transactions
Biochemical Society Transactions www.biochemsoctrans.org

Physiological function and catalytic versatility of bacterial multihaem cytochromes *c* involved in nitrogen and sulfur cycling

Jörg Simon*[1], Melanie Kern*, Bianca Hermann†, Oliver Einsle† and Julea N. Butt‡

*Institute of Microbiology and Genetics, Department of Biology, Technische Universität Darmstadt, Schnittspahnstrasse 10, 64287 Darmstadt, Germany, †Institute of Organic Chemistry and Biochemistry, University of Freiburg, Albertstrasse 21, 79104 Freiburg, Germany and ‡Centre for Molecular and Structural Biochemistry, School of Chemistry and School of Biological Sciences, University of East Anglia, Norwich Research Park, Norwich NR4 7TJ, U.K.

Abstract

Bacterial MCCs (multihaem cytochromes *c*) represent widespread respiratory electron-transfer proteins. In addition, some of them convert substrates such as nitrite, hydroxylamine, nitric oxide, hydrazine, sulfite, thiosulfate or hydrogen peroxide. In many cases, only a single function is assigned to a specific MCC in database entries despite the fact that an MCC may accept various substrates, thus making it a multifunctional catalyst that can play diverse physiological roles in bacterial respiration, detoxification and stress defence mechanisms. The present article briefly reviews the structure, function and biogenesis of selected MCCs that catalyse key reactions in the biogeochemical nitrogen and sulfur cycles.

Introduction

Bacterial MCCs (multihaem cytochromes *c*) contain at least two (but often many more) covalently bound haem groups that are attached to HBMs (haem *c*-binding motifs) (usually CX_2CH) by the enzyme CCS (cytochrome *c* synthase) [1]. MCCs carry out a diverse range of functions in bacterial energy metabolism, and, in particular, members of this protein class are involved in reactions that contribute significantly to global nitrogen, sulfur and iron cycling. Prominent examples of such processes are nitrification, respiratory ammonification of nitrate and nitrite, anammox (anaerobic ammonium oxidation), iron(III) reduction and conversion of sulfur compounds such as sulfite, thiosulfate and tetrathionate. Many well-known MCCs act as redox mediators that facilitate electron transport (either directly or indirectly) between the membranous quinone/quinol pool and primary dehydrogenases or terminal reductases organized in bacterial respiratory chains. These MCC families (for instance the NapC, NrfH, TorC, NapB, NrfB, QrcA and OhcA classes) are not within the scope of the present article (for reviews, see [2–4]). Also excluded are MCCs harbouring additional cofactors, e.g. flavin, in their active site or those MCCs that facilitate redox transformation of extracellular substrates by providing a conduit for trans-outer membrane electron transport in species such as *Geobacter* and *Shewanella* (see [5] for the structure of one such MCC). In that sense and in the context of the biogeochemical nitrogen and sulfur cycles, the present paper aims to summarize properties of MCCs that convert the important physiological substrates nitrite, hydroxylamine, hydrazine, sulfite, thiosulfate and tetrathionate. Some of the corresponding MCC enzymes have been structurally characterized in the past and were found to display conserved haem *c*-packing motifs, although their primary structures are largely unrelated [6,7]. More importantly, however, purified MCCs are frequently found to convert more than one substrate, pointing to multiple physiological roles. The present article aims to briefly review the current knowledge on MCC multifunctionality. In addition, aspects of homologous and heterologous MCC biogenesis and overproduction systems are addressed.

Physiological functions and substrate range of MCC enzymes

Prominent examples of multifunctional MCCs catalysing key reactions in the biogeochemical nitrogen and sulfur cycles are given in Table 1. Here, some of the key features of these enzymes are summarized and the fact is stressed that one has to distinguish between physiological enzyme function(s) *in vivo* and biochemical (*in vitro*) properties of purified enzymes that are mainly explored in artificial redox reaction set-ups. The classical enzyme displaying wide substrate versatility is NrfA (enzyme 1 in Table 1) which was initially described as an ammonium-producing pentahaem cytochrome *c* nitrite reductase catalysing the key reaction in respiratory nitrite ammonification [33]. In most cases, NrfA contains an active-site haem *c* group that is covalently bound by a unique CX_2CK HBM [6]. To date this HBM has only been found in NrfAs and the variants that carry an additional N-terminal

Key words: cytochrome *c* nitrite reductase, cytochrome *c* synthase, dissimilatory sulfite reduction, hydrazine oxidoreductase, hydroxylamine oxidoreductase, multihaem cytochrome *c*.
Abbreviations used: anammox, anaerobic ammonium oxidation; CCS, cytochrome *c* synthase; Hao, hydroxylamine oxidoreductase; HBM, haem *c*-binding motif; Hzo, hydrazine oxidoreductase; MCC, multihaem cytochrome *c*; OCC, octahaem cytochrome *c*; Onr, octahaem cytochrome *c* nitrite reductase; Otr, octahaem tetrathionate reductase.
[1]To whom correspondence should be addressed (email simon@bio.tu-darmstadt.de).

Table 1 | **Catalytic versatility of selected MCCs relevant to nitrogen and sulfur cycle reactions**

Enzyme (number and primary structure of HBMs)	Representative organisms and respective cytochrome *c* biogenesis system*	Reaction(s) catalysed†	Physiological function(s)	Selected reference(s)
1. Cytochrome *c* nitrite reductase NrfA (4 CX_2CH; 1 CX_2CK = HBM 1)	***Desulfovibrio vulgaris*** (I/II), ***Escherichia coli*** (I), *Salmonella enterica* (I), ***Sulfurospirillum deleyianum*** (II), ***Wolinella succinogenes*** (II)	R: nitrite→ammonium; R: hydroxylamine→ammonium; R: nitric oxide→ammonium; R: nitric oxide→nitrous oxide; R: nitrous oxide→dinitrogen; R: sulfite→sulfide; R: hydrogen peroxide→water; O: hydrazine→undefined product	Respiratory nitrite ammonification; nitrogen compound detoxification	[8–15]
2. Octahaem cytochrome *c* nitrite reductase Onr (7 CX_2CH; 1 CX_2CK = HBM 4; Tyr^{303} covalently bonded to Cys^{305})‡	***Thioalkalivibrio nitratireducens*** (I), many *Geobacter* species	R: nitrite→ammonium; R: hydroxylamine→ammonium; R: sulfite→sulfide; R: hydrogen peroxide→water	Not known, but nitrite ammonification is unlikely	[16]
3. Cytochrome *c* nitrite reductase NrfA (5 CX_2CH)	*Bdellovibrio bacteriovorus* (II), *Campylobacter jejuni* (II), *Helicobacter hepaticus* (II), *Rhodopirellula baltica* (II)	R: nitrite→ammonium; R: nitric oxide→ammonium	Respiratory nitrite ammonification; nitric oxide detoxification	[17,18]
4. Octahaem tetrathionate reductase Otr (8 CX_2CH)	***Shewanella oneidensis*** (I), many other *Shewanella* and *Geobacter* species	R: nitrite→ammonium; R: hydroxylamine→ammonium; R: tetrathionate→thiosulfate; O: thiosulfate→tetrathionate	Not known	[19,20]
5. Hydroxylamine oxidoreductase Hao (8 CX_2CH; tyrosine present§)	***Nitrosomonas europaea*** (I) and many other nitrifying bacteria	O: hydroxylamine→nitrite; O: hydrazine→dinitrogen; O: hydroxylamine→nitric oxide; R: nitric oxide→ammonium, hydroxylamine; R: nitrite→ammonium	Nitrification	[21–23]
6. Anammox-type hydroxylamine oxidoreductase (8 CX_2CH; tyrosine present)	*Candidatus* 'Brocadia anammoxidans' (?), *Candidatus* 'Kuenenia stuttgartiensis' (II), Strain KSU-1 (anammox planctomycete) (?)	O: hydroxylamine→nitric oxide, nitrous oxide; O: hydrazine→dinitrogen; R: nitric oxide→nitrous oxide; R: nitrite→nitrous oxide, nitric oxide	Channelling of hydroxylamine via NO into the anammox process (?)	[24–27]
7. Hydrazine oxidoreductase Hzo (7 CX_2CH; 1 CX_4CH = HBM 3; tyrosine present)	*Candidatus* 'Kuenenia stuttgartiensis' (II), Strain KSU-1 (anammox planctomycete) (?)	O: hydrazine→dinitrogen	Anammox	[26–28]
8. Epsilonproteobacterial Hao-type enzyme εHao (8 CX_2CH; tyrosine absent)	*Caminibacter mediatlanticus* (II), *Campylobacter concisus* (II), *Campylobacter curvus* (II), *Campylobacter fetus* (II), *Nautilia profundicola* (II)	Not examined, but nitrite reduction and hydroxylamine oxidation are likely (see the main text)	Assimilatory and/or dissimilatory nitrite ammonification	[8,29]
9. Cytochrome *c* sulfite reductase MccA/SirA (7 CX_2CH; 1 $CX_{15/17}CH$ = HBM 8)	*S. oneidensis* (I), *W. succinogenes* (II), other Proteobacteria	R: sulfite→sulfide	Respiratory sulfite reduction	[30,31]
10. Thiosulfate dehydrogenase TsdA (2 CX_2CH)	*Allochromatium vinosum* (I), *C. jejuni* (II)	O: thiosulfate→tetrathionate	Not known	[32]

*Representative organisms (in alphabetical order) were chosen according to the availability of physiological data and/or characterization of purified enzymes. Bold names indicate that a high-resolution structure model is available; (I) and (II) denote the presence of cytochrome *c* biogenesis systems I and/or II (question marks indicate uncertain or unclear assignments).

†The substrate with the highest specific activity or turnover number is underlined. If more than one product was detected, the dominant product is underlined. O, oxidation; R, reduction.

‡Numbering according to primary structure of *T. nitratireducens* Onr.

§Denotes the presence of the tyrosine residue that covalently links enzyme monomers within a homotrimer in *N. europaea* Hao.

trihaem cytochrome *c* domain [Onr (octahaem cytochrome *c* nitrite reductase); enzyme 2 in Table 1]. Crystal structures of classical NrfAs and of Onr clearly show that the lysine residue of the CX_2CK motif acts as the proximal haem *c* iron ligand and that substrates bind at the distal face of this active-site haem *c* [13,34]. The reduction potential of the lysine-ligated haem lies within the range readily accessible to histidine-ligated haems [35]. Consequently, it is proposed that the proximal lysine residue facilitates catalysis by offering greater opportunity for back-bonding to stabilize the iron–nitrite nitrogen bond that in turn weakens the N–O bonds of the substrate [34]. Subsequent protonation of the relatively electron-rich nitrite oxygens will lead to an HO–N=O adduct from which water can then be liberated in the first steps of the catalytic cycle.

Classical NrfAs and Onr are available from different bacterial sources and were shown to convert nitric oxide, hydroxylamine, hydrogen peroxide or sulfite, although less efficiently than nitrite [12,13,15,16] (Table 1). The formation of ammonium on reduction of both nitric oxide and hydroxylamine has led to the widely accepted proposal that these substrates are also intermediates during the reduction of nitrite. If this is indeed the case, then the transformation of NO to N_2O (nitrous oxide) and N_2O to N_2 catalysed by some classical NrfAs will probably occur by a distinct pathway [11]. The buried nature of the active site and the histidine–histidine ligation of neighbouring haem groups makes a bimolecular coupling of iron–nitrosyl species unlikely and nitrous oxide formation may instead follow a mechanism similar to that proposed for myoglobin [36]. The fact that *Thioalkalivibrio nitratireducens*, with an abundance of Onr, is unable to support anaerobic growth with nitrite and actually accumulates nitrite during anaerobic growth with nitrate suggests that the primary physiological role for this enzyme may not be nitrite reduction [37]. This may reflect distinct functionality of the enzyme resulting from a cross-linked tyrosine–cysteine pair within the active site that is not found in classical NrfAs (Table 1). Here it is also interesting that classical NrfA and Onr proteins are known to reduce sulfite to sulfide, a process that is isoelectronic with nitrite reduction to ammonium, so that these enzymes can be regarded as a connecting link between the nitrogen and sulfur cycles [13,15,16]. It is unclear, however, whether sulfite reduction by these enzymes plays a physiological role, even more so as sulfite usually does not induce enzyme formation. Overall, the observed catalytic versatility is supposed to provide physiological advantages to bacteria in their natural environments, and NrfA proteins have indeed been shown to confer resistance to stress exerted by reactive, toxic and/or mutagenic compounds such as nitric oxide, hydroxylamine or hydrogen peroxide [9,38,39].

Homologues of the classical NrfAs exist that harbour five CX_2CH motifs, i.e. the CX_2CK HBM is replaced by the conventional CX_2CH HBM (enzyme 3 in Table 1). NrfA from *Campylobacter jejuni* is one such enzyme that has been shown to have a physiological role in nitrite ammonification as well as in nitric oxide detoxification and stress defence [17]. Catalytically active *C. jejuni* NrfA has been recently produced in *Wolinella succinogenes*, but it is not known whether its active-site haem *c* is ligated by the HBM histidine residue or, for example, by a lysine residue located outside the HBM [18]. Site-directed mutagenesis in *Escherichia coli nrfA* replaced the lysine codon with that for histidine within the CX_2CK HBM, resulting in a protein displaying significantly lower *in vitro* nitrite-reduction rates than wild-type enzyme [40]. Similar results were also obtained with the corresponding variant of *W. succinogenes* NrfA [41]. Both variants were unable to support formate-dependent nitrite reduction of their respective host organisms. Thus, whereas the CX_2CK motif may be dispensible in terms of nitrite reductase activity, it may clearly affect the physiological competence of the enzyme. It remains to be established whether this is through its compromised rate of nitrite reduction or an adverse consequence of an altered product distribution such as the overproduction of cytotoxic nitric oxide.

Another MCC class capable of nitrite and hydroxylamine reduction was named octahaem tetrathionate reductase (Otr), since the *Shewanella oneidensis* Otr was shown to also interconvert tetrathionate and thiosulfate (enzyme 4 in Table 1). Although Otr contains eight CX_2CH motifs, the crystal structure of *S. oneidensis* Otr showed that the active-site haem *c* is actually ligated by a lysine residue that replaces the HBM histidine residue [19]. Maximal *in vitro* rates of nitrite reduction are ~400-fold lower for this enzyme than for Onr and the classical NrfAs, again demonstrating that lysine co-ordination of the active-site haem is not in itself sufficient to impart rapid nitrite-reduction rates.

The largest group of OCCs (octahaem cytochromes *c*) are those present predominantly in nitrifiers and anammox bacteria in which they catalyse hydroxylamine and/or hydrazine turnover [42] (enzymes 5–8 in Table 1). On the basis of primary and tertiary structure comparisons, an evolutionary relationship of these enzymes to the NrfA/Onr/Otr classes described above has been postulated [42]. In fact, the crystal structure of the *Nitrosomonas europaea* Hao (hydroxylamine oxidoreductase), the enzyme that oxidizes hydroxylamine to nitrite in bacterial nitrification, was shown to be similar to NrfA with haems 1–5 of NrfA being superimposable on to haems 4–8 of Hao [6]. A CX_2CH HBM co-ordinates the active-site haem and provides the proximal histidine haem ligand. However, this haem *c* is unusual in that the two typical thioether bonds are accompanied by a protein-derived tyrosine cross-link to a haem *meso* carbon. It is likely that this modulates the properties of the resulting 'P460' haem to favour oxidative, rather than reductive, transformations. On the other hand, the reduction of nitrite and nitric oxide are reported to be catalysed by *N. europaea* Hao *in vitro* using reduced Methyl Viologen as reductant [22,23]. Anammox bacteria contain different classes of Hao-type enzymes and some of them also contain the active-site tyrosine residue required for 'P460' formation [27] (enzymes 6 and 7 in Table 1). *In vivo*, such enzymes may function either as oxidizers of hydrazine [Hzo (hydrazine oxidoreductase); oxidizing hydrazine to dinitrogen which is in the terminal

step of anammox chemistry] or as nitric oxide-generating hydroxylamine oxidoreductases (nitric oxide is a substrate for hydrazine synthase, which is also an MCC). It is currently unknown which features have to be taken into account to distinguish Hao and Hzo OCCs. The higher reduction potential of the nitrite–nitric oxide redox couple compared with the hydroxylamine–nitric oxide couple may form the basis for selecting nitrite or nitric oxide as the product of hydroxylamine oxidation.

Another enigmatic OCC class (here named εHao) is found in some unusual ammonifying Epsilonproteobacteria, the majority of which lacks an NrfA homologue [8] (enzyme 8 in Table 1). Although clearly related to Hao-type OCCs, the active-site tyrosine residue is absent from εHao. It was hypothesized that this enzyme reduces nitrite to hydroxylamine in *Nautilia profundicola* [29], but might just as well perform nitrite reduction to ammonium, thereby functionally replacing NrfA. As *N. profundicola* apparently needs εHao for nitrate assimilation, the latter possibility is conceivable, since an ammonium transporter is encoded in the genome in contrast with a putative facilitator of hydroxylamine import. We have recently produced *Campylobacter fetus* εHao in *W. succinogenes* and are currently exploring its biochemical properties in reductive as well as in oxidative nitrogen compound conversions (M. Kern and J. Simon, unpublished work).

In addition to NrfA, another MCC has been described that reduces sulfite to sulfide (enzyme 9 in Table 1). This MCC was called MccA in *W. succinogenes* [30,44] and SirA in *S. oneidensis* [31]. In both bacteria, its physiological function appears to be respiratory sulfite reduction, making this enzyme functionally equivalent to sirohaem-containing dissimilatory sulfite reductases of sulfate-reducing bacteria and archaea ([31], and M. Kern, M.G. Klotz and J. Simon, unpublished work). The formation of *W. succinogenes* MccA was induced by the presence of sulfite as the sole electron acceptor and the protein was shown to contain eight haem *c* groups that all appear to have histidine–histidine ligation [30]. Seven haems are bound by CX_2CH HBMs, whereas the last haem is apparently attached to both cysteine residues of a conserved $CX_{15}CH$ motif (Table 1). To date, it is unclear whether this motif constitutes the active site of the enzyme. It is also interesting that, in contrast with the enzymes discussed above, there is no spectroscopic evidence for a haem offering a vacant or water-bound distal site to which the substrate may be readily expected to bind. It is also striking that purified *W. succinogenes* MccA was unable to reduce nitrite, since most enzymes reducing sulfite to sulfide are effective nitrite reducers [30]. Clues to the basis for this selectivity may emerge when the activity towards potential substrates such as nitric oxide and hydroxylamine have been tested and the impact of nitrite on sulfite reduction rates has been assessed. If nitrite is excluded from the active site, perhaps it is not sulfite, but rather SO_2, that is the true enzyme substrate, since SO_2 will always be present in aqueous sulfite solutions. Finally, in the context of sulfur compound conversion, another novel MCC needs to be mentioned. The dihaem cytochrome *c* thiosulfate dehydrogenase TsdA from *Allochromatium vinosum* was shown to oxidize thiosulfate to tetrathionate ([32], and C. Dahl, personal communication) (enzyme 10 in Table 1). Similar enzymes are present in a diverse range of bacteria, including some Epsilonproteobacteria.

Clearly much further work is required to understand the *in vitro* and *in vivo* activities of these MCCs such that these can be confidently predicted from genome analyses. It should also be remembered that the environment the enzymes experience *in vivo* may differ in many ways from those of the *in vitro* laboratory assay. For example, periplasmic MCCs in haloalkaliphiles may well experience pH>>7 and molar salt concentrations, and we have established that these can have a significant impact on the catalytic properties of MCCs (R. Doyle, S. Marritt and J.N. Butt, unpublished work).

The diversity of HBMs and the discovery of dedicated cytochrome *c* synthases

Haem attachment to apo-cytochromes *c* in bacteria requires a complex enzymic biogenesis system, and two distinct systems have been described and named system I (or Ccm system) and system II (or Ccs system) [44]. The key enzyme that catalyses the formation of two thioether bonds between haem *b* vinyl groups and the reduced cysteine thiol groups of the HBM is a membrane-bound CCS [also referred to as CCHL (cytochrome *c* haem lyase)]. However, the detailed molecular mechanisms of HBM and haem recognition, as well as the reaction mechanism of haem attachment by CCS, are unresolved to date. In system II, the CCS is a complex of two membrane-bound proteins (CcsA and CcsB) or a corresponding fusion protein (CcsBA) which is predominantly present in Epsilonproteobacteria [18]. System II CCSs contain conserved pairs of functionally crucial histidine residues that have been proposed to be involved in haem *b* export to the outside of the bacterial cytoplasmic membrane where cytochrome *c* maturation takes place [45,46]. The most likely candidate of the system I cytochrome *c* synthase is the membrane protein CcmF. Both CcsA and CcmF carry a tryptophan-rich signature sequence (the WWD domain) that might play a role in haem *b* binding and presentation before thioether bridge formation. Interestingly, several enzymes described in Table 1 contain unconventional HBMs (CX_2CK, CX_4CH, $CX_{15/17}CH$) or covalent modifications involving either haem *c* or conserved amino acid residues located in the vicinity of the active site of substrate conversion. In the cases of the CX_2CK and $CX_{15}CH$ HBMs, dedicated CCS isoenzymes have been described to be required for the haem-attachment process in addition to the CH_2CH-recognizing CCS [30,40,41,46]. Such special CCSs derive from the cognate maturation system and have been called NrfEFG (specific to the CX_2CK motif of *E. coli* NrfA), NrfI (specific to the CX_2CK motif of *W. succinogenes* NrfA) or CcsA1 (presumably specific to the $CX_{15}CH$ motif of *W. succinogenes* MccA). Possibly, the $CX_{15}CH$ motif of *S. oneidensis* SirA, an MccA homologue,

is handled by the NrfEFG homologues SirEFG [31]. It therefore appears that system I- and system II-containing organisms both developed dedicated CCS enzymes during evolution in order to attach haem to unconventional HBMs present in NrfA or MccA/SirA. As far as we know, these dedicated CCSs are always based on the cytochrome *c* biogenesis system that is used for handling the CX_2CH HBM. Notably, the genome sequences of several *Anaeromyxobacter*, *Bacteroides* and *Geobacter* species encode between four and six distinct CcsA homologues which might indicate the presence of more as yet unrecognized HBMs [43]. With respect to the other covalent modifications in MCCs described in Table 1 (i.e. tyrosine cross-links), it is unclear whether or not the formation of these is enzymically catalysed. In principle, such bonds may form spontaneously during protein folding and this distinction may have an impact on the success of MCC-overproduction systems that we now move on to discuss.

Bacterial MCC-overproduction systems

One prerequisite for testing the substrate versatility of MCCs is the availability of pure enzymes in sufficient amounts. Suitable purification procedures have been described for species belonging to various proteobacterial classes as well as for anammox bacteria (see Table 1 for references), but protocols are often time-consuming and rely on many purification steps that diminish enzyme yield. In addition, interesting and novel cytochromes *c* are regularly predicted from the genomes of organisms (i) that are hard to grow in reasonable amounts (such as nitrifiers and many fastidious Epsilonproteobacteria), (ii) that contain a vast amount of MCCs impairing purification of single cytochromes (for example, many *Geobacter* and *Shewanella* species), or (iii) that are not available in pure cultures (for instance, anammox bacteria). Furthermore, novel MCCs can be readily deduced from metagenomic data of environmental samples due to their HBMs. For these reasons, efficient (heterologous) cytochrome *c*-overproduction systems are desirable to facilitate enzyme purification and characterization. The most widespread means of cytochrome *c* production uses aerobically grown *Escherichia coli* cells that harbour a plasmid containing the system I-encoding *ccm* gene cluster [47–49]. Recently, an alternative overproduction system was described employing the system II bacterium *W. succinogenes* as host organism [50]. It remains to be seen which of these systems is most efficient, as comparative data are lacking, but it is quite likely that the production success for a particular MCC cannot be accurately predicted as its maturation depends on many different parameters that are not understood in detail. However, it has been shown that an MCC from a system I organism can be produced in a system II organism and vice versa [46,48,49].

Conclusions and perspectives

The current amount of genomic and metagenomic sequence data is expected to increase rapidly in the near future. It therefore seems reasonable to assume that many unknown MCC families are yet to be discovered. However, the substrate range and physiological function(s) of these enzymes will remain difficult to deduce from their primary structure due to the described catalytic multifunctionality. This situation is reminiscent of proteins of the copper-containing Amo (ammonia mono-oxygenase)/pMMO (particulate methane mono-oxygenase) family that are known to convert different substrates such as ammonia, methane, ethane, butane or hexane. Another example are molybdoenzymes that contain a molybdo-bis(pyranopterin guanine dinucleotide) cofactor and convert a highly diverse range of substrates, including formate, nitrate, arsenate, selenate and polysulfide. In order to reveal the detailed function of all of these metalloenzymes, physiological studies of bacterial strains and mutants, as well as biochemical, spectroscopic and crystallization experiments using purified enzymes, are mandatory. In this context, the direct detection of substrates and products by techniques such as gas chromatography, HPLC and MS is recommended rather than the much simpler colorimetric detection of coupled turnover of substrate(s) and redox dyes. In addition, research in this area will benefit from engineering novel cytochrome *c*-production systems that may use a wide range of organisms equipped with the required biogenesis systems. Such systems will also allow the production of MCC variants after site-directed mutagenesis which is very useful for the verification of mechanistic hypotheses based on the methods described above.

Acknowledgements

We apologize to colleagues whose work we could not cite due to space limitations.

Funding

Work in our laboratories is supported by the Deutsche Forschungsgemeinschaft [grant numbers SI 848/5-1 (to J.S.) and EI 520/5-1 (to O.E.)] and the Biotechnology and Biological Sciences Research Council [grant number BB/G009228 (to J.N.B.)].

References

1 Sharma, S., Cavallaro, G. and Rosato, A. (2010) A systematic investigation of multiheme *c*-type cytochromes in prokaryotes. J. Biol. Inorg. Chem. **15**, 559–571
2 Simon, J. and Kern, M. (2008) Quinone-reactive proteins devoid of haem *b* form widespread membrane-bound electron transport modules in bacterial respiration. Biochem. Soc. Trans. **36**, 1011–1016
3 Simon, J., van Spanning, R.J.M. and Richardson, D.J. (2008) The organisation of proton motive and non-proton motive redox loops in prokaryotic respiratory systems. Biochim. Biophys. Acta **1777**, 1480–1490
4 Pereira, I.A.C., Ramos, A.R., Grein, F., Marques, M.C., da Silva, S.M. and Venceslau, S.S. (2011) A comparative genomic analysis of energy metabolism in sulfate reducing bacteria and archaea. Front. Microbiol. **2**, 69

5 Clarke, T.A., Edwards, M.J., Gate, A.J., Hall, A., White, G.F., Bradley, J., Reardon, C.L., Shi, L., Beliaev, A.S., Marshall, M.J. et al. (2011) Structure of a bacterial cell surface decaheme electron conduit. Proc. Natl. Acad. Sci. U.S.A. **108**, 9384–9389
6 Einsle, O., Messerschmidt, A., Stach, P., Bourenkov, G.P., Bartunik, H.D., Huber, R. and Kroneck, P.M.H. (1999) Structure of cytochrome *c* nitrite reductase. Nature **400**, 476–480
7 Mowat, C.G. and Chapman, S.K. (2005) Multi-heme cytochromes: new structures, new chemistry. Dalton Trans. **7**, 3381–3389
8 Kern, M. and Simon, J. (2009) Electron transport chains and bioenergetics of respiratory nitrogen metabolism in *Wolinella succinogenes* and other Epsilonproteobacteria. Biochim. Biophys. Acta **1787**, 646–656
9 Kern, M., Volz, J. and Simon, J. (2011) The oxidative and nitrosative stress defence netweork of *Wolinella succinogenes*: cytochrome *c* nitrite reductase mediates the stress response to nitrite, nitric oxide, hydroxylamine and hydrogen peroxide. Environ. Microbiol. **13**, 2478–2494
10 Stach, P., Einsle, O., Schumacher, W., Kurun, E. and Kroneck, P.M.H. (2000) Bacterial cytochrome *c* nitrite reductase: new structural and functional aspects. J. Inorg. Biochem. **79**, 381–385
11 Costa, C., Macedo, A., Moura, I., Moura, J.J.G., LeGall, J., Berlier, Y., Liu, M.Y. and Payne, W.J. (1990) Regulation of the hexaheme nitrite/nitric oxide reductase of *Desulfovibrio desulfuricans*, *Wolinella succinogenes* and *Escherichia coli*: a mass spectrometric study. FEBS Lett. **276**, 67–70
12 van Wonderen, J.H., Burlat, B., Richardson, D.J., Cheesman, M.R. and Butt, J.N. (2008) The nitric oxide reductase activity of cytochrome *c* nitrite reductase from *Escherichia coli*. J. Biol. Chem. **283**, 9587–9594
13 Kemp, G.L., Clarke, T.A., Marritt, S.J., Lockwood, C., Poock, S.R., Hemmings, A.M., Richardson, D.J., Cheesman, M.R. and Butt, J.N. (2010) Kinetic and thermodynamic resolution of the interactions between sulfite and the pentahaem cytochrome NrfA from *Escherichia coli*. Biochem. J. **431**, 73–80
14 Rudolf, M., Einsle, O., Neese, F. and Kroneck, P.M.H. (2002) Pentahaem cytochrome *c* nitrite reductase: reaction with hydroxylamine, a potential reaction intermediate and substrate. Biochem. Soc. Trans. **30**, 649–653
15 Lukat, P., Rudolf, M., Stach, P., Messerschmidt, A., Kroneck, P.M.H., Simon, J. and Einsle, O. (2008) Binding and reduction of sulfite by cytochrome *c* nitrite reductase. Biochemistry **47**, 2080–2086
16 Tikhonova, T.V., Slutsky, A., Antipov, A.N., Boyko, K.M., Polyakov, K.M., Sorokin, D.Y., Zvyagilskaya, R.A. and Popov, V.O. (2006) Molecular and catalytic properties of a novel cytochrome *c* nitrite reductase from nitrate-reducing haloalkaliphilic sulfur-oxidizing bacterium *Thioalkalivibrio nitratireducens*. Biochim. Biophys. Acta **1764**, 715–723
17 Pittman, M.S., Elvers, K.T., Lee, L., Jones, M.A., Poole, R.K., Park, S.F. and Kelly, D.J (2007) Growth of *Campylobacter jejuni* on nitrate and nitrite: electron transport to NapA and NrfA via NrfH and distinct roles for NrfA and the globin Cgb in protection against nitrosative stress. Mol. Microbiol. **63**, 575–590
18 Kern, M., Eisel, F., Scheithauer, J., Kranz, R.G. and Simon, J. (2010) Substrate specificity of three cytochrome *c* haem lyase isoenzymes from *Wolinella succinogenes*: unconventional haem *c* binding motifs are not sufficient for haem *c* attachment by NrfI and CcsA1. Mol. Microbiol. **75**, 122–137
19 Mowat, C.G., Rothery, E., Miles, C.S., McIver, L., Doherty, M.K., Drewette, K., Taylor, P., Walkinshaw, M.D., Chapman, S.K. and Reid, G.A. (2004) Octaheme tetrathionate reductase is a respiratory enzyme with novel heme ligation. Nat. Struct. Mol. Biol. **11**, 1023–1024
20 Atkinson, S.J., Mowat, C.G., Reid, G.A. and Chapman, S.K. (2007) An octahaem *c*-type cytochrome from *Shewanella oneidensis* can reduce nitrite and hydroxylamine. FEBS Lett. **581**, 3805–3808
21 Igarashi, N., Moriyama, H., Fujiwara, T., Fukumori, Y. and Tanaka, N. (1997) The 2.8 Å structure of hydroxylamine oxidoreductase from a nitrifying chemolithotrophic bacterium, *Nitrosomonas europaea*. Nat. Struct. Biol. **4**, 276–284
22 Kostera, J., Youngblut, M.D., Slosarczyk, J.M. and Pacheco, A.A. (2008) Kinetic and product analysis of NO· reductase activity in *Nitrosomonas europaea* hydroxylamine oxidoreductase. J. Biol. Inorg. Chem. **13**, 1073–1083
23 Kostera, J., McGarry, J. and Pacheco, A.A. (2010) Enzymatic interconversion of ammonia and nitrite: the right tool for the job. Biochemistry **49**, 8546–8553
24 Schalk, J., de Vries, S., Kuenen, J.G. and Jetten, M.S.M. (2000) Involvement of a novel hydroxylamine oxidoreductase in anaerobic ammonium oxidation. Biochemistry **39**, 5405–5412
25 Shimamura, M., Nishiyama, T., Shinya, K., Kawahara, Y., Furukawa, K. and Fujii, T. (2008) Another multiheme protein, hydroxylamine oxidoreductase, abundantly produced in anammox bacterium besides the hydrazine-oxidizing enzyme. J. Biosci. Bioeng. **105**, 243–248
26 Strous, M., Pelletier, E., Mangenot, S., Rattei, T., Lehner, A., Taylor, M.W., Horn, M., Daims, H., Bartol-Mavel, D., Wincker, P. et al. (2006) Deciphering the evolution and metabolism of an anammox bacterium from a community genome. Nature **440**, 790–794
27 de Almeida, N.M., Maalcke, W.J., Keltjens, J.T., Jetten, M.S.M. and Kartal, B. (2011) Proteins and protein complexes involved in the biochemical reactions of anaerobic ammonium-oxidizing bacteria. Biochem. Soc. Trans. **39**, 303–308
28 Shimamura, M., Nishiyama, T., Shigetomo, H., Toyomoto, T., Kawahara, Y., Furukawa, K. and Fujii, T. (2007) Isolation of a multiheme protein with features of a hydrazine-oxidizing enzyme from an anaerobic ammonium-oxidizing enrichment culture. Appl. Environ. Microbiol. **73**, 1065–1072
29 Campbell, B.J., Smith, J.L., Hanson, T.E., Klotz, M.G., Stein, L.Y., Lee, C.K., Wu, D., Robinson, J.M., Khouri, H.M., Eisen, J.A. and Cary, S.C. (2009) Adaptations to submarine hydrothermal environments exemplified by the genome of *Nautilia profundicola*. PLoS Genet. **5**, e1000362
30 Hartshorne, R.S., Kern, M., Meyer, B., Clarke, T.A., Karas, M., Richardson, D.J. and Simon, J. (2007) A dedicated haem lyase is required for the maturation of a novel bacterial cytochrome *c* with unconventional covalent haem binding. Mol. Microbiol. **64**, 1049–1060
31 Shirodkar, S., Reed, S., Romine, M. and Saffarini, D. (2011) The octahaem SirA catalyses dissimilatory sulfite reduction in *Shewanella oneidensis* MR-1. Environ. Microbiol. **13**, 108–115
32 Henson, D., Sperling, D., Trüper, H.G., Brune, D.C. and Dahl, C. (2006) Thiosulphate oxidation in the phototrophic sulphur bacterium *Allochromatium vinosum*. Mol. Microbiol. **62**, 794–810
33 Simon, J. (2002) Enzymology and bioenergetics of respiratory nitrite ammonification. FEMS Microbiol. Rev. **26**, 285–309
34 Einsle, O., Messerschmidt, A., Huber, R., Kroneck, P.M.H. and Neese, F. (2002) Mechanism of the six-electron reduction of nitrite to ammonia by cytochrome *c* nitrite reductase. J. Am. Chem. Soc. **124**, 11737–11745
35 Marritt, S.J., Kemp, G.L., Xiaoe, L., Durrant, J.R., Cheesman, M.R. and Butt, J.N. (2008) Spectroelectrochemical characterization of a pentaheme cytochrome in solution and as electrocatalytically active films on nanocrystalline metal-oxide electrodes. J. Am. Chem. Soc. **130**, 8588–8589
36 Bayachou, M., Lin, R., Cho, W. and Farmer, P.J. (1998) Electrochemical reduction of NO by myoglobin in surfactant film: characterization and reactivity of the nitroxyl (NO-) adduct. J. Am. Chem. Soc. **120**, 9888–9893
37 Sorokin, D.Y., Tourova, T.P., Sjollema, K.A. and Kuenen, J.G. (2003) *Thialkalivibrio nitratireducens* sp. nov., a nitrate-reducing member of an autotrophic denitrifying consortium from a soda lake. Int. J. Syst. Evol. Microbiol. **53**, 1779–1783
38 Poock, S.R., Leach, E.R., Moir, J.W.B., Cole, J.A. and Richardson, D.J. (2002) Respiratory detoxification of nitric oxide by the cytochrome *c* nitrite reductase of *Escherichia coli*. J. Biol. Chem. **277**, 23664–23669
39 Mills, P.C., Rowley, G., Spiro, S., Hinton, J.C. and Richardson, D.J. (2008) A combination of cytochrome *c* nitrite reductase (NrfA) and flavorubredoxin (NorV) protects *Salmonella enterica* serovar Typhimurium against killing by NO in anoxic environments. Microbiology **154**, 1218–1228
40 Eaves, D.J., Grove, J., Staudenmann, W., James, P., Poole, R.K., White, S.A., Griffiths, I. and Cole, J.A. (1998) Involvement of products of the *nrfEFG* genes in the covalent attachment of haem *c* to a novel cysteine-lysine motif in the cytochrome *c*552 nitrite reductase from *Escherichia coli*. Mol. Microbiol. **28**, 205–216
41 Pisa, R., Stein, T., Eichler, R., Gross, R. and Simon, J. (2002) The *nrfI* gene is essential for the attachment of the active site haem group of *Wolinella succinogenes* cytochrome *c* nitrite reductase. Mol. Microbiol. **43**, 763–770
42 Klotz, M.G., Schmid, M.C., Strous, M., Op den Camp, H.J.M., Jetten, M.S.M. and Hooper, A.B. (2008) Evolution of an octahaem cytochrome *c* protein family that is key to aerobic and anaerobic ammonia oxidation by bacteria. Environ. Microbiol. **10**, 3150–3163

43 Hartshorne, R.S., Richardson, D.J. and Simon, J. (2006) Multiple haem lyase genes indicate substrate specificity in cytochrome *c* biogenesis. Biochem. Soc. Trans. **34**, 146–149
44 Kranz, R.G., Richard-Fogal, C, Taylor, J.S. and Frawley, E.R. (2009) Cytochrome *c* biogenesis: mechanisms for covalent modifications and trafficking of heme and for heme–iron redox control. Microbiol. Mol. Biol. Rev. **73**, 510–528
45 Frawley, E.R. and Kranz, R.G. (2009) CcsBA is a cytochrome *c* synthetase that also functions in heme transport. Proc. Natl. Acad. Sci. U.S.A. **106**, 10201–10206
46 Kern, M., Scheithauer, J., Kranz, R.G. and Simon, J. (2010) Essential histidine pairs indicate conserved haem binding in epsilonproteobacterial cytochrome *c* haem lyases. Microbiology **156**, 3773–3781
47 Arslan, E., Schulz, H., Zufferey, R, Künzler, P. and Thöny-Meyer, L. (1998) Overproduction of the *Bradyrhizobium japonicum c*-type cytochrome subunits of the *cbb*3 oxidase in *Escherichia coli*. Biochem. Biophys. Res. Commun. **251**, 744–747
48 Hoffmann, M., Seidel, J. and Einsle, O. (2009) CcpA from *Geobacter sulfurreducens* is a basic di-heme cytochrome *c* peroxidase. J. Mol. Biol. **393**, 951–965
49 Londer, Y.Y., Pokkuluri, P.R., Orshonsky, V, Orshonsky, L. and Schiffer, M. (2006) Heterologous expression of dodecaheme "nanowire" cytochromes *c* from *Geobacter sulfurreducens*. Protein Expression Purif. **47**, 241–248
50 Kern, M. and Simon, J. (2011) Production of recombinant multiheme cytochromes *c* in *Wolinella succinogenes*. Methods Enzymol. **486**, 429–446

Received 17 August 2011
doi:10.1042/BST20110713