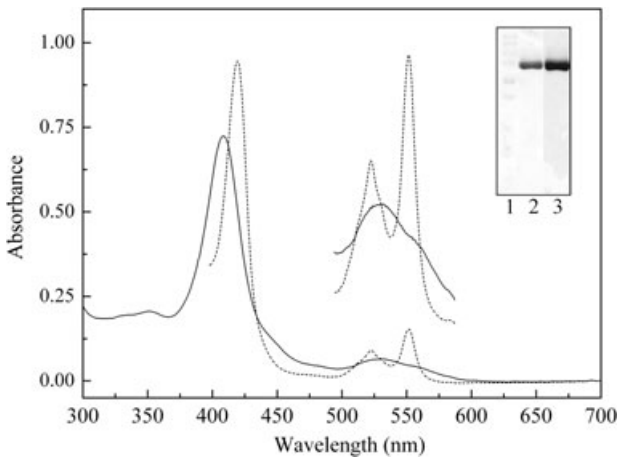

Fig. 3. UV/Vis absorption spectra of *W. succinogenes* MccA [0.8 μM in 20 mM Tris/HCl (pH 7.5), 100 mM NaCl]. Solid line, air-oxidized (as isolated) MccA with absorbance maxima at 408 and 532 nm. Broken line, MccA reduced with excess solid sodium dithionite showing absorbance maxima at 419, 522 and 552 nm. Inset, 5× amplification of the 500–580 nm region. The gel shows SDS-PAGE analysis of purified MccA (4 μg per lane). Lane 1, molecular weight markers. Lane 2, purified MccA stained for haem-dependent peroxidase activity. Lane 3, purified MccA stained with Coomassie blue.

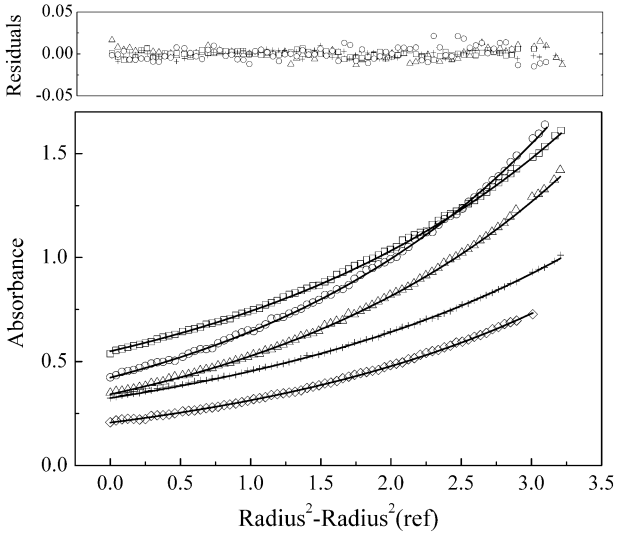

Fig. 4. Sedimentation equilibrium analysis of MccA. Samples were analysed at concentrations of 0.5 μM (diamonds), 1.0 μM (crosses), 2 μM (triangles), 10 μM (squares) and 20 μM (circles) in 20 mM Tris-HCl (pH 7.5), 100 mM NaCl. The sedimentation equilibrium response at each concentration was monitored at a single absorbance as described in *Experimental procedures*. Residuals between the experimental data and the fitted curves are displayed in the upper panel.

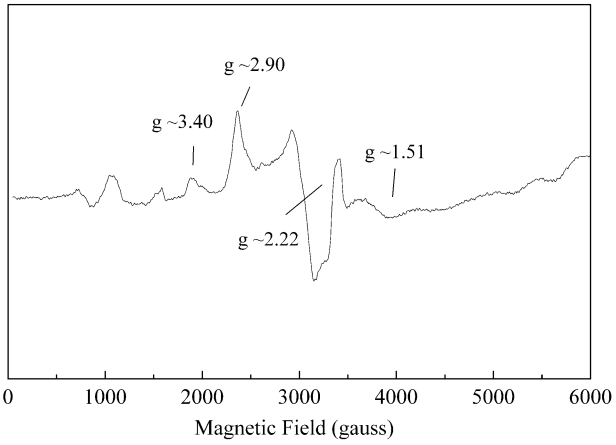

Fig. 5. Continuous-wave X-band EPR spectroscopy of MccA. The air-oxidized spectrum was collected at a temperature of 10 K and microwave power of 2 mW. The modulation amplitude was 1 mT, the microwave frequency was 9.67 GHz, and the MccA concentration was 0.2 mM in 50 mM Tris/HCl (pH 7.5) and 100 mM NaCl.

bis-histidine ligated ferric haem irons in which the planes of the imidazole rings of the axial iron ligands are nearly parallel (Walker *et al*., 1986). However, the g_z feature is asymmetrical and broad, with a half height line width of 170 G (the half height line width of the g_z feature from a rhombic signal arising from ferric haem is characteristically around 50 G) suggesting that a number of different haems with slightly different g_z values are contributing to a composite g_z feature in MccA. In addition to the rhombic signals, a ramp-shaped peak feature g ~3.40 is present. This is likely to be a 'large g_{max}' signal of the type that arises from haems in which the planes of the two imidazole rings of the axial histidine ligands are nearly perpendicular (Walker, 1999). There is no evidence of significant concentrations of high-spin haem, as reflected by the absence of intense signals at $g \sim 6$.

UV/Vis absorption spectra of the oxidized (as isolated) and dithionite-reduced MccA are typical for a *c*-type cytochrome containing low-spin ferric or ferrous haem respectively (Fig. 3). There is no absorbance feature in the region between 600 and 650 nm (oxidized sample) indicating the absence of a ligand-metal charge transfer band and thus the absence of high-spin ferric haem. Haem quantification using pyridine haemochrome spectra (α-absorbance band maximum at 550 nm) indicated a content of 8.12 (±0.7, $n = 6$) mol haem *c* per mol monomeric MccA. Mediated spectrophotometric redox potentiometry revealed an absorbance α-band centred at 554.5 nm in a +40 *minus* +280 mV redox difference spectrum (Fig. 6). This α-band was blue-shifted to 552 nm and increased in magnitude by approximately eightfold in a −300 *minus* +280 mV redox difference spectrum. The development of the fully reduced α-band peak was monitored as a function of potential. The transition from fully oxidized to fully reduced occurs over a wide 400 mV redox span that reflects the presence of multiple haems with overlapping redox potentials. There are a number of inflection points in the titre and the simplest solution that yields a satisfactory ($r^2 > 0.99$) fit is one which groups eight $n = 1$ haems into three isopotential components with

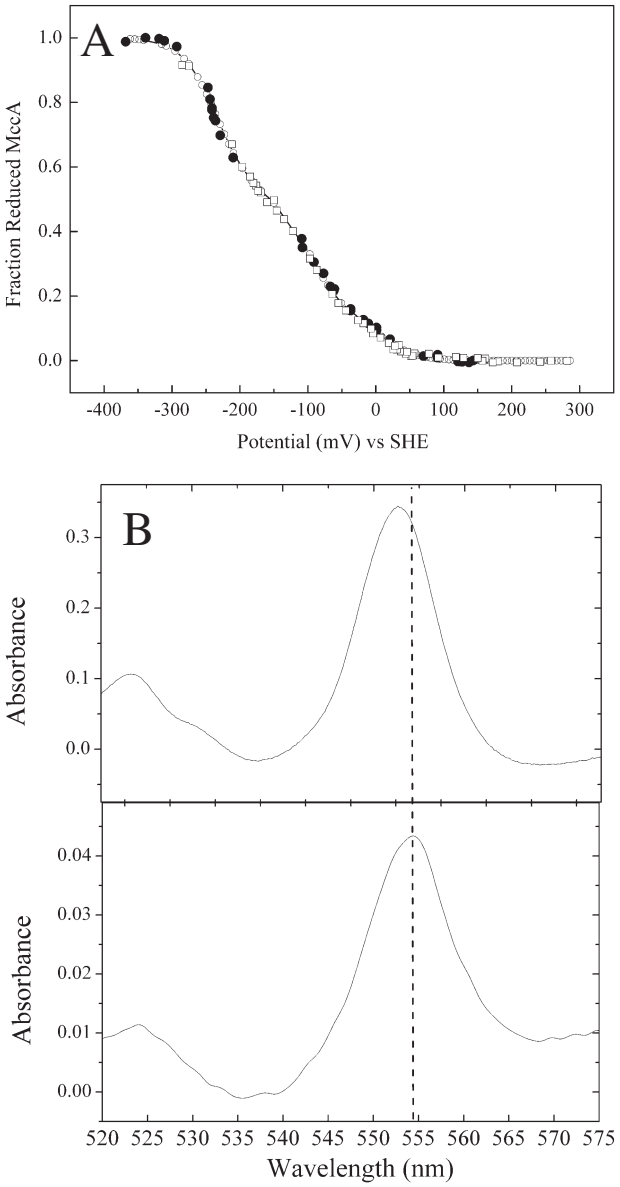

Fig. 6. Spectropotentiometric titration of MccA [3 μM in 20 mM Tris/HCl (pH 7.5), 100 mM NaCl].
A. MccA reduction (as determined from absorbance values acquired at 552 *minus* 540 nm) is plotted versus potential. Marker shape (filled circles, open squares, open circles) corresponds to three repeat experiments.
B. Redox difference spectra focusing on the α-peak absorbance maximum. Upper panel spectrum: −300 mV *minus* +280 mV (versus SHE). Lower panel spectrum: +40 mV *minus* +280 mV (versus SHE).

mid-point redox potentials of +17 mV, −117 mV and −233 mV (versus the standard hydrogen electrode, SHE), contributing 13% (1 haem), 38% (3 haems) and 49% (4 haems), respectively, to the total absorption change (Fig. 6). The midpoint potentials of all eight components are consistent with low-spin haems with *bis*-nitrogen haem iron ligation (e.g. His-His or His-Lys ligands), rather than His-Met where the electron withdrawing properties of the Met-S ligand stabilize the reduced state of the haem iron and so raise the potential. Redox difference spectra confirmed that the first component has an α-band centred at 554.5 nm, which suggests that the red-shifted absorption maximum can be assigned to a single haem *c* that has a potential domain that is more positive than the other seven haems with α-bands centred at 552 nm.

MALDI-MS was employed to examine haem attachment to individual tryptic fragments of MccA. The analysis revealed a high sequence coverage of MccA (Fig. 1, Table S1 in *Supplementary material*) and identified a minor impurity as the so-called NAP protein from *W. succinogenes* DSMZ 1740 (data bank designation WS1589, Accession No. NP_907728) (Fig. S2 and Table S1 in *Supplementary material*). Haem-containing fragments corresponding to five of the seven conventional haem *c*-binding motifs of MccA were detected by tandem mass spectrometry (MS/MS) (Fig. 1 and Table S1 in *Supplementary material*). The residual two haem *c*-binding motifs (No. 3 and 7) are situated on large tryptic fragments that presumably evaded detection. The same holds true for polypeptide containing two of the four single cysteine residues present in all known MccA primary structures (Fig. 1). Intriguingly, however, haem was found to be attached to tryptic fragments containing either the CH signature or the conserved single cysteine residue that lies 15 residues upstream of this. A fragment in which haem bridges these two fragments was not observed which is probably due its size and the tendency of such fragments to loose the haem cofactor by in-source or post-source decay during MALDI-MS (cf. Table S1A in *Supplementary material*). Separation of the tryptic digest by nano-HPLC (high-performance liquid chromatography) followed by electrospray ionization MS/MS or MALDI-MS/MS analysis gave no improvement on the results presented above (data not shown). The sensitivity of our analysis, however, is comparable to methods described previously for the mass spectrometric identification of haem-containing peptides derived from multihaem cytochromes *c* (Yang *et al*., 2005). Taken together, the results confirm the presence of an eighth, unconventionally bound haem group in MccA. Cumulative evidence suggests that this haem group is bound, in a previously unprecedented manner, via two covalent linkages to the two cysteine residues of a $CX_{15}CH$ sequence (Fig. 1). It is likely that this haem group is the one with the red-shifted absorption maximum and the most positive midpoint potential thus ensuring a directed electron flow to this site.

Physiological function of MccA

The function of MccA in *W. succinogenes* is not known. The lack of a high-spin haem in MccA argues for its function in periplasmic electron transfer rather than substrate turnover. MccA might be reduced by an iron-sulphur cluster protein like MccC (Fig. 1A) or another cytochrome. Purified MccA had no catalytic activity towards reduction of fumarate, nitrate, nitrite, thiosulphate, tetrathionate or Fe(III) complexes using various artificial electron donor substrates (not shown). It cannot be excluded, however, that the novel haem *c* group of MccA is involved in conversion of an as yet unknown substrate. None of the standard growth conditions, i.e. anaerobic respiration with nitrate, nitrite, fumarate or polysulphide as electron acceptor, led to induction of MccA production in wild-type *W. succinogenes* cells.

Maturation of W. succinogenes *MccA*

The results described thus far have firmly established that MccA binds one of its haems to a novel haem *c*-binding motif. Intriguingly, all known *mccA* genes are surrounded by open reading frames encoding potential CCHL enzymes (Hartshorne *et al*., 2006). In case of the cytochrome *c* biogenesis system II organism *W. succinogenes*, the nearby CCHL gene has been named *ccsA1* (Fig. 1). It is one of three CCHL genes encoded on the *W. succinogenes* genome (Hartshorne *et al*., 2006). A *W. succinogenes ccsA1* deletion mutant was constructed by replacing most of *ccsA1* by a kanamycin resistance gene (see *Experimental procedures* for details). In this mutant the maturation of all known *c*-type cytochromes produced under fumarate- and nitrate-respiring conditions was apparently not affected (Fig. 2, lanes 5 and 6). The mutant grew normally by respiratory nitrate ammonification with formate as electron donor indicating that the *c*-type cytochromes NrfA, NrfH and NapB are functional. Nitrite reductase activity using reduced benzyl viologen as electron donor was not altered in *W. succinogenes* Δ*ccsA1*. After deletion of the *ccsA1* gene in the genome of the MccA-producing *W. succinogenes* strain, MccA was no longer detectable by haem staining in cells harvested in the stationary growth phase (Fig. 2, lanes 3 and 4). Cells taken from the exponential growth phase, however, transiently formed detectable MccA but its amount was significantly lower than in wild-type cells (not shown). Despite a number of efforts the instability of this protein prevented purification for further biochemical analysis. The results clearly indicate that CcsA1 is involved in MccA maturation accounting for the attachment of part of the MccA haem groups. One

attractive possibility is that CcsA1 is required only for attachment of the unconventionally bound haem group, analogous to the function of NrfI in NrfA maturation (see *Introduction*) while conventional CXXCH motifs of MccA and other cytochromes *c* might be handled by the third CCHL enzyme encoded on the *W. succinogenes* genome (CcsA2) (Hartshorne *et al*., 2006). Partially unfolded MccA devoid of at least one haem group is likely to be degraded when the cells enter the stationary growth phase. At present we cannot exclude, however, that a so far unidentified haem lyase, which is not of the CcsBA type, is required for MccA maturation in addition to CcsA1. Such a scenario would be reminiscent of the unknown haem lyases that covalently attach haem to the CH signature of unusual mitochondrial *c*-type cytochromes (see *Introduction*) or to the single cysteine residue in the chloroplastic cytochrome b_6f complex (de Vitry *et al*., 2004).

Conserved mcc *gene clusters in bacteria*

A *ccsA1* homologue is present in the *mcc* gene clusters of several *Campylobacter* species (Hartshorne *et al*., 2006). In those cases where a complete genome sequence is available, the *Campylobacter ccsA1* homologue is apparently one of two distinct genes encoding similar CCHL enzymes. Interestingly, *Campylobacter* species do not contain a *nrfI* gene which is in line with the fact that the CXXCK motif in NrfA is replaced by the classical CXXCH motif.

In the genome sequences of various *Shewanella* species (containing the cytochrome *c* biogenesis system I as opposed to system II in *W. succinogenes* and *Campylobacter* spp.) a *ccmF*-type CCHL gene is found upstream of *mccA*, though on the opposite DNA strand (Hartshorne *et al*., 2006). In addition, two small open reading frames encoding proteins resembling the N- and C-terminal halves of CcmH are found in the vicinity of *mccA* (Hartshorne *et al*., 2006). These three accessory genes are similar to *nrfE*, *-F* and *-G* of *E. coli*. It is therefore likely that they also constitute a specific CCHL system that presumably is involved in MccA maturation. In *Shewanella oneidensis* MR-1 the three corresponding genes are upregulated by the presence of 10 mM disodium thiosulphate, similarly to *mccA* itself (Beliaev *et al*., 2005). Therefore, it seems that CCHL enzymes dedicated to MccA maturation evolved on the basis of the existent cytochrome *c* biogenesis system, a situation reminiscent of NrfA assembly (see above).

Conclusions

This work has introduced a novel octa-haem bacterial *c*-type cytochrome that contains an unconventionally bound haem *c* group. Evidence is presented that this haem is bound via two cysteine residues that are part of a $CX_{15\ or\ 17}CH$ sequence in various MccA proteins (see Fig. S1 in *Supplementary material*) and that the specialized haem lyase CcsA1 is required for its attachment. If this were the case, it seems reasonable to suggest that CcsA1 initially recognizes the CH signature as the histidine of the conventional CXXCH motif has been shown to be essential for covalent haem attachment in *E. coli* (Allen *et al*., 2005b). Future site-directed modification of MccA is necessary to decide whether or not the first cysteine residue of the above mentioned sequence is essential for the haem attachment process. At present, it cannot be excluded that the CH signature is sufficient to enable haem *c* attachment mediated by a CcsA1-type haem lyase; a case that would resemble haem attachment to those eukaryotic *c*-type cytochromes with a mono-cysteine motif. Site-directed modification of cysteine residues within the four classical haem *c*-binding motifs of another *W. succinogenes* cytochrome *c* (NrfH) has already demonstrated that, in one case, despite having three CXXCH and one SXXCH motif, NrfH still contained four covalently bound haem groups (Simon *et al*., 2002). This demonstrated that *W. succinogenes* is able to covalently attach haem to a mono-cysteine motif. In the light of the results presented here, it is possible that the CcsA1 haem lyase catalyses this reaction. In contrast, two cysteine residues are obligatorily required for haem attachment by system I of *E. coli* (Allen *et al*., 2002). This might now be explained by the fact that the *E. coli* genome encodes only two CCHL enzymes (CcmF and NrfE) which are thought to be specific for CXXCH and CXXCK motifs respectively. Clearly, more experiments are required in order to characterize the substrate spectrum of different CCHLs with respect to distinct haem *c*-binding motifs [$CX_{2\text{-}4}CH$, CXXCK, $(C)X_nCH$]. Unfortunately, no reports are available concerning the reaction mechanism of CCHL and no such enzyme has ever been purified.

The correlation between novel *c*-type cytochromes like MccA and dedicated haem lyases needs further investigation in an approach combining molecular biology and bioinformatics. Assuming that only one CCHL enzyme is required for haem attachment to the conventional CXXCH motif, the presence of multiple CCHL gene copies in bacterial genomes suggests haem binding to unconventional motifs. As an example, the genome of *Geobacter sulfurreducens* PCA encodes six CcsA isoenzymes. In such proteomes, the existence of novel and as yet unrecognized haem *c*-binding motifs is more than likely. The computer-based prediction of *c*-type cytochromes requires reconsideration in order to prevent overlooking potential *c*-type cytochrome encoding genes in bacterial genomes. Especially, open reading frames encoding proteins with a Sec-dependent signal sequence that are located in the vicinity

Table 1. Strains, plasmids and oligonucleotide primers used in this study.

Strain, plasmid or primer	Relevant properties, usage, nucleotide sequence (5′→3′)	Reference
Bacterial strains		
Wolinella succinogenes DSMZ 1740	Wild-type strain	DSMZ
W. succinogenes P_{frd}-*mccA*	Strain overproducing MccA	This study
W. succinogenes Δ*ccsA1*	Deletion mutant lacking the *ccsA1* gene	This study
W. succinogenes P_{frd}-*mccA* Δ*ccsA1*	Strain overproducing MccA in Δ*ccsA1* background	This study
Escherichia coli XL1-Blue	Strain used for cloning and plasmid propagation	Stratagene
Plasmids		
pFrdcat2	Derivative of pSC101. *E. coli* low-copy-number vector containing the *W. succinogenes frdCAB* genes including *frd* promoter and terminator sequences. Contains the chloramphenicol resistance gene *cat*	Simon *et al*. (1998)
pMccA	Derivative of pFrdcat2 containing an *mccA* gene fragment from *W. succinogenes* introduced via two *Avr*II restriction sites	This study
pΔccsA1::kan	Plasmid to replace part of the *ccsA1* gene in *W. succinogenes* by the *kan* gene upon double homologous recombination	This study
Primers		
pFrdcat2Fw	CCCCTAGG**TAA**ATCTCCTTGGAGCGGGGTCTCCC Forward primer used for amplification of the pFrdcat2 vector fragment; contains an *Avr*II restriction site (underlined) and the *frdB* stop codon (in bold)	Mileni *et al*. (2006)
pFrdcat2Rv2	GGCCTAGG**CAT**CTGTTTCCCCTGTGCAGTATTG Reverse primer used for amplification of the pFrdcat2 vector fragment; contains an *Avr*II restriction site (underlined) and the *frdC* start codon (in bold, complementary sequence)	This study
Ws0379Fw	GGCCTAGG**TTG**TCAGGCTGGAGTGTTTTGAAAGG Forward primer used for amplification of the *mccA* gene from genomic DNA of *W. succinogenes*. The *Avr*II restriction site is underlined. The *mccA* start codon is shown in bold	This study
Ws0379Rv	GGCCTAGGTTGGAGCTCTTTGGCGTTTCCAAGGG Reverse primer used for amplification of the *mccA* gene from genomic DNA of *W. succinogenes*. The *Avr*II restriction site is underlined	This study
ccsA1-1	CGGAATTCCGGGTCTTTCAGCAGGAACAGCG The *Eco*RI restriction site is underlined	This study
ccsA1-2	CGGGATCCATCACCGCCTCATACCACTTTGCC The *Bam*HI restriction site is underlined	This study
ccsA1-3	CGGGATCCGCCTATCGACGCCGAAAAGTGGGG The *Bam*HI restriction site is underlined	This study
ccsA1-4	ACGCGTCGACTAGCGATTTGATAGATCACTCCACC The *Sal*I restriction site is underlined	This study

of putative CCHL genes ask for careful inspection regarding putative haem-binding motifs.

Experimental procedures

Growth of W. succinogenes

All strains of *W. succinogenes* were grown at 37°C by anaerobic respiration with formate as electron donor and either fumarate or nitrate as terminal electron acceptor using medium as described previously (Lorenzen *et al*., 1993).

Construction of W. succinogenes *mutants*

Table 1 denotes the strains, plasmids and primers used in this study. The *mccA* gene is expressed in mutant *W. succinogenes* P_{frd}-*mccA* after introduction of the fumarate reductase promoter (P_{frd}) into the wild-type genome immediately upstream of *mccA* thus replacing its genuine promoter (Fig. 1). This promoter substitution was achieved upon integration of plasmid pMccA by homologous recombination between an *mccA* fragment present on the plasmid and the genomic *mccA* gene. Plasmid pMccA contains P_{frd} and the *frdC* start codon followed by an *mccA* fragment (5′-end) of 1020 bp. This fragment was obtained by PCR using primers Ws0379Fw and Ws0379Rv. Plasmid pMccA is based on plasmid pFrdcat2 (Simon *et al*., 1998). Using primers pFrdcat2Fw and pFrdcat2Rv2, a pFrdcat2 vector fragment was amplified by PCR that comprises P_{frd} and the *frdC* start codon at one end and the *frdB* stop codon at the other end. This vector fragment and the *mccA* fragment were ligated. As all primers carried *Avr*II restriction sites (CCTAGG) at their 5′-ends to facilitate ligation, two additional codons (coding for proline and arginine) were inserted immediately downstream of both the start and the stop codon. Transformation of *W. succinogenes* cells with plasmid pMccA was carried out by electroporation (Simon *et al*., 1998). Transformants were selected in medium containing formate and nitrate in the presence of chloramphenicol (12.5 mg l^{-1}). The desired inte-

gration of the plasmid into the genome was examined by PCR using suitable primer pairs. The PCR product was sequenced confirming the expected nucleotide sequence, thus excluding recombination artefacts.

W. succinogenes Δ*ccsA1* was constructed by transforming nitrate-grown wild-type cells with plasmid pΔccsA1::kan (Table 1) and selection in medium containing kanamycin (25 mg l^{-1}). Two DNA fragments (black boxes in Fig. 1) corresponding to genomic regions upstream and downstream of *ccsA1* were synthesized by PCR using primers ccsA1-1, ccsA1-2, ccsA1-3 and ccsA1-4 listed in Table 1 and genomic DNA from *W. succinogenes* as template. The primers carried suitable restriction sites for cloning at their 5′-ends. The upstream fragment (580 bp; primers ccsA1-1 and ccsA1-2) was inserted into pBR322 after digestion with *Eco*RI and *Bam*HI. Subsequently, the downstream fragment (542 bp; primers ccsA1-3 and ccsA1-4) was inserted after digestion with *Bam*HI and *Sal*I. The identity of each cloned PCR fragment was confirmed by sequencing. Finally, the kanamycin resistance gene cartridge (*kan*) was inserted after digestion with *Bam*HI. The orientation of *kan* was the same as for the *ccsA1* fragments. *W. succinogenes* Δ*ccsA1* retained 6% and 1% of the respective 5′- and 3′-ends of *ccsA1*. Strain *W. succinogenes* P_{frd}-*mccA* Δ*ccsA1* was constructed upon transformation of *W. succinogenes* Δ*ccsA1* with pMccA (see above).

Purification of MccA from W. succinogenes P_{frd}-mccA

All steps were performed at 4°C and the presence of MccA was routinely checked by staining SDS polyacrylamide gels for covalently bound haem (Goodhew *et al*., 1986). *W. succinogenes* P_{frd}-*mccA* cells were suspended in buffer A containing 20 mM Tris/HCl (pH 7.5) and disrupted by French press treatment (three passages at 1000 psi). The obtained cell homogenate was centrifuged (1 h at 150 000 *g*) and the soluble cell fraction (supernatant) was loaded onto a DEAE-Sepharose CL-6B anion exchange column (Pharmacia Biotech) previously equilibrated with buffer A. Under these conditions, MccA did not bind to the column and eluted upon washing with buffer A. Fractions containing MccA were combined and dialysed against buffer B containing 20 mM KPO_4^{2-} (pH 7.5) using a 30 kDa cut-off membrane (Amicon). The concentrated dialysate was loaded onto a hydroxyapatite column (Ultrogel, Sigma) equilibrated with buffer B. After washing with buffer B, bound proteins were eluted using a linear phosphate gradient (20 mM to 1 M KPO_4^{2-}, pH 7.5). Fractions containing MccA eluted at approximately 0.5–0.6 M KPO_4^{2-}. The fractions were combined, concentrated by ultrafiltration (30 kDa cut-off membrane) and loaded onto a Superdex S200 gel filtration column (Pharmacia Biotech), previously equilibrated with buffer B containing 100 mM KCl. Fractions containing MccA were combined, dialysed against buffer A and loaded onto an analytical Mono-Q Sepharose column (Pharmacia Biotech) equilibrated with buffer A. The column was developed by washing with buffer A. MccA did not bind to the matrix and MccA-containing fractions were pooled, concentrated and dialysed against 20 mM Tris/HCl (pH 7.5), 100 mM NaCl. The concentration of purified MccA was estimated using the Soret absorbance maximum at 408 nm using the experimentally determined absorption extinction coefficient of 900 mM^{-1} cm^{-1}.

Mass spectrometry

Purified MccA was dialysed into 10 mM ammonium bicarbonate buffer and lyophilized. Matrix DHBs (2,5-dihydroxy benzoic acid:2-hydroxy-5-methoxy benzoic acid = 10:1) (Bruker Daltonik, Bremen) was prepared using acetonitrile/0.1% trifluoroacetic acid (TFA) 1:2, v/v as solvent. One microlitre of matrix solution was mixed with 1 μl of dissolved MccA (10 μM in 25 mM ammonium bicarbonate buffer) and dried in air. A protein calibration mixture (1 μM; #208241, Bruker Daltonik, Bremen) was used for external and internal calibration. The same MccA sample was digested with trypsin (sequencing grade, Sigma-Aldrich) using an enzyme to substrate ratio of 1:40 by weight. Digestion was stopped after incubation at 37°C for 24 h using formic acid (10%, v/v). One microlitre of digested sample was crystallized with 1 μl of matrix [3 g l^{-1} α-cyano-4-hydroxycinnamic acid in 70% (v/v) acetonitrile/0.5% (v/v) TFA]. Prior to analysis the crystals were washed briefly with ice-cold 5% (v/v) formic acid to reduce salt content. A peptide mass mixture (Applied Biosystems, MDS Sciex) was spotted for external calibration. The protein mass determination was performed with the Voyager DE-PRO instrument (Applied Biosystems, MDS Sciex). Spectra were recorded in the linear mode (25 kV acceleration voltage, 92.5% grid voltage, 750 ns delay time). Spectra were smoothed, noise-filtered, labelled and calibrated using Data Explorer software (Applied Biosystems, MDS Sciex). For the mass region above 60 000 the external and internal calibration ($n = 3$) results in an accuracy of approximately ±100 Da. The digested sample was acquired on the Ultraflex TOF/TOF (Bruker Daltonik, Bremen) in the reflectron mode. The MS spectrum was subjected to monoisotopic labelling and external calibration using Flex Analysis software (Bruker Daltonik, Bremen). The calibration yields an accuracy of 50 p.p.m. Additionally, MS/MS spectra were acquired to verify haem modifications (mass accuracy: precursor = 50 p.p.m.; fragments = 0.5–0.7 Da). Further data interpretation and visualization was performed with Biotools software (Bruker Daltonik, Bremen). MS spectra were analysed with Mascot™ (Perkins *et al*., 1999).

Spectroscopic characterization of MccA

Low temperature continuous-wave parallel mode EPR spectroscopy was performed at X-band frequency, using an ER200D spectrometer. The microwave bridge and electromagnet were interfaced to an EMX control (Bruker Biospin). A dual-mode X-band cavity (Bruker, type ER116DM) was used, where sample temperature was modulated using an ESR-900 liquid helium flow cryostat.

UV/Vis absorption spectra were acquired at 15°C on an Aminco SLM DW2000 spectrophotometer. Mediated spectropotentiometry was performed as described previously (Dobbin *et al*., 1999). A saturated solution of quinhydrone (hydroquinine/benzoquinone 1:1 complex; $E_0' = +295$ mV) was used as redox standard for the Pt-Ag/AgCl combination microelectrode. The mediators employed (all at 10 μM

final concentration) were diaminodurene ($E_0' = +250$ mV), phenazine methosulphate ($E_0' = +80$ mV), phenazine ethosulphate ($E_0' = +55$ mV), juglone ($E_0' = +30$ mV), duroquinone ($E_0' = +5$ mV), menadione ($E_0' = -80$ mV), anthraquinone 2,6-disulphonate ($E_0' = -185$ mV), anthraquinone 2-sulphonate ($E_0' = -225$ mV) and benzyl viologen ($E_0' = -360$ mV). Reduction potentials were referenced to SHE. Sodium dithionite and potassium ferricyanide were used as reductant and oxidant respectively. An equilibration period of 10 min was allowed before spectra were acquired in the range of 500–700 nm. Redox titrations were fitted with a variable number of $n = 1$ Nernstian components using a customized Tablecurve program that allows the midpoint redox potential, the number of redox components, and the contribution of each component to float as appropriate.

For quantification of the number of covalently bound *c*-type haems, conversion into pyridine derivatives was achieved by incubating protein (3 μM) with pyridine (2.1 M) and NaOH (75 mM) in water at room temperature for 15 min. Sodium dithionite and potassium ferricyanide were added to separate aliquots of the resulting solution such that the final concentrations of protein, reductant and oxidant were 2.5 μM, 1.5 mM and 750 μM respectively. Haem content was subsequently determined by using a difference molar absorption coefficient of 19.1 mM^{-1} cm^{-1} for the pyridine ferrohaemochrome *minus* the pyridine ferrihaemochrome at 550 nm (Berry and Trumpower, 1987).

Analytical ultracentrifugation

Sedimentation-equilibrium experiments were performed in a Beckman Optima XL-I analytical ultracentrifuge equipped with scanning absorbance optics and an An50 Ti rotor. 0.5, 1, 2, 10 and 20 μM MccA samples dissolved in 20 mM Tris/HCl (pH 7.5), 100 mM NaCl, were loaded into charcoal-filled Epon double-sector cells fitted with quartz windows. Reference sectors were filled with buffer, and centrifugation was performed at 5500 r.p.m. and 20°C. Absorbance profiles of each sample were collected every 4 h at 410, 420, 435, 530 and 571 nm for the 0.5, 1, 2, 10 and 20 μM MccA samples respectively. Once equilibrium conditions were reached (confirmed by the absorbance profile remaining constant between data obtained 4 h or more apart), five profiles were collected for each sample. A partial specific volume for MccA of 0.721 ml g^{-1} was calculated from the amino acid sequence using the program SEDNTERP and data collected at equilibrium were analysed with the software ULTRASCAN 7.2. The data from each sample was initially fitted to a single component model to give an average molecular weight at each concentration. Subsequently, all five sets of data were fitted to a monomer/trimer equilibrium model using $K_d = [\text{monomer}]^3/[\text{trimer}]$. To obtain a dissociation constant for the trimeric MccA complex, the molecular weight obtained from mass spectrometry was used. The absorbance coefficients of MccA at each wavelength measured were determined using the UV/Vis absorbance profile.

Miscellaneous procedures

Analyses of cell fractions and chromatography samples were performed by SDS-PAGE. Slab gels of 12% (w/v) polyacrylamide were employed for the resolution of the proteins, with samples being loaded via a stacking gel of 5% (w/v) polyacrylamide. The samples were prepared for electrophoresis by incubation with 3 M urea and 90 mM SDS at room temperature for 30 min. Gels were stained with Coomassie brilliant blue R-250. The presence of *c*-type cytochromes was tested for using a haem-linked peroxidase staining method (Goodhew *et al*., 1986). Protein concentration was determined using the BCA method with bovine serum albumin as standard (Smith *et al*., 1985). For N-terminal sequence determination, a 10 μM solution of MccA was heated at 90°C for 20 min with 0.7 M 2-mercaptoethanol/35 mM SDS. The protein was resolved by SDS-PAGE and electroblotted onto PVDF membrane. Sequence cycles were acquired on an Applied Systems Procise Sequencer by Mike Weldon (Protein and Nucleic Acid Chemistry Facility, Cambridge University).

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. CLUSTAL W alignment of 18 different MccA primary structures known as of January 2007.

Fig. S2. Representative MALDI-TOF mass spectra of purified MccA using external (A) or internal calibration (B).

Fig. S3. Representative mass spectra of tryptic fragments obtained from the MccA sample.

Table S1. Protein fragments detected by MALDI-MS in the tryptic digest of the MccA preparation.

Table S2. Average MccA masses from six independent MALDI-MS experiments using external or internal calibration.

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