DISS. ETH No. 15962

Dynamics of heme delivery and protein-protein interactions during cytochrome *c* maturation

A dissertation submitted to the

SWISS FEDERAL INSTITUTE OF TECHNOLOGY

ZÜRICH

for the degree of

Doctor of Natural Sciences

presented by

UMESH AHUJA

M.Sc (Hons) Microbiology, Panjab University, India

born 10.02.1976

citizen of India

accepted on the recommendation of

Prof. Dr. Linda Thöny-Meyer, examiner

Prof. Dr. Markus Aebi, co-examiner

2005

ACKNOWLEDGEMENTS

First and the foremost, I would like to express my special gratitude to Linda for her whole hearted support that she conferred through out my stay in her laboratory. She had been extremely instrumental in shaping every aspect of my thesis work and without her keen interest, remarkable scientific expertise and friendly supervision my thesis would not have seen this day of light. I feel proud and privileged to have her as my adviser and also to get to know such a loving person. Sincerely, I can never thank her enough for giving me this wonderful opportunity.

During my stay in Linda's laboratory, I came in contact with several fascinating people with whom I shared great comradeship. I would like to start thanking first Elizabeth for introducing me not only to the routine laboratory work but giving me a better insight into the Swiss culture and their way of doing things with efficiency and precision. I thank Qen for sharing her experimental skills, which really helped me in the long run. I thank Melissa for being a good company and invoking my interest in crystallography, which later helped me a lot while growing my own crystals of solCcmH.

A special thanks goes to Olaf and Martin with whom I shared most part of my stay in the laboratory sharing ideas and discussing damned experiments at 3° clock coffee breaks. I really enjoyed having them around and will miss the good time we shared together. Next on my list are Daniel and Daniela for all their contributions to my work and for bringing liveliness in B26 after everyone had left.

I would also like to thank people from Wolf's laboratory especially Brit and Kristin and Ackim from Dimroth's group for their support at many levels through out my work.

I would also like to thank my collaborators: Oliver Einsle, Georg-August-universität, Göttingen; Lars Hederstedt, Lund university, Sweden and Anna Rozhkova and Rudi Glockshuber, ETH Zurich.

I am especially grateful to the institute management, in particular, Alain for always lending his ears to whatever and whenever problems transpired. Thank you very much for all the quick-fix solutions and valuable suggestions. In this same breath I would also like to thank Jacques for his superb IT services, especially, for my ever-troubling laptop. I would also like to thank Palmira, Paul, Stephan, Chiara and Silvia for their efforts in smooth running of the institute.

I thank Markus Aebi for his keen interest in my work and critically reviewing my dissertation.

Last but certainly not the least; I would like to let my family know that how much they mean to me and that without their support and encouragement I would have not come that far. I especially like to thank my wife Preeti for her unconditional support she bestowed on me all the time. I am greatly indebted to them, for which I can never thank them enough.

I dedicate my thesis to my loving family and my wife.

TABLE OF CONTENTS

ABSTRACT **1**

ZUSAMMENFASSUNG **3**

CHAPTER 1

Synopsis

Cytochrome *c* maturation pathway of *Escherichia coli* 13

How is heme transported across the membrane? 14

Role of CcmC in heme delivery 15

A dual function for CcmC? 18

Role of CcmD in heme delivery 19

Role of CcmE in heme delivery 21

Role of CcmF in heme delivery 23

Current working model for the heme delivery process during cytochrome *c* maturation 24

Outlook 26

References 30

CHAPTER 2

Dynamic features of a heme delivery system for cytochrome *c* maturation **37**

SUMMARY 38

INTRODUCTION 39

EXPERIMENTAL PROCEDURES 42

Bacterial strains and growth conditions 42

Screening under anaerobic respiratory growth on TMAO 42

P1 phage transduction 42

Construction of plasmids and site-directed mutagenesis 43

Random mutagenesis by error prone PCR 44

Random mutagenesis by using a repair deficient strain 44

DNA sequencing 44

Cell fractionation 45

Biochemical methods 45

Heme binding assays 47

Co-immunoprecipitation 49
RESULTS 50
Rationale of the screen 50
Generation and analysis of random *ccmC* mutants 52
Analysis of *ccmC* site-directed mutants 57
Analysis of *ccmC* mutants in the tryptophan-rich motif 61
Analysis of *ccmC* mutants in the absence of *ccmD* 62
CcmC does not bind CcmF 65
Analysis of a CcmCE fusion protein 66
DISCUSSION 69
REFERENCES 75
CHAPTER 3
CcmD is involved in complex formation between CcmC and the heme chaperone CcmE during cytochrome *c* maturation 79
SUMMARY 80
INTRODUCTION 81
EXPERIMENTAL PROCEDURES 83
Bacterial strains and growth conditions 83
Construction of plasmids and site-directed mutagenesis 83
DNA sequencing 86
Co-purification with histidine-tagged proteins 86
Co-purification with strep-tagged protein 89
Cell fractionation 89
Biochemical methods 89
Heme binding assays 90
Tricine gels 90
Co-immunoprecipitation 90
Pulse chase experiment 91
Formation of spheroplasts and inside out (ISO) vesicles and proteinase K treatment 91
Treatment of membrane vesicles with various chaotropic agents 92
RESULTS 93

Functional analysis of truncated CcmD derivatives 93
Involvement of CcmD in heme delivery 98
Role of CcmD in ternary complex formation 101
Influence of CcmD on CcmE stability 104
Topology of CcmD 104
DISCUSSION 107
REFERENCES 110
CHAPTER 4
The membrane anchors of the heme chaperone CcmE and the periplasmic thioredoxin CcmG are functionally important 113
SUMMARY 114
INTRODUCTION 115
EXPERIMENTAL PROCEDURES 116
Bacterial strains and growth conditions 116
Construction of plasmids and site-directed mutagenesis 116
DNA sequencing 118
Cell fractionation 118
Biochemical methods 119
Co-immunoprecipitation 120
RESULTS 121
Functional analysis of chimerical CcmE 121
Functional analysis of chimerical CcmG 127
Is the membrane anchor essential for the function of CcmE and CcmG? 129
Heme transfer and co-immunoprecipitation of soluble CcmE with CcmC 134
DISCUSSION 136
REFERENCES 140
Publications 143
Curriculum vitae 145

ABSTRACT

C-type cytochromes are an ubiquitous class of electron transfer proteins that contain heme as a covalently attached cofactor. Covalent attachment of the heme is a posttranslational process and requires the activity of several protein components. In the γ-proteobacterium *E. coli* cytochrome *c* maturation is accomplished in the presence of eight membrane proteins (CcmA-H) encoded by the *ccm* operon (cytochrome c maturation). The hallmark of cytochrome *c* maturation is the formation of two thioether bonds between the vinyl groups of heme and the cysteine residues of apo-cytochrome *c*. The apo-cytochrome *c* and heme are both synthesized in the cytoplasm but must be transported to the periplasm for the covalent heme attachment. While it is known that apo-cytochrome *c* translocation across the plasma membrane takes place in the presence of the SecYEG translocon, the transport mechanism of heme is unknown. CcmC can bind heme and also catalyzes the covalent attachment of heme to the apo-CcmE. The covalent attachment of heme to apo-CcmE is stimulated in the presence of the small membrane protein CcmD. In this work various biochemical and genetic approaches were undertaken to further characterize and understand the individual contribution of CcmC, CcmD and CcmE in the heme delivery pathway for cytochrome *c* maturation.

An extensive biochemical analysis of several random mutants obtained from a specially designed genetic screen, and also of a CcmC-E fusion protein, revealed a new phenotype that affected cytochrome *c* maturation without affecting holo-CcmE formation. This suggested that heme transfer from holo-CcmE to apo-cytochrome *c* was in some way influenced by CcmC. In a parallel protein-protein interaction study between CcmC, CcmE and CcmF, it was found that no ternary complex existed between these three proteins while CcmE was found to be associated with both CcmC and CcmF individually. This led to the idea of a dynamic

movement of CcmE between two physically separated sub-complexes containing CcmC in one and CcmF in the other.

The role of CcmD was analyzed both genetically and biochemically. The functional domain of CcmD was mapped to its central hydrophobic membrane region and to part of the C-terminal domin including a net positive charge. CcmD was found to associate with both CcmC and CcmE and to constitute a ternary complex together with CcmC and CcmE. A topological analysis suggested that CcmD is an integral interfacial membrane protein with both its N- and C-terminus protruding in the cytoplasm, which contradicted the previously proposed model.

The importance of the CcmE membrane anchor was elucidated by replacing it with the membrane anchor of CcmG. This chimerical version of CcmE was affected neither in holo-CcmE formation nor in holo-cytochrome *c* formation, although protein levels of this chimera were much weaker than that of wild-type CcmE. Contrary to this, the soluble version of CcmE, which lacked the membrane anchor, was abundant in the periplasm, but poorly complemented cytochrome *c* maturation. The chimerical and soluble CcmE were both found to interact with CcmC, suggesting that the site of interaction resided in the periplasmic domain of CcmE. The lack of complementation by soluble CcmE may be due to inefficient heme transfer to apo-cytochrome *c* via CcmF.

ZUSAMMENFASSUNG

C-Typ Cytochrome sind weit verbreitete Elektronentransport-Proteine, welche den Kofaktor Häm kovalent gebunden haben. Die kovalente Bindung des Häms an das Protein findet nach der Translation statt und benötigt die Aktivität diverser Enzyme. Im γ-Proteobacterium *E. coli* wird die Cytochrom *c* Reifung von acht Membranproteinen, CcmA-H, katalysiert. Diese werden vom *ccm* Operon (cytochrome c maturation) codiert. Die Reaktion führt zu zwei kovalenten Thioether Bindungen zwischen den Vinyl Gruppen von Häm und zwei Cystein Resten von Apo-Cytochrom *c*. Sowohl das Apo-Cytochrom *c* als auch Häm werden im Cytoplasma synthetisiert und müssen anschliessend für die kovalente Bindung ins Periplasma transportiert werden. Während das Apo-Cytochrom *c* von der allgemeinen Sekretionsmaschinerie SecYEG ins Periplasma transportiert wird, ist es noch unbekannt, wie das Hämmolekül ins Periplasma gelangt. Eines der Proteine der Cytochrom *c* Reifung, CcmC, bindet Häm und katalysiert die kovalente Bindung des Häms an ein weiteres Protein der Cytochrom *c* Reifung, Apo-CcmE. Die kovalente Bindung von Häm an Apo-CcmE wird in Gegenwart des kleinen Membranproteins CcmD stimuliert. In dieser Arbeit wurden diverse biochemische und genetische Methoden gebraucht, um die Bedeutung der Proteine CcmC, CcmD und CcmE in der Häm-Übertragung besser zu verstehen.

In einer extensiven biochemischen Analyse diverser unspezifisch erhaltener CcmC Mutanten und eines künstlichen CcmC-E Fusionsproteins wurde ein neuer Phänotyp entdeckt. Dieser Phänotyp bestand darin, dass die Cytochrom *c* Reifung defekt war ohne dass die Holo-CcmE Bildung beeinträchtigt wurde. Dies galt als Indiz dafür, dass CcmC den Transfer von Häm vom Holo-CcmE zum Apo-Cytochrom *c* beeinflusst. Parallel dazu konnte mittels Protein-

Protein Interaktionsstudien kein Komplex bestehend aus CcmC, CcmE und CcmF nachgewiesen werden. CcmE allerdings interagierte mit beiden Proteinen, CcmC und CcmF. Daraus entwickelte sich die Idee, dass sich CcmE dynamisch zwischen CcmC und CcmF bewegt und jeweils Subkomplexe bildet, um Häm dem Apo-Cytochrom *c* zu zuführen.
Auch die Rolle von CcmD wurde sowohl genetisch als auch biochemisch analysiert. Die funktionell wichtige Domäne von CcmD wurde in ihrer hydrophoben, membran-durchspannenden Region lokalisiert, wobei auch wichtig, ist dass die C-terminale Domäne von CcmD positive Ladungen hat. Es wurde gezeigt, dass CcmD mit CcmC und CcmE interagiert und dass alle drei zusammen gemeinsam in einem ternären Komplex vorliegen. Die Topologie von CcmD wurde bestimmt. Entgegen bisherigen Annahmen wurde gezeigt, dass sowohl der N- als auch der C- Terminus von CcmD cytoplasmatisch sind.
Der Membrananker von CcmE wurde durch den Membrananker von CcmG ersetzt, um die Bedeutung dieser Domäne zu analysieren. Das resultierende chimäre CcmE Protein war weder in der Holo-CcmE-noch in der Holo-Cytochrom *c* Bildung beeinträchtigt und dies obwohl deutlich weniger davon exprimiert wurde. Im Gegensatz dazu wurde eine lösliche Version von CcmE ohne Membrananker, im Periplasma gut exprimiert, konnte aber die Cytochrom *c* Reifung nur schlecht komplementieren. Sowohl das chimäre als auch das lösliche CcmE interagierten mit CcmC, was darauf hindeutet, dass die Affinität für CcmE in der periplasmischen Domäne von CcmE liegt. In Anwesenheit von löslichem CcmE ist die Reifung von Cytochrom *c* wahrscheinlich deswegen ineffizient, weil der Häm Transfer zum Apo-Cytochrom *c* mittels CcmF kaum funktioniert.

CHAPTER 1

Synopsis

C-type cytochromes are ubiquitous heme-containing electron transfer proteins found in a wide variety of prokaryotic and eukaryotic organisms (Moore, 1990; Scott, 1996). A distinctive feature of all *c*-type cytochromes is the presence of a heme group covalently linked to the polypeptide via two (rarely, one) thioether bonds to a strictly conserved CXXCH motif (Fig.1). The heme cofactor is redox active, and thus these proteins are typically found in respiratory or photosynthetic electron transport chains. Cytochromes *c* were among the first proteins that were used extensively in the biophysical studies of protein folding (Myer, 1968; Ulmer and Kagi, 1968) and since then have been a popular subject for study in general areas of redox reactions and protein chemistry. Despite decades of work on these proteins the general interest in these proteins has not diminished, and only quite recently another interesting aspect of these proteins has come to light: the biogenesis of cytochromes *c*. The process involves posttranslational events that lead to the covalent attachment of heme to the apo-protein. This includes the targeting of the pre-apo-protein and the heme cofactor to the correct subcellular compartment, the processing of the targeting sequence and the formation of thioether bonds between the heme vinyl groups and the cysteines of the highly conserved CXXCH heme binding motif of the apo-cytochrome *c* (Thony-Meyer, 2002). In nature three distinct systems for the biogenesis of the *c*-type cytochromes have evolved (Kranz *et al.*, 1998; Page *et al.*, 1998; Thony-Meyer, 1997; Xie and Merchant, 1998). System I is present in α-, γ- and some β-proteobacteria, *Deinococcus*, and plant and protozoal mitochondria. System II is found in most β-, δ-, and ε-proteobacteria, cytophagales, aquaficales and plant and algal chloroplasts. System III operates in fungal, vertebrate and invertebrate mitochondria (Fig. 2-4). The evidence for these three maturation pathways is based on genetic and biochemical work from microbial models such as *Escherichia coli* (Thony-Meyer *et al.*, 1995),

Rhodobacter capsulatus (Beckman *et al.*, 1992), *Paracoccus denitrificans* (Page and Ferguson, 1995), and *Bradyrhizobium japonicum* (Ramseier *et al.*, 1991) of system I, *Chlamydomonas reinhardtii* (Xie and Merchant, 1996), *Bacillus subtilis* (Le Brun *et al.*, 2000; Schiott *et al.*, 1997; Schiott *et al.*, 1997), and *Bordetella pertussis* (Beckett *et al.*, 2000) of system II and *Saccharomyces cerevisiae* (Dumont *et al.*, 1987) and *Neurospora crassa* (Drygas *et al.*, 1989) of system III. In archaea, fewer system I components are found with *ccmC* and *ccmF* always present while *ccmE* is never found. Despite a common underlying goal these three systems are very distinct. They employ different sets of protein components, many of which are genetically different. A few but not all of the participating protein components share some common features. One of the striking features among the proteins involved in heme delivery is the presence of a tryptophan-rich motif and the conserved histidines in CcmC and CcmF (system I) and CcsA/ResC (system II) (Table 1).

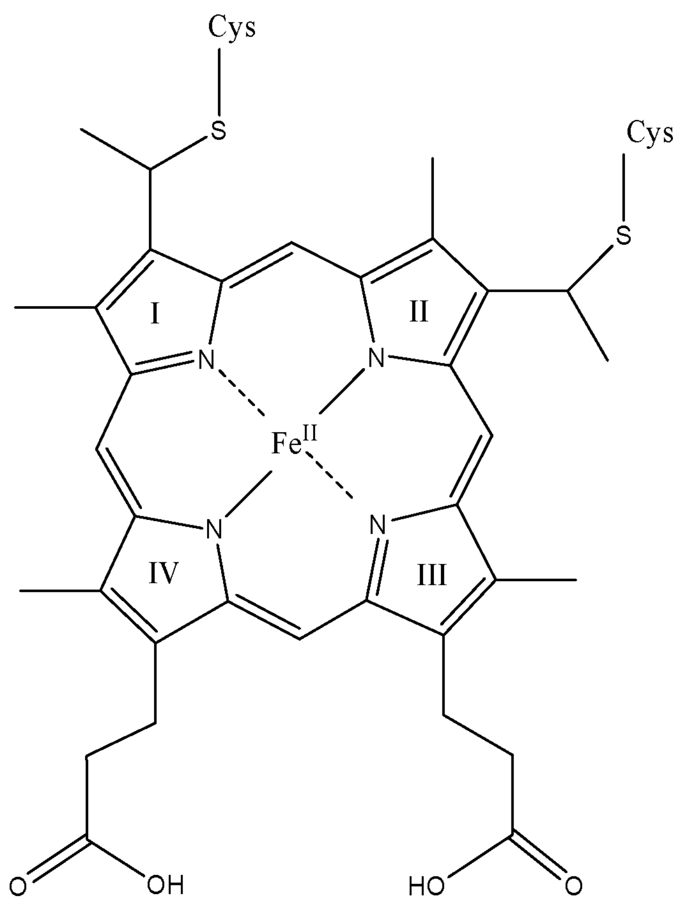

Fig. 1 Heme-peptide linkage. The Cysteine (Cys) residues in the heme-binding motif of apo-cytochrome *c* are linked to the vinyl side chains of the tetrapyrrole via thioether bonds.

Table 1: Proteins involved in different cytochrome *c* maturation pathways with their characteristic features (modified from Thöny-Meyer, 2000).

System (representative)	Protein	Characteristic sequence motifs
System I (*E. coli*)	DsbA, DsbB	CXXC
	DsbD/DipZ	CXXC and S/TPC
	CcmA	Walker-A and -B motifs
	CcmB	FXXDXXDGSL
	CcmC	WGXXWXWDXRLTS
	CcmD	
	CcmE	LPDLFR and LAKHDE
	CcmF	WGGXWFWDPVEN
	CcmG	WCXXC
	CcmH	LRCXXC
	CcmI/CycH[1]	
System II (*Synechocystis/Bacillus*)	CcsA/ResC	WXXXWXWDPKET
	CcsB/ResB	Y/FXS/TXW/YF/Y-X_{13} -C
	CcsC/CcdA	G-X_5-S/TPC-X_5-P and G-X_7-S/TPC
	ResA	CXXC
	BdbD	CXXC
	BdbC	CXXC
System III (*Saccharomyces cerevisiae*)	Cytochrome *c* heme lyase (CCHL)	CPV
	Cytochrome c_1 heme lyase (CC_1HL)	CPV
	Cyc2p	FAD binding domain

[1] In *E. coli* CcmI is the C-terminal half of CcmH, whereas in other species it is a separate protein that is known as CycH.

The process of cytochrome *c* maturation in system I

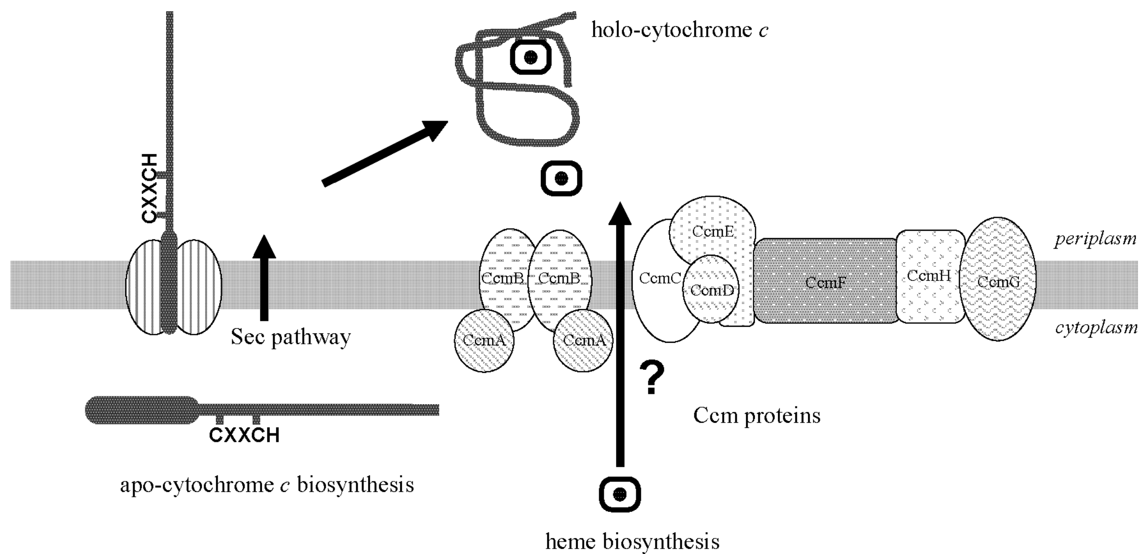

Fig. 2 Schematic representation of cytochrome *c* maturation system I. The system is found in α- γ- and some β-proteobacteria, *Deinococcus*, plant and protozoal mitochondria. In bacteria, the maturation process occurs in the periplasm, while it occurs in the intermembrane space of plant and protozoal mitochondria. The pre-apo-cytochrome *c* and the heme are synthesized in the cytoplasm and transported across the membrane (as shown by the arrows). In the periplasm, the heme is covalently attached to the apo-cytochrome *c* in the presence of Ccm proteins.

The process of cytochrome *c* maturation in system II

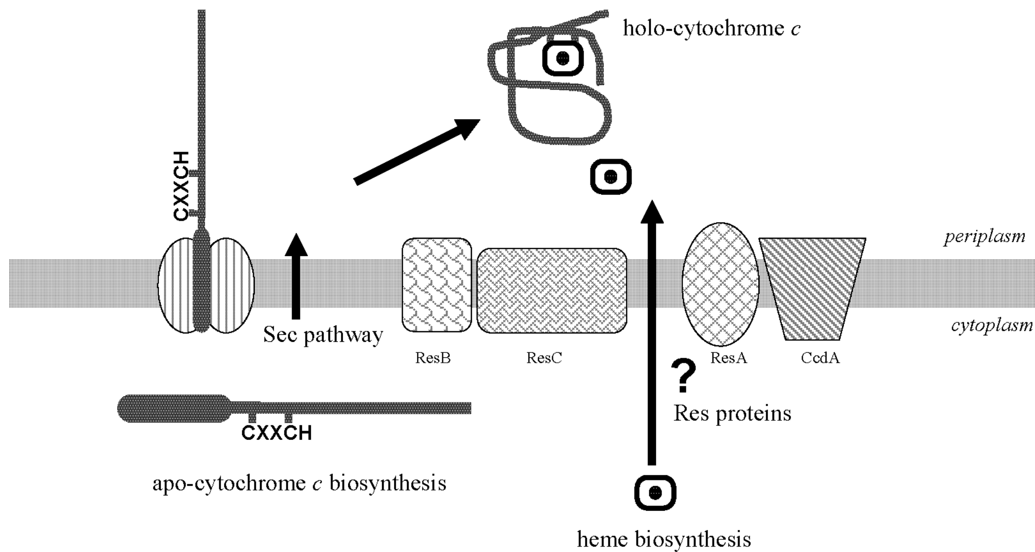

Fig. 3 Schematic representation of cytochrome *c* maturation system II. The system is found in δ-, ε- and most β-proteobacteria, and in plant and algal chloroplasts. In bacteria (as shown above) the maturation process occurs in the periplasm, while it occurs in the lumen of plant and algal chloroplasts. The pre-apo-cytochrome *c* and the heme are synthesized in the cytoplasm and transported across the membrane (as shown by arrows). In the periplasm, the heme is covalently attached to the apo-cytochrome *c* in the presence of the Res proteins and CcdA.

The process of cytochrome *c* maturation in system III

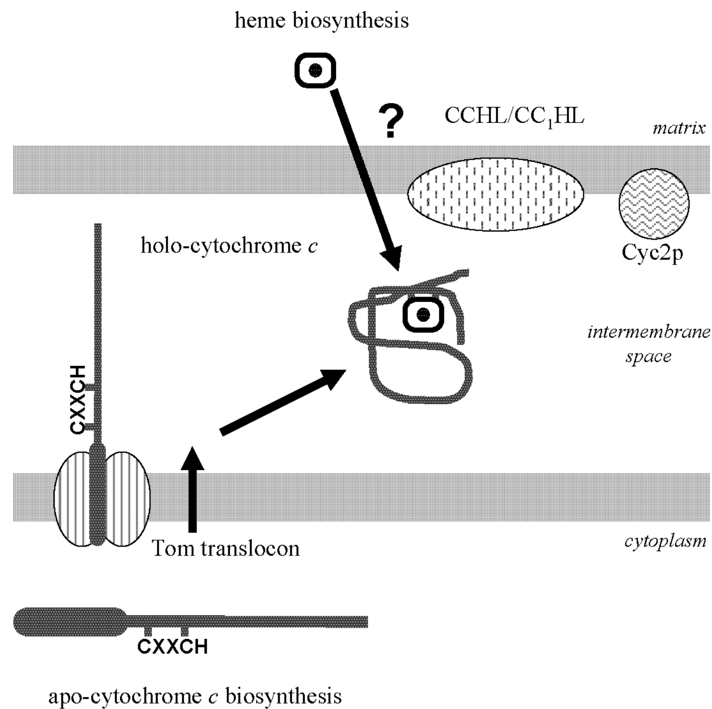

Fig. 4 Schematic representation of cytochrome *c* maturation system III. The system is found in fungal and metazoal mitochondria; heme ligation takes place in the intermembrane space of mitochondria involving the protein cytochrome *c* heme lyase (CCHL) or cytochrome c_1 heme lyase (CC_1HL).

Cytochrome *c* maturation pathway of *Escherichia coli*

In the Gram-negative bacterium *Escherichia coli*, eight genes (*ccmABCDEFGH*; *ccm* for cytochrome c maturation) have been identified, which are essential for the maturation of *c*-type cytochromes (Grove *et al.*, 1996; Thony-Meyer *et al.*, 1995). Their gene products comprise an ABC-transporter for a so far unknown substrate (CcmA, CcmB), heme transfer proteins required for covalent linkage of heme to CcmE (CcmC and CcmD), a heme chaperone (CcmE), a putative heme lyase (CcmF), and the subunits of a periplasmic, thioreduction pathway (CcmG, CcmH) (Table I). A schematic representation of the maturation pathway in *E. coli* is presented in Fig. 2. According to current knowledge the CcmC protein is known to be essential and sufficient for transfer and covalent attachment of heme to the heme chaperone, CcmE (Schulz *et al.*, 1999). The heme transfer to apo-CcmE is stimulated in the presence of a small membrane protein, CcmD (Schulz *et al.*, 2000). CcmE binds heme transiently and delivers it to apo-cytochrome *c* in the presence of the CcmF protein (Ren *et al.*, 2002). CcmF together with the CcmH protein is believed to form a heme lyase complex (Ren *et al.*, 2002), where CcmH is proposed to reduce the internal cysteine disulfides in the heme binding motif of the apo-cytochrome *c* prior to heme ligation (Fabianek *et al.*, 1999). CcmG is a thioredoxin-like protein that is believed to reduce the cysteines of CcmH and thus generate a pool of reduced CcmH protein during the maturation pathway (Fabianek *et al.*, 1998).

In this work emphasis is placed upon the heme delivery pathway and the proteins (directly and/or closely) associated with it. In particular the proteins CcmC, CcmD and CcmE have been examined in more detail, and form a central theme of my work presented in chapters 1-3,

respectively. A comprehensive discussion on the heme delivery pathway is presented; based on previous and current knowledge a new model has been proposed.

How is heme transported across the membrane?

Heme is synthesized in the cytoplasm of *E. coli*, and the process of heme synthesis has been extensively reviewed elsewhere (O'Brian and Thony-Meyer, 2002). Free heme is an amphiphilic molecule which tends to stack at physiological pH and readily associates with the membranes. It is toxic to cells due to its innate peroxidase activity (Van den Berg *et al.*, 1988). Heme is therefore always found associated with a carrier protein. The last step in the heme biosynthesis pathway is the insertion of iron (II) into protoporphyrin IX, which is catalysed by ferrochelatase (Beale, 1996). Once the heme molecule is synthesized it must either (i) passively diffuse through the membrane to be available for cytochrome *c* maturation or (ii) use an energy-driven transporter, much like the Sec translocon used for the transport of apo-cytochrome *c*, to be translocated across the membrane or (iii) an energy-independent transporter. The former possibility is difficult to envisage, as free heme is rarely available and is toxic to the cells. Among the Ccm proteins expressed from the *ccm* operon, CcmA and CcmB, which have sequence and motif similarities to known ABC-transporters, have been proposed to be a likely candidate for the heme translocation (Goldman *et al.*, 1997; Goldman *et al.*, 1998; Thony-Meyer, 1997). However, alternative evidence has accumulated suggesting that CcmAB do not constitute a heme transporter. A *ccmA* deletion strain was deficient in cytochrome *c* maturation but was competent in maturation of *b*-type cytochromes
in the periplasm of *E. coli* (Goldman *et al.*, 1996; Throne-Holst *et al.*, 1997). Lack of heme uptake in everted membrane vesicles of a wild-type and a *ccmA* deletion strain in an *in vitro*

experiment further indicated that the CcmA and CcmB proteins do not function as a heme transporter (Cook and Poole, 2000). Interestingly, CcmA and CcmB have been shown to influence holo-CcmE formation; holo-CcmE does not form in their absence. Overexpression of CcmC from a plasmid could restore holo-CcmE formation in *ccmA* and *ccmB* mutant strains, but not holo-cytochrome *c* maturation (Schulz *et al.*, 1999). These results indicated that CcmAB probably participate in cytochrome *c* maturation by transporting a molecule other than heme, which is involved in improving heme transfer to apo-CcmE and subsequent cytochrome *c* maturation. Therefore, the molecular mechanism of heme transport remains unknown and in addition stimulates the question about another substrate required for cytochrome *c* maturation, transported by the CcmAB constituted ABC-transporter.

Role of CcmC in heme delivery

One of the early and essential steps in the maturation pathway is the transfer and covalent attachment of heme to the cytochrome *c* maturation-specific heme chaperone, CcmE (Schulz *et al.*, 1998). Subsequently, heme is transferred from the holo-CcmE intermediate to apo-cytochrome *c*. It has been shown that covalent binding of heme to apo-CcmE requires the activity of the CcmC maturation factor (Schulz *et al.*, 1999). An *E. coli ccmC* in-frame deletion mutant is deficient in the production of *c*-type cytochromes (Throne-Holst *et al.*, 1997), most likely because heme cannot be transferred to CcmE (Schulz *et al.*, 1999).

E. coli CcmC is an integral membrane protein of which the predicted topology was derived from the known topologies of its homologues in *Rhodobacter capsulatus* and *Pseudomonas fluorescens* (Gaballa *et al.*, 1998; Goldman *et al.*, 1998). *E. coli* CcmC shares 41% identity and 58% similarity with its *R. capsulatus* homologue HelC and 52% identity and 70%

similarity with its *P. fluorescens* homologue CcmC. According to the experimentally established membrane topology of these proteins, CcmC contains six transmembrane helices (Fig. 1 chapter 1), separated by two cytoplasmic and three periplasmic helices (Ren and Thony-Meyer, 2001). CcmC contains a conserved tryptophan-rich signature motif (WGXφWXWDXRLT, where φ is an aromatic amino acid residue) (Kranz *et al.*, 1998; Thony-Meyer *et al.*, 1994; Thony-Meyer, 1997; Xie and Merchant, 1996) that resides in the second periplasmic domain and shares a high degree of similarity with the tryptophan-rich motif of CcmF, NrfE and the ResC/CcsA homologues of the type II cytochrome *c* maturation system (Thony-Meyer, 2000). In addition, two absolutely conserved histidines are present in the first and the third periplasmic domains of CcmC. Mutational analysis of these motifs of the *E. coli* CcmC protein revealed that they are functionally important (Schulz *et al.*, 2000). The presence of hydrophobic residues within the tryptophan-rich motif was initially thought to provide a platform for the binding of heme with the two conserved histidines serving as axial heme ligands (Goldman *et al.*, 1998). It has been shown that CcmC can bind heme and that the tryptophan-rich motif is involved in direct protein-protein interaction with CcmE (Ren and Thony-Meyer, 2001). In short, one can easily distinguish that CcmC plays a central role in heme delivery to apo-CcmE. The mechanism of heme delivery to apo-CcmE via CcmC can be divided into two sub-steps. First, CcmC recruits/binds heme and in the second step transfers heme to CcmE. In either step the molecular mechanism of these processes is far from clear. There are several open questions that need to be answered in order to delineate the exact role of CcmC. First, the nature of the heme-CcmC interaction needs to be described. Is the “heme binding” that was observed by affinity of CcmC to hemin agarose meaningful? How is heme recruited by CcmC and translocated/flipped across the membrane bilayer? Is

heme delivered to CcmC in the periplasm? Are there any accessory proteins or factors assisting CcmC? Which kinds of residues are involved in heme binding? In the second stage, it would be imperative to address the other aspect of CcmC function i.e. heme transfer and covalent ligation of heme to apo-CcmE. Again, several questions arise, such as: how is heme presented to CcmE? What can be learned and interpreted from the genetic and biochemical characterization of existing mutants? Are there any accessory proteins involved in the process? Apart from the amino acids in the tryptophan-rich motif, CcmC contains several other highly conserved residues, which have never been studied for their possible contribution to the heme binding and/or delivery during cytochrome *c* maturation. In a first part of this work, a genetic screen was designed and tested in a Δ*ccmC* mutant strain background. It was based on the anaerobic respiratory growth of *E. coli* in the presence of TMAO as a terminal electron acceptor and allowed the discrimination of the cytochrome *c* maturation competent versus deficient cells based on the colony size and color. Several mutants were obtained, but unfortunately, none were affected in heme binding and/or delivery to CcmE. Interestingly, the mutants obtained were affected in cytochrome *c* formation without disruption in the formation of holo-CcmE. This was a novel observation and implicated CcmC to be involved in a process which influences the transfer of heme from holo-CcmE to the apo-cytochrome *c*. This possibility was further tested and supported by two different sets of experiments: (i) a functional CcmCE fusion polypeptide was shown to have a similar phenotype; (ii) CcmC did not co-precipitate with CcmF neither in the presence nor in the absence of wild-type CcmE. This contradicted the idea of the formation of a CcmCEF super complex and led to propose a model that favored the shuttling of CcmE between two membrane complexes: on the one hand that containing CcmC, CcmE and most probably CcmD and on the other hand of CcmF,

CcmH and CcmE. In retrospect, the main purpose of this work, i.e. the elucidation of the mechanism by which CcmC binds and delivers heme, still remains unknown. In a fresh approach one could try to find out the fate of the heme molecule once it leaves ferrochelatase. A co-immunoprecipiation experiment with ferrochelatse and CcmC would be the most logical test to begin such a study.

A dual function for CcmC?

Several *ccmC* mutants from different organisms have been described, which were not only affected in cytochrome *c* maturation, but also exhibited other phenotypes. Particularly interesting was the analysis of *P. fluorescens ccmC* mutants, which showed that CcmC is required for the production of pyoverdines, which are fluorescent siderophores (Gaballa *et al.*, 1996; Gaballa *et al.*, 1998). In *Paracoccus denitrificans*, a *ccmC* mutant, in addition to deficiencies in cytochrome *c* maturation and siderophore production, showed intolerance to rich medium (Page and Ferguson, 1999). The connection between these processes is not known. In the pathogenic bacterium *Legionella pneumophila*, CcmC was found to be required for cytochrome *c* production, growth in low-iron conditions, and at least for some forms of intracellular infection of eukaryotic hosts (Viswanathan *et al.*, 2002). Taken together it is unclear whether these unique phenotypes are directly associated with CcmC function or are consequences of holo-cytochrome *c* deficiency. In the former case it would then mean that CcmC has dual functional roles, thus adding another layer of complexity and being yet more fascinating to study.

Role of CcmD in heme delivery

It has been shown previously that covalent binding of heme to an essential histidine (H130) of apo-CcmE requires the activity of the CcmC maturation protein. This activity as well as the level of the CcmE polypeptide in the membranes was significantly enhanced in the presence of the CcmD protein (Schulz *et al.*, 1999). CcmD is essential for cytochrome *c* maturation, but a *ccmD* mutant phenotype can be bypassed by overexpression of the CcmCE proteins (Schulz *et al.*, 1999). CcmD is a small polypeptide of 69 amino acid residues with three domains: (i) an N-terminal domain consisting of residues 1-13 with partly polar side chains, (ii) a hydrophobic membrane anchor comprising residues 14-37 of an entirely hydrophobic nature, and (iii) a C-terminal, soluble domain that is rich in charged residues with predominantly positive charges. This domain structure and charge distribution is very well conserved in CcmD homologues of other bacteria, whereas individual amino acid residues are not. The *ccmD* gene is found downstream of *ccmC* in most bacteria that use system I for cytochrome *c* maturation. Due to low sequence homology it is difficult to otherwise locate *ccmD*, if not linked to the other *ccm* genes. The membrane topology of CcmD has been assumed to be like that of its *Rhodobacter capsulatus* homologue, HelD. In this case the C-terminus was proposed to reside in the cytoplasm based on the activity of a LacZ fusion and on the inactivity of a corresponding PhoA fusion (Goldman *et al.*, 1997). In addition, the absence of positive charges N-terminal of the predicted transmembrane helix is in agreement with an N-terminus-out and C-terminus-in topology.

In this work, by co-immunoprecipitation and co-purification experiments it could be shown that CcmD physically interacted with CcmC while interaction with CcmE was only detectable by co-immunoprecipitation. This discrepancy could be suggestive of the nature of interaction

between the two proteins. Additionally, the presence of CcmD in a co-purification experiment was shown to support a ternary complex consisting of CcmCDE. A deletion analysis of individual domains revealed that the central hydrophobic domain of CcmD is essential for its function. Moreover, the C-terminal, cytoplasmic domain seems to require a net positive charge in order to be functional. A double arginine (R44AR48A) mutant of CcmD induced enhanced assembly of both apo- and holo-CcmE in the membranes while severely affecting cytochrome *c* formation indicating the importance of these charges. The biochemical topology analysis, in a proteinase K protection assay in spheroplasts, indicated that CcmD is an integral interfacial membrane protein with its N- and C-termini extruding into the cytoplasm. Interactions of CcmD with either ferrochelatase, the last heme biosynthetic enzyme, or directly with heme were not detectable. From a pulse chase experiment the likelihood of CcmD influencing the kinetics of heme attachment to apo-CcmE could be excluded. Thus in the light of the existing biochemical characterization the function for CcmD can be to modulate the protein-protein interaction between CcmC and CcmE by assisting CcmE to properly attach and/or detach heme upon transfer via CcmC. It is also possible that CcmD exerts some kind of regulatory role by controlling the heme uptake. One idea that can be possibly envisaged is the use of the net positive charge in regulating various protein-protein contacts during heme delivery. The N-terminus of CcmE contains a net positive charge that could be easily repulsed and subsequently detached from the CcmCE complex upon addition of CcmD. It is thus difficult at the moment to assign a specific function to CcmD but it is definitive that the future work on CcmD would be focussed on the positively charged residues of the C-terminus. Since we have all the desired mutants of *ccmD* it would be possible in the

first instance to test this hypothesis through the test of co-immunoprecipitation and co-purification.

Role of CcmE in heme delivery

CcmE plays a pivotal role during cytochrome *c* maturation. It has been designated a “heme chaperone” since it binds heme at a strictly conserved histidine (H130) transiently, yet covalently, and subsequently delivers it to the CXXCH motif of apo-cytochrome *c* (Schulz *et al.*, 1998). Over the years a lot of interest in this unusual heme chaperone has been generated, thus leading to an extensive genetic and biochemical characterization *in vivo* and *in vitro* (Daltrop *et al.*, 2002; Enggist *et al.*, 2003; Enggist and Thony-Meyer, 2003; Ren and Thony-Meyer, 2001; Schulz *et al.*, 1999). *E. coli* CcmE is a 159 amino acid protein with an N-terminal membrane anchor (1-29 residues) and a soluble periplasmically oriented domain (30-159 residues). The solution structure of the soluble domain of the *E. coli* and the *Shewanella putrefaciens* apo-CcmE proteins have been solved by NMR (Arnesano *et al.*, 2002; Enggist *et al.*, 2002). The structure of the soluble apo-protein revealed that the protein consists of two domains that are flexibly oriented relative to each other. The bulk of the soluble apo-protein forms a rigid core consisting of a six-stranded β-barrel (residues I34-K129) which is capped by a short α-helix. The rigid core is followed by a structurally less well-defined C-terminal domain (D131-S159) with a short single helical turn in the tail of the peptide. The strictly conserved H130 sits at the interface of the two domains. A putative heme-binding site at the surface of the β-barrel, mainly containing hydrophobic resides in the vicinity of H130, has been proposed, while the flexible C-terminal domain has been implicated either in shielding

heme or being involved in protein-protein interactions with other proteins (Enggist *et al.*, 2002; Enggist and Thony-Meyer, 2003). Soluble CcmE lacking the N-terminal membrane spanning domain was shown to poorly complement cytochrome *c* maturation, which suggests that the membrane anchor contributes to the function of CcmE. The contribution of the CcmE membrane anchor could be envisaged either in imparting specificity to the protein, as CcmE is known to interact with CcmC, CcmD and CcmF, or it could simply allow the soluble active domain to assemble correctly on the membrane bi-layer and thus be structurally important. It may still be possible that the membrane anchor fulfils both of these functions. To test this, a chimerical version of CcmE (CcmG'-'E) was constructed where the membrane anchor of CcmE was replaced with that of CcmG. CcmG, like CcmE, is a monotopic membrane protein with its C-terminal active domain extruding into the periplasmic space (Fabianek *et al.*, 1998) and thus made a good candidate for the construction of the chimerical version. The chimerical version displayed parity with wild-type CcmE in an interaction study with CcmC although it was severely affected in heme uptake, which also resulted in poor complementation for cytochrome *c* maturation. The soluble CcmE also interacted with CcmC like its wild-type counterpart, but displayed differential heme uptake with low efficiency in the minimal situation when only CcmC was co-expressed, and with high efficiency when co-expressed with CcmABCD or the entire set of Ccm proteins. No matter how efficient the formation of soluble holo-CcmE was, poor complementation of cytochrome *c* maturation was observed. Two things can thus be concluded: (i) the membrane anchor of CcmE is important, especially in heme transfer to apo-cytochrome *c* and (ii) CcmAB and CcmD are important for heme transfer to apo-CcmE. Another interesting finding of this work is the interaction of chimerical CcmE with CcmC, although the interaction point can be exclusively mapped to the

periplasmic domain; the participation of the membrane anchor acting in synergy with the periplasmic domain can not be completely ruled out. Also, it remains to be seen whether or not CcmG can also interact with CcmC or other known partners of CcmE. This will be crucial as CcmG has been shown to not interact with its more likely partner CcmH (unpublished personal observation and Anna Rozhkova, personal communication).

Role of CcmF in heme delivery

CcmF is required for a step after heme binding to CcmE, i.e., for the transfer of heme from CcmE to apocytochrome *c*. CcmF has been proposed to be the bacterial cytochrome *c* heme lyase; however, no biochemical evidence confirming this role has yet been reported. Like CcmC, CcmF also possesses a tryptophan-rich motif and has been shown to be an essential cytochrome *c* maturation factor in *E. coli* (Grove *et al.*, 1996; Schulz *et al.*, 1998; Throne-Holst *et al.*, 1997). This protein, by analogy to its homologue from *Rhodobacter capsulatus* (Goldman *et al.*, 1998), has 11 trans-membrane helices, and its tryptophan-rich motif resides in the third periplasmic domain. Besides the tryptophan-rich motif, four histidines have been described to be conserved in presumably periplasmic domains (H92, H173, H261 and H303) (Goldman *et al.*, 1998). A fifth, most probably cytoplasmic, invariant histidine, H491 was noticed. Since, in CcmC, two invariant histidines have been proven to be important for the function of the protein, it was assumed that conserved histidines of CcmF could also play an essential role for ligation of heme to apo-cytochrome *c*. If CcmF is a heme lyase of system I cytochrome *c* maturation, it is expected to release heme from the heme chaperone CcmE and to transfer it to apo-cytochrome *c*. This implies that CcmE and CcmF must interact directly. In addition, CcmF is also expected to interact with either apo-cytochrome *c*, or with a factor

that binds and recruits apo-cytochrome *c*. The former type of interaction would be transient, whereas the latter might be steady. A candidate for such a factor is CcmH, for various reasons (Fabianek, 1999). In the co-immunoprecipitation experiments it could be shown that CcmF directly interacted with CcmE and CcmH but not with apo-cytochrome *c* (Ren *et al.*, 2002). This fitted very well with the hypothesis that predicted CcmF to catalyze heme detachment from holo-CcmE and subsequently to catalyze the heme attachment to the reduced cysteine thiols of the apo-cytochrome *c* with the help of the CcmH. Additionally, it was demonstrated that the conserved histidines H173, H261, H303 and H491 and the tryptophan-rich signature motif of the CcmF protein family were required for the function.

Current working model for the heme delivery process during cytochrome *c* maturation

The new working model has been adapted to fit the results obtained from my work detailed in chapters 1-3. In the new model CcmC and CcmD form a CcmE-specific docking complex where CcmC delivers and covalently attaches heme onto apo-CcmE. CcmD either stabilizes the CcmC-CcmE contact or allows holo-CcmE to be released from the complex upon heme attachment or can perform both. The role of the substrate exported by the CcmAB constituted ABC-transporter, in a CcmC-mediated heme attachment to apo-CcmE, is elusive as the molecular mechanism of heme attachment is not known. Once apo-CcmE is charged with heme it is then ready to associate with another sub-complex comprising CcmF and CcmH which is named the heme lyase complex. CcmF and most probably CcmH are required for detachment of heme from holo-CcmE and stereospecific insertion and covalent ligation of heme into the apo-cytochrome *c*. The schematic representation of the model is presented in Figure 5.

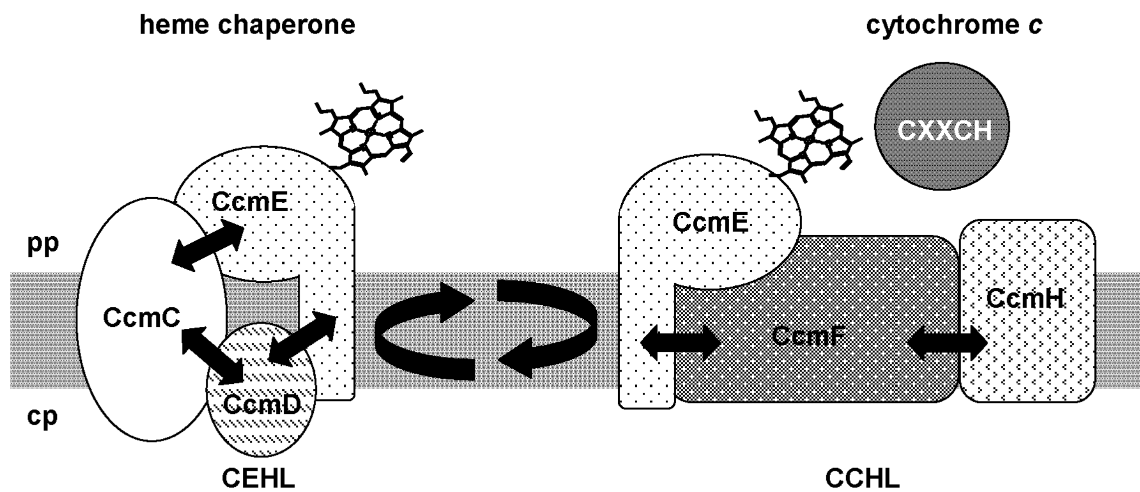

Fig. 5 Current view of heme delivery during cytochrome *c* maturation. Heme delivery occurs via CcmCD to CcmE and further via CcmF/CcmH to apo-cytochrome *c*. Direct protein-protein interactions shown by co-immunoprecipitation are indicated by *black arrows*. CEHL, CcmE heme lyase; CCHL, cytochrome *c* heme lyase; pp, periplasm; cp, cytoplasm.

Outlook

Heme delivery during cytochrome *c* maturation in *E. coli* has in recent years become a topic of great interest and scrutiny (Ahuja and Thony-Meyer, 2003, 2005; Arnesano *et al.*, 2002; Daltrop *et al.*, 2002; Daltrop *et al.*, 2002; Enggist *et al.*, 2002; Ren and Thony-Meyer, 2001). The process of heme delivery can be sub-divided into two phases, in the first phase heme is recruited and covalently attached to the heme chaperone, CcmE and in the second phase it is then transferred to apo-cytochrome *c*. In both phases of the heme delivery a different set of Ccm proteins are involved, while CcmE seems to be the only known common intermediate. Several key questions were addressed in this work. Most notable were the role of CcmC in heme uptake and transfer to CcmE, involvement of CcmD and the membrane anchor of CcmE in heme delivery.

A genetic screen developed here to obtain *ccmC* mutants affected in either heme uptake or heme transfer was largely unsuccessful. A fresh attempt to obtain more mutants needs to be undertaken since the screen was not complete. A similar genetic approach could also be extended to other *ccm* genes. In some cases (*ccmD*, *ccmE*, *ccmH*) it has already been applied and shown to be functional. A genetic screen in a *ccmE* mutant background might in part answer a vital question of heme transfer and covalent attachment which was addressed here in the context of CcmC. A biochemical or biophysical assay will have to be developed to delineate the nature of heme binding to CcmC. From this work, various lines of evidence have suggested the shuttling nature of CcmE between CcmC and CcmF constituted sub complexes. Further work, combining genetic and biochemical approaches should be undertaken to further substantiate or refute this hypothesis.

Recently, in an *in vitro* test, it has been shown that holo-CcmE can be matured at very low levels in the absence of CcmC (Daltrop *et al.*, 2002). It is yet to be tested if the presence of

CcmC and perhaps CcmD in a similar assay can stimulate holo-CcmE formation. Characterization of CcmD in the present work has confirmed its stimulatory effect on heme transfer to CcmE as previously observed (Schulz *et al.*, 1999). The functional domain of CcmD was mapped to its C-terminal part with a net positive charge on it. The nature of the function of the positively charged residues and whether or not they are involved in any modulation during heme transfer by altering different protein-protein contacts remains to be examined. One idea to test this hypothesis could be by performing a co-purification of wild-type CcmE with mutant CcmD protein where the positively charged residues (of CcmD) in the C-terminal region namely, the arginines, are changed to negatively charged residues such as glutamic acid or aspartic acid. In this situation the electrostatic repulsion would cease and would allow CcmE to be pulled down with CcmD. In a reverse approach, positively charged residues in the N-terminus of CcmE can be mutated to negatively charged residues, and the protein can then be tested for co-purification with wild-type CcmD. Another interesting aspect of CcmD characterization was its topology which in a way has also strengthened the importance of CcmD in regulating heme delivery process. An ongoing collaboration with Dr. Maria Johansson, from the Swedish NMR center at Göteborg University, to solve the solution structure would yield more insights into the structure and function of CcmD in the future. The presence and/or formation of membrane complexes cooperating during cytochrome *c* maturation process was predicted and in part demonstrated in this work. There are still some unanswered questions related to it, for example, CcmCDE have been shown to form a ternary complex. What is the stoichiometry of these proteins within the complex? The demonstration of co-purification of CcmC with CcmB (Christensen, O. unpublished work) raises another question: whether or not the existing ternary complex could be extended to include CcmAB?

So far in the co-purification experiments only two or at the most three proteins were tested. The idea of the presence of a larger complex can only be tested if more Ccm proteins are included. Techniques such as Blue-native PAGE or use of cross-linkers could be quite useful in this direction.

A consequence of the domain swapping work with CcmE to investigate the role of its membrane spanning domain is studing CcmG in new light of results presented here (Chapter III). Any outcome indicative of a role other than redox control would then change the existing idea of cytochrome *c* maturation and would direct the future course of work in this field.

Interaction studies of *E. coli* apo-cytochrome *c* with any of the Ccm proteins in the past, and also undertaken in this work, have been unsuccessful, which may be attributed in part to the transient nature of the interaction and to the instability of apo-cytochrome *c* in the absence of the complete maturation machinery. Recently, it has been shown that heme can be efficiently attached to a CXXCH heme binding motif which was tagged to maltose binding protein (MBP) in the presence of the *ccmA-H* gene products (Braun *et al.*, 2005). The resultant holo-protein was identical to holo-cytochrome *c* in terms of both biophysical and biochemical features. The tagged protein was stable in the apo-form and could be easily purified using an amylose affinity column. For all these reasons mentioned, MBP-CXXCH thus makes an ideal candidate for interaction studies with the Ccm proteins.

While this discussion was being written, the nature of the heme-histidine (H130) bond of holo-CcmE came to light by NMR (Lee, *et al.*, 2005). According to the discovery heme is either attached via its 2-vinyl or 4-vinyl to the β carbon of the $N^{\delta1}$ of H130. With this proof of a covalent bond, the emphasis should now turn to assign definitive point of heme attachment to either of the two vinyls. Furthermore, the structure of holo-CcmE is still a challenge, as no

crystals of *E. coli* holo-CcmE could be obtained so far. A comprehensive approach should be followed to crystallize holo-CcmE from different organisms including extremophiles.

Other areas within the field of cytochrome *c* maturation, not discussed in this thesis, are worth studying. These include the investigation of role of CcmAB constituted ABC-transporter and its putative substrate, the redox control pathway involving any of CcmH, CcmG, DsbA and DsbD and determination of structures of the Ccm proteins.

In summary, the study of cytochrome *c* maturation in *E. coli* is still at its infancy with some progress has been made through my work. There are certainly new and exciting discoveries to be made and new insights to be gained.

References

Ahuja, U., and Thony-Meyer, L. (2003) Dynamic features of a heme delivery system for cytochrome *c* maturation. *J Biol Chem* **278**: 52061-52070.

Ahuja, U., and Thony-Meyer, L. (2005) CcmD Is Involved in Complex Formation between CcmC and the Heme Chaperone CcmE during Cytochrome *c* Maturation. *J Biol Chem* **280**: 236-243.

Arnesano, F., Banci, L., Barker, P.D., Bertini, I., Rosato, A., Su, X.C., and Viezzoli, M.S. (2002) Solution structure and characterization of the heme chaperone CcmE. *Biochemistry* **41**: 13587-13594.

Beale, S.I. (1996) Biosynthesis of hemes in *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology (Neidharth, F.C., Curtiss, R., III, Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M., and Umbarger, H. C., eds) pp. 731-748, American Society for Microbiology Press, Washington, D. C.

Beckett, C.S., Loughman, J.A., Karberg, K.A., Donato, G.M., Goldman, W.E., and Kranz, R.G. (2000) Four genes are required for the system II cytochrome *c* biogenesis pathway in *Bordetella pertussis*, a unique bacterial model. *Mol Microbiol* **38**: 465-481.

Beckman, D.L., Trawick, D.R., and Kranz, R.G. (1992) Bacterial cytochromes *c* biogenesis. *Genes Dev* **6**: 268-283.

Braun, M., Rubio, I.G., and Thony-Meyer, L. (2005) A heme tag for in vivo synthesis of artificial cytochromes. *Appl Microbiol Biotechnol* **67**: 234-239.

Cook, G.M., and Poole, R.K. (2000) Oxidase and periplasmic cytochrome assembly in *Escherichia coli* K-12: CydDC and CcmAB are not required for haem-membrane association. *Microbiology* **146 (Pt 2)**: 527-536.

Daltrop, O., Allen, J.W., Willis, A.C., and Ferguson, S.J. (2002) In vitro formation of a *c*-type cytochrome. *Proc Natl Acad Sci U S A* **99**: 7872-7876.

Daltrop, O., Stevens, J.M., Higham, C.W., and Ferguson, S.J. (2002) The CcmE protein of the *c*-type cytochrome biogenesis system: unusual in vitro heme incorporation into apo-CcmE and transfer from holo-CcmE to apocytochrome. *Proc Natl Acad Sci U S A* **99**: 9703-9708.

Drygas, M.E., Lambowitz, A.M., and Nargang, F.E. (1989) Cloning and analysis of the *Neurospora crassa* gene for cytochrome *c* heme lyase. *J Biol Chem* **264**: 17897-17906.

Dumont, M.E., Ernst, J.F., Hampsey, D.M., and Sherman, F. (1987) Identification and sequence of the gene encoding cytochrome *c* heme lyase in the yeast *Saccharomyces cerevisiae*. *Embo J* **6**: 235-241.

Enggist, E., Thony-Meyer, L., Guntert, P., and Pervushin, K. (2002) NMR structure of the heme chaperone CcmE reveals a novel functional motif. *Structure (Camb)* **10**: 1551-1557.

Enggist, E., Schneider, M.J., Schulz, H., and Thony-Meyer, L. (2003) Biochemical and mutational characterization of the heme chaperone CcmE reveals a heme binding site. *J Bacteriol* **185**: 175-183.

Enggist, E., and Thony-Meyer, L. (2003) The C-terminal flexible domain of the heme chaperone CcmE is important but not essential for its function. *J Bacteriol* **185**: 3821-3827.

Fabianek, R.A., Hennecke, H., and Thony-Meyer, L. (1998) The active-site cysteines of the periplasmic thioredoxin-like protein CcmG of *Escherichia coli* are important but not essential for cytochrome *c* maturation in vivo. *J Bacteriol* **180**: 1947-1950.

Fabianek, R.A. (1999) New insights into the redox pathway of bacterial cytochrome *c* maturation: CycY/CcmG and CcmH. Ph.D thesis, Eidgenössische Technische Hochschule, Zurich, Switzerland.

Fabianek, R.A., Hofer, T., and Thony-Meyer, L. (1999) Characterization of the *Escherichia coli* CcmH protein reveals new insights into the redox pathway required for cytochrome *c* maturation. *Arch Microbiol* **171**: 92-100.

Gaballa, A., Koedam, N., and Cornelis, P. (1996) A cytochrome *c* biogenesis gene involved in pyoverdine production in *Pseudomonas fluorescens* ATCC 17400. *Mol Microbiol* **21**: 777-785.

Gaballa, A., Baysse, C., Koedam, N., Muyldermans, S., and Cornelis, P. (1998) Different residues in periplasmic domains of the CcmC inner membrane protein of *Pseudomonas fluorescens* ATCC 17400 are critical for cytochrome *c* biogenesis and pyoverdine-mediated iron uptake. *Mol Microbiol* **30**: 547-555.

Goldman, B.S., Gabbert, K.K., and Kranz, R.G. (1996) Use of heme reporters for studies of cytochrome biosynthesis and heme transport. *J Bacteriol* **178**: 6338-6347.

Goldman, B.S., Beckman, D.L., Bali, A., Monika, E.M., Gabbert, K.K., and Kranz, R.G. (1997) Molecular and immunological analysis of an ABC transporter complex required for cytochrome *c* biogenesis. *J Mol Biol* **268**: 724-738.

Goldman, B.S., Beck, D.L., Monika, E.M., and Kranz, R.G. (1998) Transmembrane heme delivery systems. *Proc Natl Acad Sci U S A* **95**: 5003-5008.

Grove, J., Tanapongpipat, S., Thomas, G., Griffiths, L., Crooke, H., and Cole, J. (1996) *Escherichia coli* K-12 genes essential for the synthesis of *c*-type cytochromes and a third nitrate reductase located in the periplasm. *Mol Microbiol* **19**: 467-481.

Kranz, R., Lill, R., Goldman, B., Bonnard, G., and Merchant, S. (1998) Molecular mechanisms of cytochrome *c* biogenesis: three distinct systems. *Mol Microbiol* **29**: 383-396.

Le Brun, N.E., Bengtsson, J., and Hederstedt, L. (2000) Genes required for cytochrome *c* synthesis in *Bacillus subtilis*. *Mol Microbiol* **36**: 638-650.

Lee, D., Pervushin, K., Bischof, D., Braun, M., and Thöny-Meyer, L. (2005) Unusual heme-histidine bond in the active site of a chaperone. *J Am Chem Soc* **127**: 3716-7

Moore, G.R., pettigrew, G. W. (1990) *Cytochrome c: Evolutionary, Structural and Physiological Aspects*. New york: Springer-Verlag.

Myer, Y.P. (1968) Conformation of cytochromes: Effect of urea, temperature, extrinsic ligands, and pH variation on the conformation of horse heart ferricytochrome *c*. *Biochemistry* 7: 765-776.

O'Brian, M.R., and Thony-Meyer, L. (2002) Biochemistry, regulation and genomics of haem biosynthesis in prokaryotes. *Adv Microb Physiol* **46**: 257-318.

Page, M.D., and Ferguson, S.J. (1995) Cloning and sequence analysis of *cycH* gene from *Paracoccus denitrificans*: the *cycH* gene product is required for assembly of all *c*-type cytochromes, including cytochrome c_1. *Mol Microbiol* **15**: 307-318.

Page, M.D., Sambongi, Y., and Ferguson, S.J. (1998) Contrasting routes of *c*-type cytochrome assembly in mitochondria, chloroplasts and bacteria. *Trends Biochem Sci* **23**: 103-108.

Page, M.D., and Ferguson, S.J. (1999) Mutational analysis of the *Paracoccus denitrificans c*-type cytochrome biosynthetic genes *ccmABCDG*: disruption of *ccmC* has distinct effects suggesting a role for CcmC independent of CcmAB. *Microbiology* **145 (Pt 11)**: 3047-3057.

Ramseier, T.M., Winteler, H.V., and Hennecke, H. (1991) Discovery and sequence analysis of bacterial genes involved in the biogenesis of *c*-type cytochromes. *J Biol Chem* **266**: 7793-7803.

Ren, Q., and Thony-Meyer, L. (2001) Physical interaction of CcmC with heme and the heme chaperone CcmE during cytochrome c maturation. *J Biol Chem* **276**: 32591-32596.

Ren, Q., Ahuja, U., and Thony-Meyer, L. (2002) A bacterial cytochrome *c* heme lyase. CcmF forms a complex with the heme chaperone CcmE and CcmH but not with apocytochrome *c*. *J Biol Chem* **277**: 7657-7663.

Schiott, T., Throne-Holst, M., and Hederstedt, L. (1997) *Bacillus subtilis* CcdA-defective mutants are blocked in a late step of cytochrome *c* biogenesis. *J Bacteriol* **179**: 4523-4529.

Schiott, T., von Wachenfeldt, C., and Hederstedt, L. (1997) Identification and characterization of the *ccdA* gene, required for cytochrome *c* synthesis in *Bacillus subtilis*. *J Bacteriol* **179**: 1962-1973.

Schulz, H., Hennecke, H., and Thony-Meyer, L. (1998) Prototype of a heme chaperone essential for cytochrome *c* maturation. *Science* **281**: 1197-1200.

Schulz, H., Fabianek, R.A., Pellicioli, E.C., Hennecke, H., and Thony-Meyer, L. (1999) Heme transfer to the heme chaperone CcmE during cytochrome *c* maturation requires the CcmC protein, which may function independently of the ABC-transporter CcmAB. *Proc Natl Acad Sci U S A* **96**: 6462-6467.

Schulz, H., Pellicioli, E.C., and Thony-Meyer, L. (2000) New insights into the role of CcmC, CcmD and CcmE in the haem delivery pathway during cytochrome *c* maturation by a complete mutational analysis of the conserved tryptophan-rich motif of CcmC. *Mol Microbiol* **37**: 1379-1388.

Scott, R.A., Mauk, A. G. (1996) *Cytochrome c: A multidisciplanary Approach.* Sausalito, CA: University Science Books.

Thony-Meyer, L., Ritz, D., and Hennecke, H. (1994) Cytochrome *c* biogenesis in bacteria: a possible pathway begins to emerge. *Mol Microbiol* **12**: 1-9.

Thony-Meyer, L., Fischer, F., Kunzler, P., Ritz, D., and Hennecke, H. (1995) *Escherichia coli* genes required for cytochrome *c* maturation. *J Bacteriol* **177**: 4321-4326.

Thony-Meyer, L. (1997) Biogenesis of respiratory cytochromes in bacteria. *Microbiol Mol Biol Rev* **61**: 337-376.

Thony-Meyer, L. (2000) Haem-polypeptide interactions during cytochrome *c* maturation. *Biochim Biophys Acta* **1459**: 316-324.

Thony-Meyer, L. (2002) Cytochrome *c* maturation: a complex pathway for a simple task? *Biochem Soc Trans* **30**: 633-638.

Throne-Holst, M., Thony-Meyer, L., and Hederstedt, L. (1997) *Escherichia coli ccm* in-frame deletion mutants can produce periplasmic cytochrome *b* but not cytochrome *c*. *FEBS Lett* **410**: 351-355.

Ulmer, D.D., and Kagi, J.H. (1968) Hydrogen-deuterium exchange of cytochrome *c*: Effect of oxidation state. *Biochemistry* **7**: 2710-2717.

Van den Berg, J.J., Kuypers, F.A., Qju, J.H., Chiu, D., Lubin, B., Roelofsen, B., and Op den Kamp, J.A. (1988) The use of *cis*-parinaric acid to determine lipid peroxidation in human erythrocyte membranes. Comparison of normal and sickle erythrocyte membranes. *Biochim Biophys Acta* **944**: 29-39.

Viswanathan, V.K., Kurtz, S., Pedersen, L.L., Abu-Kwaik, Y., Krcmarik, K., Mody, S., and Cianciotto, N.P. (2002) The cytochrome *c* maturation locus of *Legionella pneumophila* promotes iron assimilation and intracellular infection and contains a strain-specific insertion sequence element. *Infect Immun* **70**: 1842-1852.

Xie, Z., and Merchant, S. (1996) The plastid-encoded *ccsA* gene is required for heme attachment to chloroplast *c*-type cytochromes. *J Biol Chem* **271**: 4632-4639.

Xie, Z., and Merchant, S. (1998) A novel pathway for cytochromes *c* biogenesis in chloroplasts. *Biochim Biophys Acta* **1365**: 309-318.

CHAPTER 2

Dynamic features of a heme delivery system for cytochrome *c* maturation

Umesh Ahuja and Linda Thöny-Meyer

Institut für Mikrobiologie, Department Biologie

Eidgenössische Technische Hochschule,

Wolfgang Pauli Strasse 10, CH-8093 Zürich, Switzerland

Journal of Biological Chemistry 278 (58): 52061-52070 (2003)

SUMMARY

In *Escherichia coli* heme is delivered to cytochrome *c* in a process involving eight proteins encoded by the *ccmABCDEFGH* operon. Heme is transferred to the periplasmic heme chaperone CcmE by CcmC, and from there to apo-cytochrome *c*. The role of CcmC was investigated by random as well as site-directed mutagenesis. Important amino acids were all located in periplasmic domains of the CcmC protein that has six membrane-spanning helices. Besides the tryptophan-rich motif and two conserved histidines new residues were identified as functionally important. Mutants G111S and H184Y had a clear defect in CcmC-CcmE interaction, did not transfer heme to CcmE and lacked *c*-type cytochromes. Conversely, mutants D47N, R55P and S176Y were affected neither in interaction with nor in delivery of heme to CcmE, but produced less than 10% *c*-type cytochromes. A strain carrying a CcmCE fusion had a similar phenotype, suggesting that CcmC is important not only for heme transfer to CcmE but also for its delivery to cytochrome *c*. Co-immunoprecipitation of CcmC with CcmF was not detectable even though CcmE co-precipitated individually with CcmC and CcmF. This contradicts the idea of CcmCEF super complex formation. Our results favor a model that predicts CcmE to shuttle between CcmC and CcmF for heme delivery.

Keywords: co-immunoprecipitation / cytochrome *c* maturation / heme chaperone / membrane proteins / mutagenesis

INTRODUCTION

The covalent attachment of heme to the CXXCH signature motif of apo-cytochromes is a critical step during cytochrome *c* biosynthesis. The process of cytochrome *c* maturation involves the formation of two thioether bonds between the vinyl groups of heme and the cysteinyl residues of apo-cytochrome *c*. Three distinct systems for the biogenesis of *c*-type cytochromes have evolved in different classes of organisms, which are annotated as system I, II and III (reviewed in (1-4)). System I is present in α- and γ-proteobacteria, *Deinococcus*, archaea and protozoal and plant mitochondria. System II prevails in gram positive bacteria, cyanobacteria, some β-, δ- and ε-proteobacteria and in plant and algal chloroplasts. System III cytochrome *c* maturation operates in fungal and metazoal mitochondria. The γ-proteobacterium *Escherichia coli* requires eight cytoplasmic membrane proteins encoded by the *ccmABCDEFGH* operon (5,6). These proteins catalyze the covalent attachment of heme to the conserved CXXCH motifs of apo-cytochromes *c*. In *E. coli*, *c*-type cytochromes are synthesized exclusively under anaerobic respiratory conditions, under which the expression of the *ccm* genes is also induced (7-9). One of the key steps in the maturation pathway is the transfer and covalent attachment of heme to the cytochrome *c* maturation-specific heme chaperone, CcmE (10); subsequently, heme is transferred from the holo-CcmE intermediate to apo-cytochrome *c*. It has been shown earlier that covalent binding of heme to an essential histidine (H130) of apo-CcmE requires the activity of the CcmC maturation factor (11). CcmC is an integral membrane protein whose topology was derived from that of its homologues in *Rhodobacter capsulatus* and *Pseudomonas fluorescens* (12,13). *E. coli* CcmC shares 41% identity and 58% similarity with its *R. capsulatus* homologue HelC and 52%

identity and 70% similarity with its *P. fluorescens* homologue. According to the experimentally established membrane topology of these proteins, CcmC contains six transmembrane domains, separated by two cytoplasmic and three periplasmic domains (14) (Fig. 1). An *E. coli ccmC* in-frame deletion mutant is deficient in the production of *c*-type cytochromes (15), most likely because heme cannot be transferred to CcmE (11). Several *ccmC* mutants from different organisms have been described, which were not only affected in cytochrome *c* maturation, but also exhibited other phenotypes. Particularly interesting was the analysis of *P. fluorescens ccmC* mutants, which showed that CcmC is required for the production of pyoverdines, a fluorescent siderophore (13,16). In *Paracoccus denitrificans*, a *ccmC* mutant in addition to deficiencies in cytochrome *c* maturation and siderophore production showed intolerance to rich medium (17). The connection between these processes is not known. In the pathogenic bacterium *Legionella pneumophila*, CcmC was found to be required for cytochrome *c* production, growth in low-iron conditions, and at least for some forms of intracellular infection of eukaryotic hosts (18).CcmC contains a conserved tryptophan-rich signature motif (WGXφWXWDXRLT, where φ is an aromatic amino acid residue) (1,3,19,20) that resides in the second periplasmic domain (Fig. 1) and shares a high degree of similarity with the tryptophan-rich motif of CcmF, NrfE and the CcsA homologues of type II cytochrome *c* maturation (21). In addition, two absolutely conserved histidines are present in the first and third periplasmic domains of CcmC. Mutational analysis of these motifs of the *E. coli* CcmC protein revealed that they are functionally important (22). The accumulation of hydrophobic residues within the tryptophan-rich motif was initially thought to provide a platform for the binding of heme with the two conserved histidines serving as axial heme ligands (12). Only recently it was shown that CcmC can bind heme and that the

tryptophan-rich motif is involved in a direct interaction with CcmE (14). Apart from the amino acids in this motif, CcmC contains several other highly conserved residues, which have never been studied so far regarding their role in heme binding and/or delivery during cytochrome *c* maturation. In this study we asked whether additional domains or amino acids are required for the function of CcmC. This is important because CcmC undergoes protein-protein interaction with at least CcmE, but most likely also with CcmD, a small cytoplasm oriented membrane protein (22), and perhaps also with the ABC transporter subunits CcmAB (23). Even though CcmC seems to bind heme (14), no mutants with defects in heme binding have been described so far. If CcmC is involved in heme transport across the membrane, one might find residues with membrane internal or even cytoplasmic location that are necessary for heme binding. Here, we explored on the one hand a strategy to generate random *ccmC* mutants deficient in cytochrome *c* maturation. These were identified in a genetic screen based on the colony phenotype under anaerobic respiratory growth conditions, when *E. coli* synthesizes up to six different *c*-type cytochromes (24,25). Their expression depends on the availability of terminal electron acceptors such as nitrate, nitrite and TMAO in the growth medium. Any block of cytochrome *c* maturation would affect the cells ability to respire anaerobically on such terminal electron acceptors. Our screen allowed the identification of cytochrome *c* maturation deficient mutants in a background strain that lacks DMSO reductase (26). This approach to identify new and important residues in CcmC was unbiased, which is important because of the high number of highly conserved residues. On the other hand, we also introduced alanines at invariant and highly conserved positions that had neither been mutated before nor hit by the random mutagenesis approach.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions—Bacterial strains and plasmids used in this study are listed in Table I. Bacteria were grown aerobically in LB medium or anaerobically in minimal salts medium (25) supplemented with 0.4% glycerol, 40 mM fumarate and 5 mM sodium nitrate as the terminal electron acceptor. Antibiotics were added at the following final concentrations: ampicillin, 200 μg/ml; chloramphenicol, 25 μg/ml. If necessary, cells were induced with 0.1% arabinose at mid-exponential growth phase.

Screening under anaerobic respiratory growth on TMAO— EC28Δ*dms* cells carrying wild-type or mutant *ccmC* on a plasmid were screened on modified minimum salt medium containing 33 mM KH_2PO_4, 60 mM K_2HPO_4, 7.5 mM $(NH)_2SO_4$, 1.7 mM Tri-Na-citrate, 0.4 mM $MgSO_4$, 67.6 μM Na_2SeO_3, 100 μM Na_2MoO_4, 10% LB medium, 0.4% glycerol and 10 mM TMAO as the terminal electron acceptor. Chloramphenicol was added at a final concentration of 25 μg/ml. The plates were incubated for 48 hours at 37°C inside an anaerobic jar under argon.

P1 phage transduction— The Δ*dmsABC::kan* marker of strain DSS301 (kindly provided by Dr. J. Weiner) (26) was transduced into the Δ*ccmC* mutant EC28 and MC1061 (15) by P1 transduction (27), resulting in EC28Δ*dms* and MC1061Δ*dms*, respectively.

Construction of plasmids and site-directed mutagenesis—*E. coli* strains DH5α (28) and XL1-Blue (29) were used as host for clonings. The plasmids pEC816, pEC817, pEC818, pEC819, pEC820, pEC828 and pEC829 expressing CcmC with the mutations W114A, R128A, L82A, W180A, W125I and D126A, respectively, were constructed by the QuickChange^{TM} site-directed mutagenesis (Stratagene, Amsterdam, NL) using pEC486 as a template and the primers listed in Table II. pEC821 expressing a C-terminally truncated CcmC$^{\Delta N223\text{-}K245}$-$H_6$ was constructed by inverse PCR, using pEC486 as a template and the following set of divergent primers: the 5' primer contained the coding sequence for six histidines upstream of the stop codon and a *Kpn*I restriction site whereas the 3' primer contained a *Kpn*I restriction site. The PCR product was digested with *Kpn*I and self-ligated to obtain pEC821. pEC799 expressing CcmD and CcmE was constructed by amplifying a 680 bp *Nde*I-*Eco*RI by PCR fragment with the primers ccmDn*Nde*I and ccmEc*Eco*RI, using pEC86 as a template. This fragment was digested with *Nde*I- and *Eco*RI- and ligated into the 5.4kb *Nde*I- and *Eco*RI- digested pISC2. pEC827 expressing hexa histidine tagged CcmC along with CcmD and CcmE was constructed by the QuickChange^{TM} site-directed mutagenesis (Stratagene, Amsterdam, NL) using pEC406 as a template. For the construction of a plasmid expressing CcmF, the *ccmF* gene was amplified by PCR using a forward primer containing the *Nde*I site and a reverse primer with an *Eco*RI site. A 2-kb *Nde*I-*Eco*RI fragment containing *ccmF* was then cloned behind the arabinose promoter of pISC2 (30), resulting in pEC825. Plasmid pEC826 expressing the CcmCE fusion protein was constructed by QuickChange^{TM} site-directed mutagenesis with a primer pair that eliminates the *ccmC* stop codon in pEC409 by addition of an adenine nucleotide. In plasmid pEC409 the *ccmC* stop codon overlaps with start codon of *ccmE*. The plasmids pEC803, pEC804, pEC805, pEC806,

pEC807, pEC808, pEC812, pEC815 and pEC824 expressing CcmC with V177G, D47N, R55P, W119R, S176Y, L183P, D126GH184Y, H184Y and G111S respectively, were obtained from the random mutagenesis as described below.

Random mutagenesis by error prone PCR—Mutations were introduced randomly into the 756-bp *ccmC* gene encoding a C-terminal histidine tag (-H_6). PCR was performed with 10 ng DNA template and the forward and reverse primers pACYC184 1613-1629 and pACYC184 4011-3941, respectively. The reaction was carried out in the presence of 200 μM dATP, dCTP, dTTP, 20 μM dGTP and 180 μM dITP, or 200 μM dATP, dGTP, dTTP, 20 μM dCTP and 180 μM dITP for 25 cycles. The PCR products were digested with *Nco*I and *Bfm*I and ligated into *Nco*I- and *Bfm*I- digested pEC486. The ligated product was transformed into the EC28Δ*dms* strain.

Random mutagenesis by using a repair deficient strain—Random mutagenesis of the *ccmC* gene was carried out in the Stratagene XL1-Red mutator strain (Statagene, CA). Plasmid pEC486 containing wild-type *ccmC* was transformed into *E. coli* XL1-Red. This strain lacks three key enzymes for DNA repair, which leads to a 5000-fold increase in the mutation rate. DNA extracted from approx. 1000 colonies was extracted and electroporated into the EC28Δ*dms* strain.

DNA sequencing—All constructs and mutants derived from PCR products were confirmed by DNA sequence analysis (Microsynth, Balgach, Switzerland). In plasmid pEC820 and pEC829

spontaneous silent mutations were found at nucleotide positions 678 and 360 of *ccmC* resulting in conservative L226L and G120G changes, respectively.

Cell fractionation—For the preparation of subcellular fractions bacterial cultures were grown anaerobically in the presence of nitrate. Periplasmic proteins obtained from 1200 ml cultures were treated with polymyxin sulfate as described previously (14). For the preparation of membrane proteins 600 ml of anaerobically grown bacterial culture were harvested, and membrane proteins were extracted as described previously (14).

Biochemical methods—Protein concentrations were determined using the Bradford assay (Bio-Rad) and BSA as a standard. Holo-cytochrome c_{550} and holo-CcmE formation was analyzed qualitatively by immunoblot and heme staining (22). Soluble cytochrome *c* (c_{550}) in the periplasmic fractions was quantified by absorption difference spectroscopy using a $\varepsilon_{550-536nm}$ of 23.2 $mM^{-1}cm^{-1}$ (30). Immunoblot analysis of the His-tagged CcmC-H_6 versions and of cytochrome c_{550}-H_6 was performed using monoclonal tetra-His antibodies (Qiagen) at a dilution of 1:2000. Antibodies directed against a the CcmE peptide (10) or a combination of the three CcmF peptides $P_{410}LVRWGRDRPRKIRN_{424}$, $S_{508}VERDVRMKSGDSVD_{522}$ and $D_{630}PRYRKRVSPQKTAPE_{645}$ were used at a dilution of 1:5000 and 1:10,000 respectively. Signals were detected using goat anti-mouse IgG (for His-tag detection) or goat anti-rabbit IgG (for detection of other antigens) conjugated to alkaline phosphatase (Bio-Rad) as secondary antibodies and CSPD (Roche Diagnostics) as substrate.

TABLE I: Bacterial strains and plasmids used in this study

Bacterial strains or plasmids	Relevant genotype/phenotype	Reference
Escherichia coli		
DH5α	*SupE*44 Δ*lacU*169 (Φ80*lacZΔ*M15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	(28)
MC1061	*hsdR mcrB araD*139 Δ(araABC-leu)*7679* Δ*lacX74 galU galK rpsL thi*	(37)
EC06	MC1061 Δ*ccmA-H*::*kan*	(5)
EC28	MC1061 Δ*ccmC*	(15)
DSS301	TG1 Δ*dmsABC::kan*	(26)
MC1061Δ*dms*	MC1061 Δ*dmsABC::kan*	This work
EC28Δ*dms*	EC28 Δ*dmsABC::kan*	This work
XL1-Red	*endA*1 *gyrA*96 *thi*-1 *hsdR*17 *supE*44 *relA*1 *lac mutD*5 *mutS mutT Tn*10(*tet*^{r})	(38)
XL1-Blue	*recA*1 *endA*1 *gyrA*96 *thi*-1 *hsdR*17 *supE*44 *relA*1 *lac* [*F´ proAB lacI*^{q}*Z*Δ*M*15*Tn*10(*tet*^{r})]	(29)
Plasmids		
pRJ3290	*B. japonicum cycA-*H_6 cloned in pISC2, Ap^R	(39)
pEC406	*ccmCDE* cloned into pACYC184, Cm^R	(11)
pEC409	*ccmCE* cloned into pACYC184, Cm^R	(11)
pEC412	*ccmE* cloned into pISC-2, Ap^R	(10)
pEC486	*ccmC-*H_6 cloned into pACYC184, Cm^R	(22)
pEC799	*ccmDE* cloned into pISC2, Ap^R	This study
pEC803	*ccmC*^{V177G}-H_6 cloned into pACYC184, Cm^R	This study
pEC804	*ccmC*^{D47N}-H_6 cloned into pACYC184, Cm^R	This study
pEC805	*ccmC*^{R55P}-H_6 cloned into pACYC184, Cm^R	This study
pEC806	*ccmC*^{W119R}-H_6 cloned into pACYC184, Cm^R	This study
pEC807	*ccmC*^{S176Y} -H_6 cloned into pACYC184, Cm^R	This study
pEC808	*ccmC*^{L183P}-H_6 cloned into pACYC184, Cm^R	This study
pEC812	*ccmC*$^{D126G,\ H184Y}$-H_6 cloned into pACYC184, Cm^R	This study

pEC815	*ccmC*^{H184Y}-H_6 cloned into pACYC184, Cm^R	This study
pEC816	*ccmC*^{W114A}-H_6 cloned into pACYC184, Cm^R	This study
pEC817	*ccmC*^{R128A}-H_6 cloned into pACYC184, Cm^R	This study
pEC818	*ccmC*^{L82A}-H_6 cloned into pACYC184, Cm^R	This study
pEC819	*ccmC*^{W180A}-H_6 cloned into pACYC184, Cm^R	This study
pEC820	*ccmC*$^{Y139A, L226L}$-H_6 cloned into pACYC184, Cm^R	This study
pEC821	*ccmC*$^{\Delta N223-K245}$-H_6 cloned into pACYC184, Cm^R	This study
pEC824	*ccmC*^{G111S}-H_6 cloned into pACYC184, Cm^R	This study
pEC825	*ccmF* cloned into pISC2, Ap^R	This study
pEC826	*ccmC-ccmE* fusion cloned into pACYC184, Cm^R	This study
pEC827	*ccmC*-H_6*DE* cloned into pACYC184, Cm^R	This study
pEC828	*ccmC*^{W125I}-H_6 cloned into pACYC184, Cm^R	This study
pEC829	*ccmC*^{G120G,D126A}-H_6 cloned into pACYC184, Cm^R	This study

Heme binding assays—150 μg total membrane proteins were solubilized at 4°C for 1 h in 1 ml of solubilization buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5 mM PMSF, and 0.5% lauroyl sarcosine). After centrifugation, the supernatant was treated with hemin agarose (Sigma) or aminododecyl agarose (Sigma) as described previously for CcmC (14). Agarose beads were washed three times with solubilization buffer and twice with PBS to remove unbound material, then treated with 2 x SDS loading dye (containing 50 mM DTT) and incubated at room temperature for 20 min. The mixture was subjected to 15% SDS-PAGE. Proteins in the gel were visualized by immunoblot analysis.

TABLE II: Oligonucleotide primers used for plasmid construction and sequencing

Primer	Bases	Nucleotide sequence (5´-3´)	Usage
*ccmC*L82A-f	31	ggcagcgtttattggcgcggtctggcagatg	Quick change primer
*ccmC*L82A-r	31	catctgccagaccgcgccaataaacgctgcc	Quick change primer
*ccmC*W114A-f	34	ggttaccggctctgcagcgggaaaaccgatgtgg	Quick change primer
ccmCW114-r	34	ccacatcggttttcccgctgcagagccggtaacc	Quick change primer
*ccmC*R128A-f	34	tgggtatgggatgcagcgctgacttctgaactgg	Quick change primer
*ccmC*R128A-r	34	ccagttcagaagtcagcgctgcatcccataccca	Quick change primer
*ccmC*Y139A-f	34	ggtgctgctgtttttggcggtgggtgtgattgcc	Quick change primer
ccmCY139Ar	34	ggcaatcacacccaccgccaaaaacagcagcacc	Quick change primer
*ccmC*W125I*Cla*I-f	43	ggcacctggtgggtaatcgatgcacgtctgacttctga actgg	Quick change primer
*ccmC*W125I*Cla*I-r	43	ccagttcagaagtcagacgtgcatcgattacccacca ggtgcc	Quick change primer
*ccmC*D126A*Kpn*I-f	44	ccgatgtggggtacctggtgggtatgggctgcacgtc tgacttc	Quick change primer
*ccmC*D126A*Kpn*I-r	44	gaagtcagacgtgcagcccatacccaccaggtaccc cacatcgg	Quick change primer
ccmCΔ223-245-His *Kpn*I	56	ggggtacctcagtggtggtggtggtggtggtgacgca tccgcatcagcgtcagcgtggc	Forward primer for cloning pEC821
*Kpn*I pACYC184 pos. 3541-71	35	ggggtacctcatatcgtcaattattacctccacgg	Reverse PCR primer for cloning pEC821
*ccmC*W180A-f	38	cattactccgtggagtgggcgaacaccctgcatcagg g	Quick change primer
*ccmC*W180A-r	38	ccctgatgcagggtgttcgcccactccacggcgtaat g	Quick change primer

ccmD$_N$ *Nde*I	26	cggatccatatgacccctgcatttgc	Forward PCR primer for cloning pEC799
ccmE$_C$ *Eco*RI	24	gggaattctcatgatgctgggtcc	Reverse PCR primer for cloning pEC799
ccmC-His-f	57	gaaaagaggccgtaaaacaccaccaccaccaccact gatgacccctgcatttgcttcc	Quick change primer
ccmC-His-r	57	ggaagcaaatgcaggggtcatcagtggtggtggtgg tggtgtttacggcctcttttc	Quick change primer
ccmF$_N$ *Nde*I	29	ggcgatatgcatatgatgccagaaattgg	Forward primer for cloning pEC825
ccmF$_C$ *Eco*RI,	27	cgggaattctcatacggcctccggcgc	Reverse PCR primer for cloning pEC825
pACYC184 1613-1629	17	cggcaccgtcaccctgg	Forward PCR primer
pACYC184 4011-3941	20	cccccgttttcaccatgggc	Reverse PCR primer with *Nco*I site
*ccmC***E*-f	30	gaaaagaggccgtaaaatgaatattcgccg	Quick change primer
*ccmC***E*-r	30	cggcgaatattcattttacggcctcttttc	Quick change primer

Co-immunoprecipitation—0.5 mg membrane proteins were solubilized and precipitated with 5 µl undiluted anti-CcmE, 5 µl undiluted anti-CcmF, or 10 µl of anti-His-tag antibodies according to the method described previously (14). The proteins were subjected to SDS PAGE, transferred to a polyvinylidene difluoride membrane and probed with antisera against the tetra-His-tag, CcmE or CcmF peptides as indicated.

RESULTS

Rationale of the screen—In the *ccmC* in frame deletion mutant EC28Δ*dms* (ΔA63-ΔG156; Δ*dmsABC*::*kan*) the predicted trans-membrane helices II , III and IV of CcmC together with the periplasmic domain II containing the tryptophan-rich motif are deleted. A Δ*ccmC*Δ*dmsABC* double mutant was constructed by P1 transduction of a Δ*dmsABC::kan* allele from a mutant kindly provided by Dr. J. Weiner. DmsABC constitute a dimethyl sulfoxide (DMSO) reductase that catalyzes the reduction of DMSO and trimethylamine *N*-oxide (TMAO) under anaerobic conditions. The *dms* mutant cannot grow anaerobically on minimal medium with glycerol and DMSO (26). However, the TMAO reductase allows *E. coli* to grow by anaerobic respiration with TMAO as a terminal electron acceptor. TMAO reductase is a hetro-dimeric enzyme composed of TorA, a periplasmic reductase containing molybdenum as cofactor and TorC, a membrane bound penta-heme *c*-type cytochrome. During anaerobic TMAO respiration electrons are transferred from the menaquinone pool to TorC and then delivered to TorA, which in turn reduces TMAO to trimethylamine (31). TorC requires the cytochrome c maturation (ccm) system composed of the CcmABCDEFGH proteins to insert the hemes covalently. Any block of TorC maturation is expected to abolish anaerobic growth on TMAO, when the alternative pathway for electron transfer via DMSO reductase is excluded. Hence, the Δ*ccmC*Δ*dms* mutant had a strong anaerobic growth phenotype: in contrast to the wild type, which formed large red colonies on TMAO plates, the mutant grew poorly and was white. The remaining growth capability of this mutant may be due to yet another DMSO reductase encoded by a second set of *dmsABC* genes that are expressed constitutively (32). When the mutant was transformed with a plasmid expressing *ccmC*-H_6 encoding wild-type, His-tagged CcmC, the cells were red and formed normal size

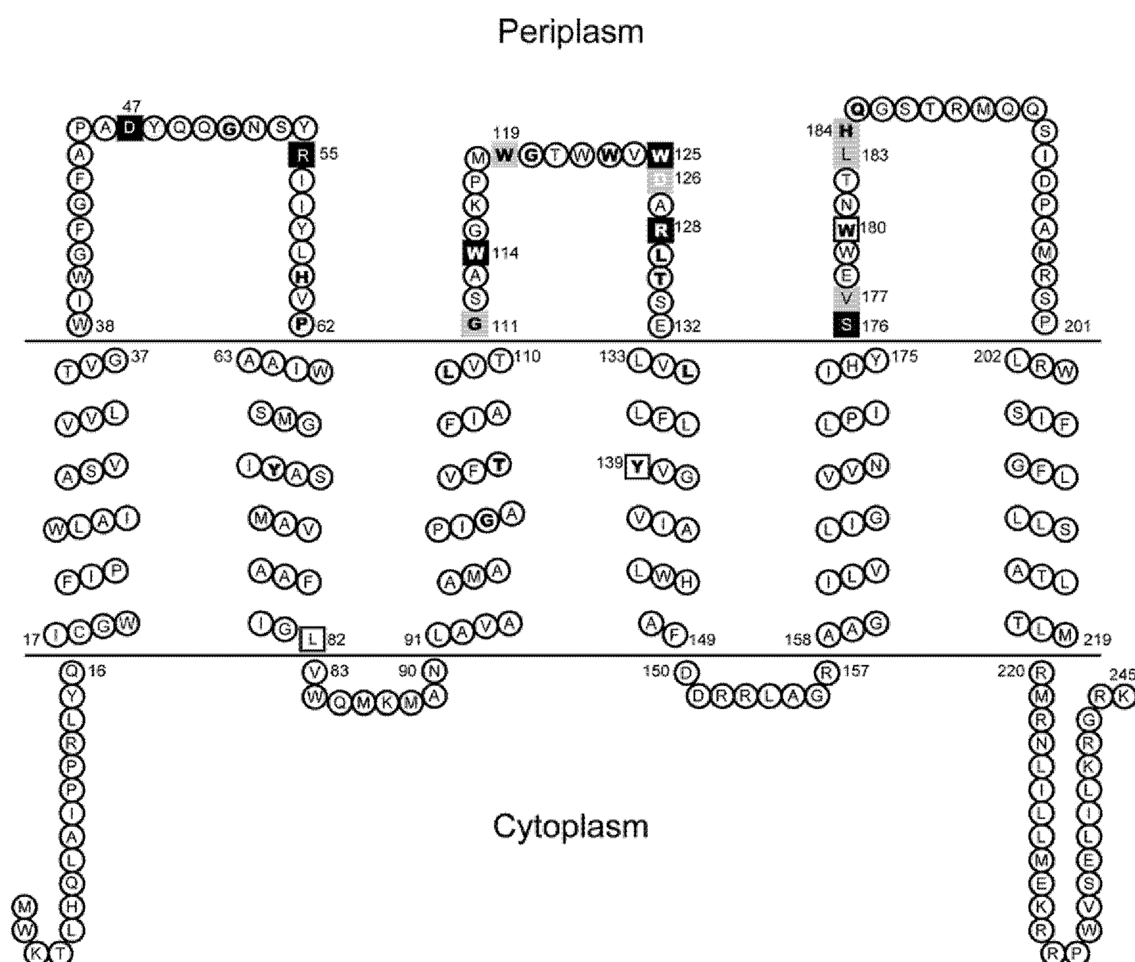

Fig. 1. Proposed topology model for *E. coli* CcmC. The topology model was constructed by using the program HMMTOP (40). It predicts six transmembrane helices, four cytoplasmic and three periplasmic domains. Conserved histidines and the W-rich motif are located in the periplasm. Amino acid residues characterized in this study are boxed. Black boxes represent amino acids for which a new phenotype was found (class II mutants). Grey boxes represent amino acid residues important for heme delivery to CcmE. Open boxes represent amino acid residues that were altered without an effect. Residues shown in bold are 100% conserved in CcmC homologues from the following species, derived from whole genome sequences deposited at NCBI: *Escherichia coli, Haemophilus influenzae, Yersinia pestis, Sinorhizobium meliloti, Agrobacterium tumefaciens, Rickettsia conorii, Rickettsia prowazekii, Pasteurella multicoda, Pseudomonas aeruginosa, Salmonella enterica, Salmonella typhimurium, Pantoea citrea, Paracoccus denitrificans, Bradyrhizobium japonicum, Rhodobacter capsulatus, Pseudomonas putida, Shewanella putrefaciens, Rhodobacter sphaeroides, Vibrio cholerae, Brucella melitensis, Mesorhizobium loti, Caulobacter crescentus, Xylella fastidiosa, Deinococcus radiodurans, Arabidopsis thaliana.*

colonies, but when an empty vector was used as control, they were small and white. This phenotype was the basis for a color screen for *ccmC* mutants.

Generation and analysis of random ccmC mutants— Random mutations were introduced into plasmid-borne *ccmC* described above by (i) error-prone PCR and (ii) the use of a repair-deficient strain for plasmid propagation. Based on the red/white phenotypes we screened 14905 colonies. 241 white candidates were tested for expression of full-length CcmC polypeptide by immunoblot using anti-His-tag antibodies. 35 mutants were found to produce normal levels of the protein. This step allowed excluding all mutants in which stop codons had been introduced accidentally or which produced unstable protein. The plasmids were isolated and sequenced. We found 14 single, 10 double, and four triple mutants. The following single mutations were identified (Table III): D47N, R55P (2x), G111S (2x), W119R (2x), S176P, S176Y, V177G (3x), L183P and H184Y. Despite the high number of redundant hits at individual amino acids from independent experiments, some residues that had been altered previously were not mutated, suggesting that we had not reached saturation. Redundant mutants were found for both mutagenesis procedures. Surprisingly, in seven cases a wild-type sequence was found along with a high plasmid copy number, as was evident from the increased amount of plasmid DNA and also from particularly strong signals in the immunoblot (data not shown). These mutants were generated from the repair-deficient strain. It is possible that the mutant phenotype imparted to the strains harboring high-copy plasmids was due to a dominant negative effect exerted by over-expressed CcmC on cytochrome *c* maturation. Such mutants were not pursued for further analysis. However, when wild-type

TABLE III: Classification of CcmC mutants

Class	Method	Mutant	% amino acid identity[a]	Level of CcmC[b]	Level of CcmE[b]	Co-IP[b]	Holo-CcmE[b]	Holo-cytochrome *c* formation (%)[c]	Colony phenotype[d]
Wild type				++	++	+++	+++	+++ (100)	red
Class I	Random	G111S	100	++	++	-	-	-	white
	Random	W119R	100	++	++	+	++	+ (0.31)	white
	Site directed	D126A	100	++	++	++	++	+ (8.2)	white
	Random	V177G	96	++	++	++	++	+ (1.2)	white
	Random	L183P	96	++	++	++	++	-	white
	Random	H184Y	100	++	++	-	-	-	white
Class II	Random	D47N	92	++	++	+++	+++	+ (6.9)	white
	Random	R55P	84	++	++	+++	+++	+ (6)	white
	Site directed	W114A	100	++	++	+++	+++	+++ (40)	pink
	Site directed	W125I	100	++	++	+++	+++	++ (19.5)	pink
	Site directed	R128A	100	++	++	+++	+++	+ (13.7)	pink
	Random	S176Y	88	++	++	++	++	-	white
Class III	Site directed	L82A	96	++	++	+++	+++	+++ (103)	red
	Site directed	Y139A	100	++	++	+++	+++	+++ (84.9)	red
	Site directed	W180A	100	++	++	+++	+++	+++ (61.5)	red

[a] Identity based on the sequences derived from the organisms listed in the legend of Fig. 1.

[b] Levels were established from the signal intensities in the immunoblots and the heme stains presented in Figs. 2-4.

[c] Spectroscopically determined concentration of soluble *c*-type cytochromes as described in Experimental Procedures.

[d] Growth phenotype observed on TMAO plates under anaerobic conditions.

ccmC was over-expressed from the pACYC184 vector relative to the other chromosomally expressed *ccm* genes, a dominant negative effect was not observed (data not shown).

Eight single mutants (Table III) were selected for a detailed analysis; among them all except one (H184Y) mapped to residues that had not been investigated previously. H184Y was included in our analysis for a comparison with the previously described mutant H184A (22). The seven newly identified, important amino acids D47, R55, G111, W119, S176, V177 and L183 were all located in periplasmic domains of CcmC (Fig. 1). To test the mutants for loss of cytochrome *c* maturation, they were grown anaerobically with nitrate as the sole electron acceptor. *E. coli* has multiple respiratory nitrate reductases (33-35) and one of them, the NarGHI enzyme does not contain *c*-type cytochromes. Hence, the organism can facultatively produce holo-cytochrome *c* as part of an electron transport system to the periplasmic nitrate reductase, the NapABC system, and *ccm* mutants can grow without suffering from a selective pressure when cytochrome *c* maturation is blocked. This implies that formation of revertants or second-site suppressors is not enforced. In our complementation experiments we overexpressed exogenous *Bradyrhizobium japonicum* cytochrome c_{550} for better monitoring the effect of a mutation in CcmC. The Δ*ccmC*Δ*dmsABC* mutant strain was co-transformed with a plasmid expressing from the vector promoter a His-tagged *B. japonicum* cytochrome c_{550} plus a plasmid encoding either the His-tagged wild-type or mutant CcmC protein (Fig. 2A). Cytochrome *c* formation was assayed by heme staining (Fig. 2A, upper panel) and immunoblot (lower panel) of periplasmic fractions. The apo-cytochrome is degraded rapidly when heme is not incorporated (30). Quantification of *c*-type cytochromes was done by absorption difference spectroscopy. None of the mutants produced wild-type levels of *c*-type cytochromes. The mutants D47N (*lane 3*) and R55P (*lane 4*) localized in the first and V177G

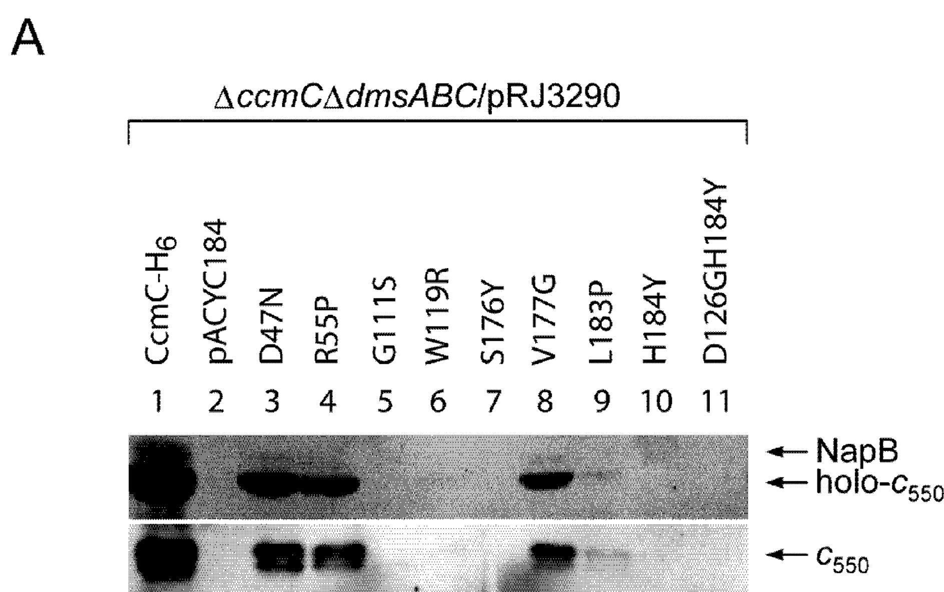

Fig. 2. Functional analysis of random *ccmC* mutants. *A*, The Δ*ccmCdms* double mutant EC28 *dmsABC::kan* was co-transformed with plasmid pRJ3290 (encoding *B. japonicum* cytochrome c_{550}-H_6) and pEC486 (wild-type CcmC-H_6; *lane 1*), pACYC184 (empty vector; *lane 2)*, pEC804 (D47N; *lane 3*), pEC805 (R55P; *lane 4*), pEC824 (G111S; *lane 5*), pEC806 (W119R; *lane 6*), pEC807 (S176Y; *lane 7*), pEC803 (V177G; *lane 8*), pEC808 (L183P; *lane 9*), pEC815 (H184Y; *lane 10*), or pEC815 (D126GH184Y; *lane 11*). Cells were grown anaerobically in the presence of 5 mM sodium nitrate. Upper panel: periplasmic proteins (200 μg per lane) were separated by 15% SDS-PAGE and stained for covalently bound heme. Lower panel: immunoblot of TCA-precipitated periplasmic protein (50 μg per lane) probed with antiserum against the His-tag of cytochrome c_{550}. NapB is a native periplasmic soluble *c*-type cytochrome expressed under these conditions.
The positions of NapB and cytochrome c_{550} are indicated on the right.

(*lane 8*) in the third periplasmic domain were the least affected in the cytochrome *c* formation when compared to the other random mutants. Mutant W119R (*lane 6*) and mutant L183P (*lane 9*) produced extremely low but detectable levels of holo-cytochrome *c*. Mutant G111S, S176Y, H184Y and the double mutant D126GH184Y lacked detectable amounts of cytochrome *c* (*lanes 5,7,10* and *11*). The H184Y phenotype matched that of the previously described mutant H184A (22). In contrast, in comparison with the D126GH184Y double mutant, the single change of D126A in the tryptophan-rich motif had resulted in functional

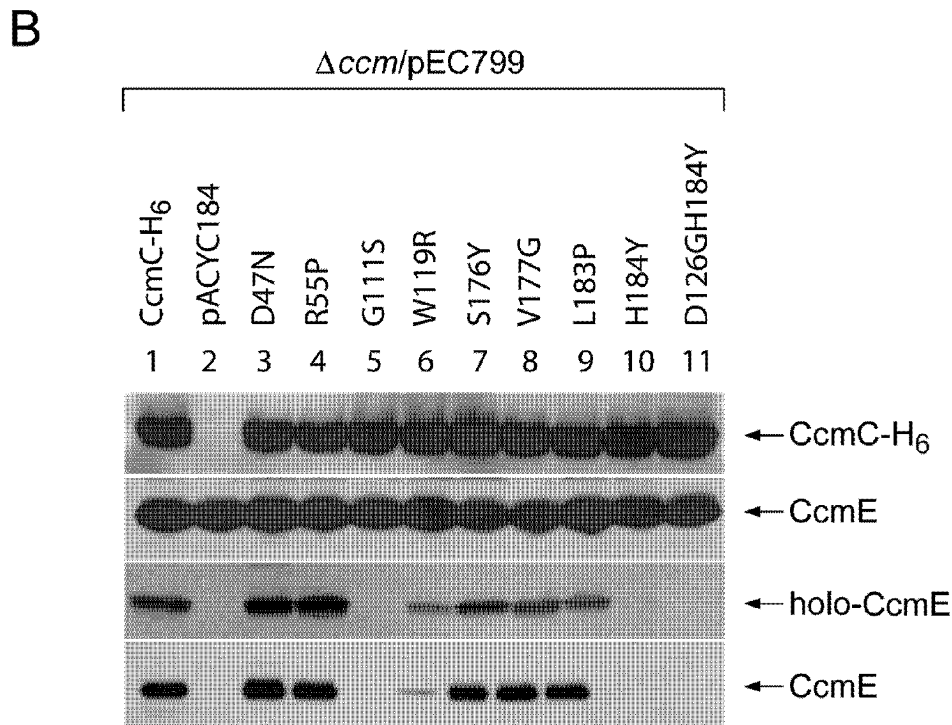

Fig. 2. Functional analysis of random *ccmC* mutants. *B*, The Δ*ccmA-H* mutant EC06 was co-transformed with plasmid pEC799 (encoding CcmDE) and the same plasmids as in A. Cells were grown anaerobically in the presence of 5 mM sodium nitrate and membranes were prepared. Proteins were separated by 15% SDS-PAGE. First panel: immunoblot of membrane proteins (100 μg per lane) probed with antiserum against the His-tag. Second panel: immunoblot of membrane proteins (100 μg per lane) probed with antiserum against CcmE. Third panel: heme stain of the same membrane proteins (100 μg per lane). Fourth panel: the same membrane proteins (500 μg per lane) were immunoprecipitated with tetra-His antibodies, and co-precipitating CcmE was detected with anti-CcmE serum. The positions of CcmC, apo- and holo-CcmE are indicated on the right.

CcmC (22). The reduced *minus* oxidized spectra were recorded for the quantification of soluble type *c* cytochromes. The value for the wild type was assigned 100%, and relative values of the various *ccmC* mutants were calculated accordingly.

Compared with the wild-type cytochrome *c* level (100%), mutants D47N, R55P, W119R and V177G produced only 6.9%, 6%, 0.3% and 1.2% cytochrome *c*, respectively, whereas,

cytochrome *c* formed in mutant L183P was below the limit of detection. As the random mutants affected cytochrome *c* maturation in varying degrees, it was necessary to investigate whether or not the mutations had (i) destabilized the protein in the membrane, (ii) affected CcmC-CcmE interaction, or (iii) affected the ability of CcmC to bind heme. The known function of CcmC is the delivery of heme to the heme chaperone CcmE, from where heme then is transferred in a second step to the apo-cytochrome. Hence, we isolated membranes from cells deleted in the entire *ccm* operon (EC06) and containing the plasmid pEC799 encoding CcmD and CcmE plus a plasmid expressing either the wild-type or a mutant *ccmC* allele. The levels of the CcmC (Fig. 2B, first panel) and CcmE polypeptides (Fig. 2B, second panel) were probed by immunoblot analysis and found to be the same for all strains; the control strain containing only CcmE but no CcmC (lane 2) showed no reaction. Holo-CcmE formation determined by heme stains (Fig. 2B, third panel) was normal in mutants D47N (*lane 3*) and R55P (*lane 4*), whereas mutants W119R, S176Y, V177G and L183P (*lanes 6-9*) contained less holo-CcmE than the wild type (*lane 1*). Mutants G111S (*lane 5*), H184Y (*lane 10*) and the double mutant D126GH184Y (*lane 11*) lacked holo-CcmE completely. We also investigated the effect of the mutations on the physical interaction between CcmC and CcmE (Figure 2B, bottom panel). A co-immunoprecipitation experiment revealed that mutants D47N (*lane 3*) and R55P (*lane 4*) co-precipitated CcmC and CcmE similar to the wild type (*lane 1*). Mutants S176Y, V177G and L183P (*lanes 7-9*) showed slightly reduced and mutant W119R drastically reduced levels of co-precipitated CcmE. For mutants G111S (*lane 5*), H184Y (*lane10*) and the double mutant D126GH184Y (*lane 11*) no CcmC-CcmE co-precipitation was found. We also tested the effect of the mutations on affinity binding to

hemin-agarose as it has been described previously (14), but no significant differences were observed (data not shown).

Analysis of ccmC site-directed mutants—CcmC contains a number of highly conserved residues, of which those localized in the core of the tryptophan-rich motif ($W_{119}G_{120}XXW_{123}XW_{125}D_{126}$) and the histidines at position 60 and 184 have been altered previously (22). Here, we focused on additional conserved residues of the CcmC protein that had failed to be picked up in our random screen: the conserved amino acids L82 and Y139 in transmembrane segment II and IV, respectively, W114 and R128 in an extension of the W-rich motif, and W180 in the third periplasmic domain were changed to alanine. In addition, the C-terminal 23 residues were deleted to test the role of this cytoplasmic domain. The Δ*ccmC*Δ*dmsABC* mutant strain was complemented for cytochrome *c* biosynthesis in the presence of plasmid pRJ3290 encoding the *B. japonicum* cytochrome c_{550} plus a plasmid encoding either the wild-type or a mutant *ccmC* allele, and cytochrome *c* formation was tested by heme staining, immunoblot (Fig. 3A) and absorption difference spectra (Table III). Most affected in cytochrome *c* maturation were the mutants R128A (*lane 5*) and W114A (*lane 4*) on either side of the W-rich motif, whereas the other mutants in residues predicted to be in transmembrane domains (*lanes 3* and *6*) were almost like the wild type. When the C-terminal 23 residues were deleted, a drastic effect on cytochrome *c* formation was observed (*lane 8*). In a next experiment the presence of CcmC and CcmE in the membrane, their interaction and the efficiency of heme delivery to CcmE were investigated as described for the random mutants. The levels of the CcmC and CcmE polypeptides in the membrane and the formation of holo-

CcmE in cells deleted in the entire *ccm* operon (EC06) and containing the plasmid pEC799 expressing *ccmDE* plus a plasmid with either the wild-type or a *ccmC-H*$_6$ mutant allele were determined. The results presented in Figure 3B (first and second panel) show that all mutants produced normal levels of CcmC and CcmE except for the C-terminal CcmC deletion, in which the levels of CcmC but not CcmE were decreased. Even though this cytoplasmic domain is not well conserved, it seems to be important for the stability of the protein.

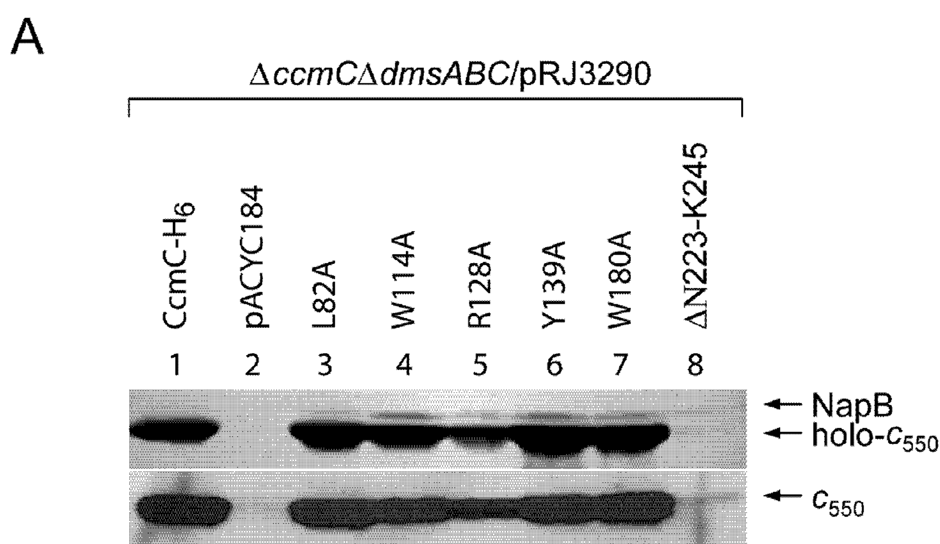

Fig. 3. Functional analysis of site-directed *ccmC* mutants. *A*, the Δ*ccmC*Δ*dms* double mutant EC28 Δ*dms* was co-transformed with plasmid pRJ3290 (encoding His-tagged *B. japonicum* cytochrome c_{550}) and pEC486 (encoding wild-type CcmC-H$_6$; *lane 1*), pACYC184 (empty vector; *lane 2)*, pEC818 (L82A; *lane 3*), pEC816 (W114A; *lane 4*), pEC817 (R128A; *lane 5*), pEC820 (Y139A; *lane 6*), pEC819 (W180A; *lane 7*), or pEC821 (ΔN223-K245; *lane 8*). Cells were grown anaerobically in the presence of 5 mM sodium nitrate. Upper panel: periplasmic proteins (200 μg per lane) were separated by 15% SDS-PAGE and stained for covalently bound heme. Lower panel: immunoblot of TCA precipitated periplasmic proteins (50 μg per lane) probed with antiserum against the His-tag. The positions of NapB and cytochrome c_{550} are indicated on the right.

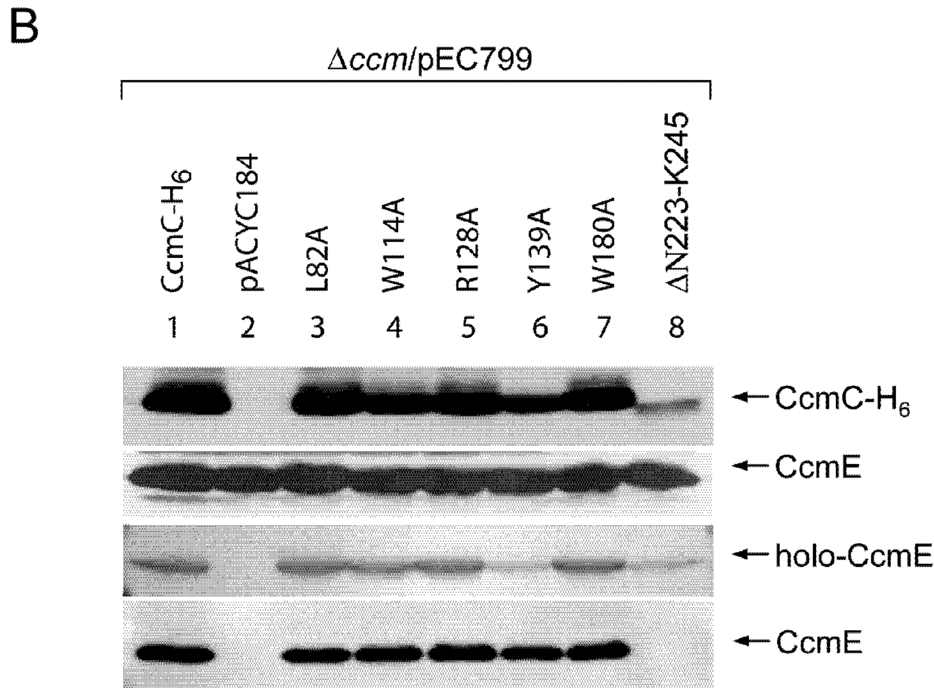

Fig. 3. Functional analysis of site-directed *ccmC* mutants. *B*, the Δ*ccmA-H* mutant EC06 was co-transformed with plasmids pEC799 (encoding CcmDE) and the same plasmids as in *A*. Cells were grown anaerobically in the presence of 5 mM sodium nitrate. First panel: immunoblot of membrane proteins (100 μg per lane) probed with antiserum against the His-tag. Second panel: immunoblot of membrane proteins (100 μg per lane) probed with antiserum against CcmE. Third panel: heme stain of the same membrane proteins (100 μg per lane) after separation by 15% SDS-PAGE. Fourth panel: membrane proteins (500 μg per lane) from the indicated strains were immunoprecipitated with tetra-His antibodies, separated by 15% SDS-PAGE, and immuno-detected with anti-CcmE serum. The position of CcmC, CcmE and holo-CcmE is indicated on the right.

Mutants L82A (Fig. 3B, third panel, *lane 3*), W114A (*lane4*), R128A (*lane 5*) and W180A (*lane 7*) produced normal (wild-type) levels (*lane 1*), whereas mutant Y139A (*lane 6*) produced less holo-CcmE. The C-terminal deletion mutant was strongly affected in heme delivery, as very little holo-CcmE was detected, most likely due to the small amount of CcmC

polypeptide. The physical interaction between CcmC and CcmE was also investigated (Figure 3B, bottom panel). None of the site-directed mutants except the C-terminal deletion mutant was affected in the physical interaction between the two proteins.

We also tried with a two-fold higher concentration of the C-terminally truncated CcmC mutant protein to co-precipitate CcmE but failed to detect it (data not shown). Next we investigated the effect of the mutations on the affinity of CcmC to hemin-agarose. No significant differences between the wild-type and the mutants were observed (data not shown). We also detected some heme binding of the truncated CcmC, but the signal was comparatively weak, which could be due to the instability of the protein. In summary, cytochrome *c* maturation of the site-directed mutants was not affected drastically in any case, and only the C-terminal deletion mutant had a clear deficiency. The site-directed mutant with the strongest effect on cytochrome *c* maturation (R128A) still produced more cytochrome *c* than the least-affected random mutant (D47N). This could explain why mutations in the strongly conserved residues had not been picked up in our screen. Thus, our screen preferentially detects mutants severely affected in cytochrome *c* maturation. In fact, when the site-directed mutants were grown under the conditions of our screening procedure, none of them were white, confirming that they produced intermediate levels of *c*-type cytochromes.

Analysis of ccmC mutants in the tryptophan-rich motif—The importance of the tryptophan-rich motif for CcmC function has been well documented. It is involved in the physical interaction between CcmC and CcmE (14). In previous work mutants in residues W119, W125 and D126 had affected the cytochrome *c* formation slightly, whereas mutants in G120, T121, W122 and W123 had produced wild-type levels of cytochromes *c* (22). In this work we

were interested to investigate the individual contribution of the conserved amino acids in the extended W-rich motif to cytochrome *c* maturation. We thus included the new mutants W114A, W119R and R128A and compared them with W125I and D126 in a detailed analysis. The mutants were used to complement cytochrome *c* biosynthesis in the Δ*ccmC*Δ*dmsABC* strain (Fig. 4A). While the changes W114A and W125I affected cytochrome *c* maturation only slightly, the R128A, D126A and in particular W119R had more severe consequences, with W119R producing less than 1% of the *c*-type cytochromes seen in a wild type (Table III). The influence of the mutation on the presence of the CcmC and CcmE polypeptides in the membrane as well as the ability of the mutants to interact with CcmE and to deliver heme were investigated (Fig. 4B). We analyzed the levels of CcmC peptide, CcmE peptide and holo-CcmE in cells deleted in the entire *ccm* operon (EC06) and containing the plasmid pEC799 expressing *ccmDE* plus a plasmid encoding either wild-type or a mutant CcmC-H_6. The results (Fig. 4) show that all mutants produced normal levels of CcmC and CcmE. Mutants W119R and D126A (*lanes 3* and *5*, respectively) produced lower levels of holo-CcmE, although the polypeptide was synthesized normally, a finding which is in agreement with an earlier study (22). However, in a co-precipitation experiment the interaction of CcmE with mutant W119R is clearly disturbed (Fig. 4B, bottom panel, *lane 3*).

Analysis of ccmC mutants in the absence of ccmD— In all our experiments holo-CcmE formation was investigated in the presence of CcmD. CcmD is a small monotopic membrane protein, which had been shown to influence heme ligation to apo-CcmE (22). In particular, it had been observed that the presence of CcmD can suppress a *ccmC* mutant phenotype prevailing in the absence of CcmD. Here, we investigated the *ccmC* mutants which had

affected cytochrome *c* maturation drastically without apparently affecting holo-CcmE formation. We analyzed levels of CcmC polypeptide, CcmE polypeptide and holo-CcmE in cells deleted in the entire *ccm* operon (strain EC06) and containing the plasmid pEC412 encoding CcmE plus a plasmid encoding either the wild-type or a mutant CcmC-H_6. In this situation CcmD was absent. The immunoblots presented in Fig. 5 show that the mutants produced normal levels of CcmC and CcmE protein (top and middle panel, respectively). In mutant W114A (*lane 2*), D47N (*lane 8*) and R55P (*lane 9*) small amounts of holo-CcmE were detectable, whereas none of the other point mutants produced visible amounts of holo-CcmE (Fig. 5, bottom panel). We included mutant W119A (*lane 7*), which had been already described previously (22) and does not carry a His-tag, not only as an internal control, but also for a better comparison with our new point mutant W119R. Our results confirm the earlier observation that CcmD is indeed required for the efficient charging of apo-CcmE with heme and that certain residues within CcmC contribute to this activity in specific ways.

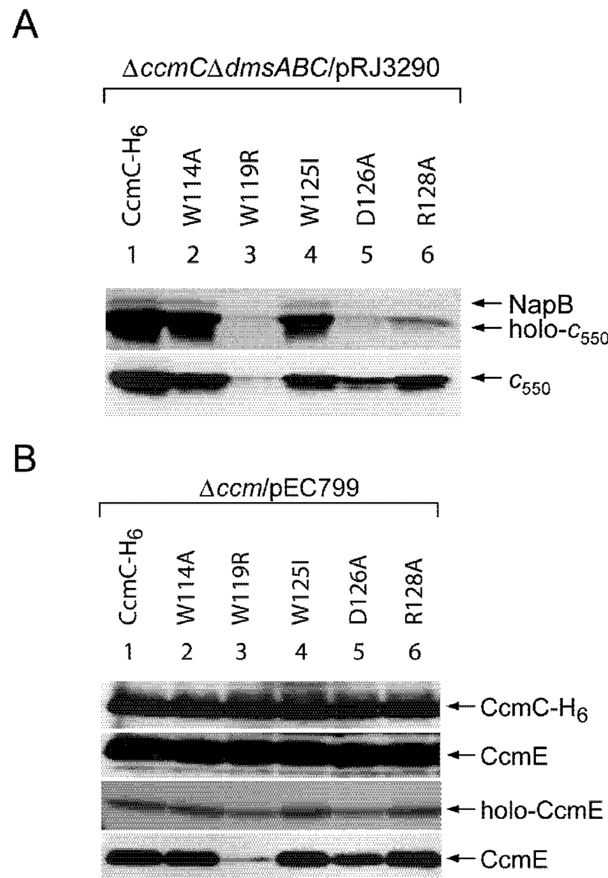

Fig. 4. Functional analysis of *ccmC* mutants in tryptophan-rich motif. *A*, The Δ*ccmC*Δ*dms* double mutant EC28 Δ*dms* was co-transformed with plasmid pRJ3290 (encoding His-tagged *B. japonicum* cytochrome c_{550}) and pEC486 (wild-type CcmC-H_6; *lane 1*), pEC816 (W114A; *lane 2*), pEC806 (W119R; *lane 3*), pEC828 (W125I; *lane 4*), pEC829 (D126A; *lane 5*), or pEC817 (R128A; *lane 6*). Cells were grown anaerobically in the presence of 5 mM sodium nitrate. Upper panel: periplasmic proteins (200 µg per lane) were separated by 15% SDS-PAGE and stained for covalently bound heme. Lower panel: immunoblot of TCA-precipitated periplasmic proteins (50 µg per lane) probed with antiserum against the His-tag. The positions of NapB and cytochrome c_{550} are indicated on the right. *B*, the Δ*ccmA-H* mutant EC06 was co-transformed with plasmids pEC799 (encoding CcmDE) and the same plasmids as in *A*. Cells were grown anaerobically in the presence of 5 mM sodium nitrate. First panel: immunoblot of membrane proteins (100 µg per lane) probed with antiserum against the His-tag. Second panel: immunoblot of membrane proteins (100 µg per lane) probed with antiserum against CcmE. Third panel: heme stain of the same membrane proteins (100 µg per lane) after separation by 15% SDS-PAGE. Fourth panel: membrane proteins (500 µg per lane) from the indicated strains were immunoprecipitated with tetra-His antibodies, separated by 15% SDS-PAGE, and immuno-detected with anti-CcmE serum. The position of CcmC, CcmE and holo-CcmE is indicated on the right.

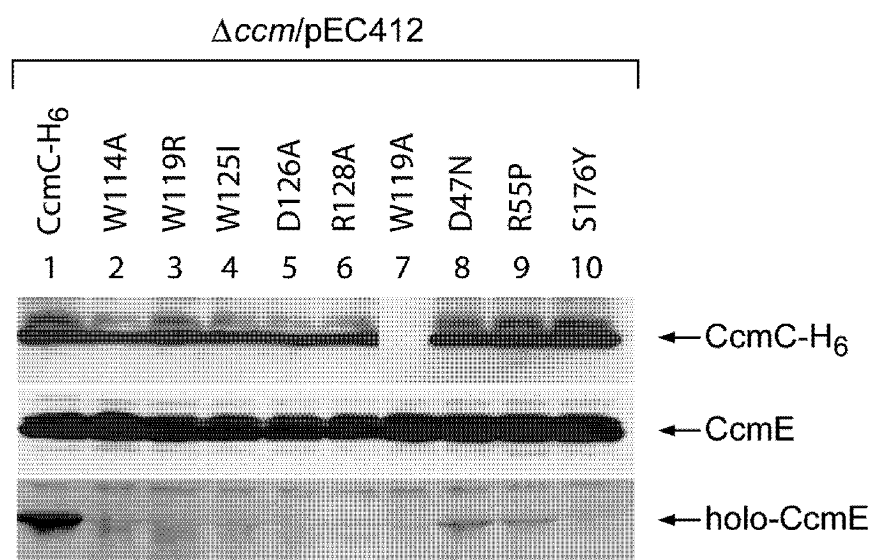

Fig. 5. Functional analysis of *ccmC* mutants in absence of *ccmD*. The Δ*ccmA-H* mutant EC06 was co-transformed with plasmid pEC799 (encoding CcmDE) and pEC486 (wild-type CcmC-H_6; *lane 1*), pEC816 (W114A; *lane 2*), pEC806 (W119R; *lane 3*), pEC828 (W125I; *lane 4*), pEC829 (D126A; *lane 5*), pEC817 (R128A; *lane 6*), pEC806 (W119R; *lane 7*), pEC804 (D47N; *lane 8*), pEC805 (R55P; *lane 9*), or pEC807 (S176Y; *lane 10*). Cells were grown anaerobically in the presence of 5 mM sodium nitrate. Proteins were separated by 15% SDS-PAGE. First panel: immunoblot of membrane proteins (100 μg per lane) probed with antiserum against the His-tag. Second panel: immunoblot of membrane proteins (100 μg per lane) probed with antiserum against CcmE. Third panel: heme stain of the same membrane proteins (100 μg per lane). The positions of CcmC, apo- and holo-CcmE are indicated on the right.

CcmC does not bind CcmF—One of the most striking findings of this mutant analysis was the discovery of a new phenotype of mutants D47N, R55P and S176Y with a deficiency in cytochrome *c* formation despite normal levels of apo- and holo-CcmE and CcmC-CcmE interaction (Table III). One plausible explanation for this might be a direct interaction with another factor catalyzing the detachment of heme from holo-CcmE and its subsequent transfer

to apo-cytochrome *c*. CcmF had previously been shown to directly interact with CcmE, and it was postulated to be a cytochrome *c* heme lyase (36). As CcmE interacts with both CcmC and CcmF, we were interested to test the formation of a ternary complex between CcmC, CcmE and CcmF. We tested by co-immunoprecipitation whether or not CcmC could physically interact with CcmF in the presence or absence of CcmE. Proteins from membrane extracts were precipitated with anti-CcmF antibodies. Subsequent immunoblot analysis of the precipitates revealed that the CcmC polypeptide did not co-precipitate with CcmF, no matter whether or not CcmE was present in the system. The inverse precipitation, i.e. using anti-histidine tag antibodies for precipitation of His-tagged CcmC and detection for co-precipitating CcmF revealed no specific signals (data not shown).

Analysis of a CcmCE fusion protein— To investigate the idea that CcmE shuttles between CcmC and CcmF and that certain CcmC mutants might prevent dissociation of CcmE from CcmC, we genetically fused the N-terminus of CcmE to the C-terminus of CcmC. We expected to obtain a functional fusion protein because both termini are predicted to be in the cytoplasm. We thus tested whether we could find the same phenotype as we had obtained in mutants with a defect in the cytochrome *c* maturation but not in the formation of holo-CcmE. We first tested heme delivery to CcmE in the fusion protein. The CcmCE fusion protein was charged with heme, but in addition, accumulation of a degradation product corresponding in size to the wild-type holo-CcmE was also observed (Fig. 6A). Additionally, we found that heme was preferentially delivered to free apo-CcmE rather than to the apo-CcmCE fusion protein (*lane 2*). To assess cytochrome *c* formation, Δ*ccmC* (EC28) and Δ*ccmE* (EC65) cells were complemented with the plasmid pEC826 encoding the CcmCE fusion or plasmid

pEC409 encoding CcmC and CcmE individually. We included plasmid pRJ3290 encoding exogenous *B. japonicum* cytochrome c_{550} for a better detection of low amounts of holo-cytochrome *c*. The fusion protein could neither efficiently complement the Δ*ccmC* nor the Δ*ccmE* mutant background as compared to the controls where CcmC and CcmE were expressed individually in the respective backgrounds. The low amounts of holo-cytochrome *c* formation in the mutant strains expressing the fusion protein might be attributed either to a low activity of the fusion protein or to the activity exerted by the cleaved product obtained from the fusion protein (Fig. 6B). As the CcmCE fusion protein cannot efficiently complement cytochrome *c* maturation, this represents another line of support for the idea that CcmE must be mobile for efficient heme delivery.

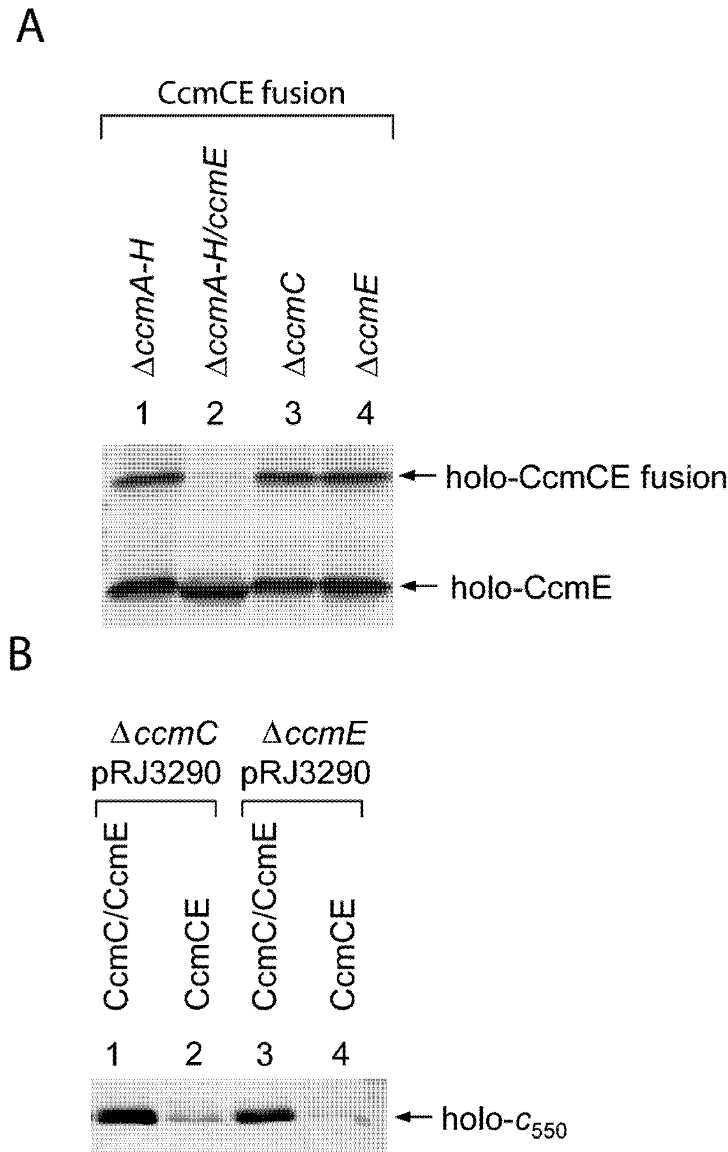

Fig. 6. Functional analysis of the CcmCE fusion protein. *A*, Heme stain of membrane proteins (100 μg per lane) separated by 15% SDS-PAGE from cells grown anaerobically in the presence of 5 mM sodium nitrate of the following strains carrying plasmid pEC826 (encoding the CcmCE fusion): *lane 1*, Δ*ccmA-H* mutant EC06; *lane 2*, EC06/pEC412 (encoding CcmE); *lane 3*, Δ*ccmC* mutant EC28; *lane 4*, Δ*ccmE* mutant EC65. The positions of the holo-CcmCE fusion protein and holo-CcmE are indicated on the right. *B*, Cytochrome *c* maturation of cells grown anaerobically in the presence of 5 mM sodium nitrate of the following strains expressing a histidine-tagged *B. japonicum* cytochrome c_{550} from pRJ3290: *lane 1*, EC28 (Δ*ccmC*)/pEC409 (encoding CcmC and CcmE; *lane 2*, EC28/pEC826 (encoding the CcmCE fusion); *lane 3*, EC65 (Δ*ccmE*)/pEC409; *lane 4* EC65/EC826. Periplasmic proteins (200 μg per lane) were separated by 15% SDS-PAGE and stained for covalently bound heme.

DISCUSSION

Heme delivery is an essential step of cytochrome *c* maturation. In *E. coli* the heme chaperone CcmE plays a crucial role in this process by binding heme transiently in a covalent manner that is still not understood in detail. However, it is known that CcmC, the subject of the present study, is needed for heme transfer to CcmE at the periplasmic side of the membrane. The signature motif WGXXWXWD of CcmC and the two conserved histidines H60 and H184 have been shown to play an important role for the interaction with CcmE (14). Yet, the number of additional highly conserved residues within the 245-amino-acid polypeptide was too high for a rational selection of specific residues for site-directed mutagenesis. Strikingly, many of the best-conserved residues map to the three periplasmic loops, whereas the six transmembrane helices are generally hydrophobic, and the small cytoplasmic parts are the least conserved. The genetic and biochemical analysis of CcmC has confirmed the role of the protein in heme delivery to CcmE, but it is still unclear whether CcmC translocates heme across the membrane or picks it up on the periplasmic side of the membrane, either from the outer leaflet or from a heme transporter, for docking it specifically to the heme chaperone CcmE. A random mutagenesis of *ccmC* was initiated to answer two questions: (i) Can cytoplasmic or membrane-integral amino acids be identified as essential structures for CcmC function? If CcmC was a heme permease, an inward-oriented heme binding site might be identified. In our study we could not identify essential residues in these domains. Site-directed mutations of L82 on the cytoplasmic side of the transmembrane helix II and Y139 in the middle of transmembrane helix IV to alanines had no effect on CcmC function. A deletion of the C-terminal cytoplasmic extension led to an assembly or stability problem and only low levels of truncated CcmC’ were obtained. However, the protein was useful for heme transfer

to CcmE, but not for cytochrome *c* maturation. (ii) Are additional, not strictly conserved amino acids essential for CcmC function? Expected mutant phenotypes ranged from a complete block in heme binding, heme delivery and CcmC-CcmE interaction to partially functioning proteins. For example, was it possible to obtain a mutant that interacted with heme and CcmE but was unable to transfer heme to CcmE? This could indicate an active participation of CcmC in the chemistry of heme attachment to CcmE. This phenotype was not found among our mutants.The genetic screen applied to learn more about CcmC was designed such that it can be used later for other *ccm* genes as well. The combination of a *dms* with a *ccm* mutant led to a colony phenotype that could be easily distinguished from the wild type. In fact, the colony phenotype in our screen proved to be a very sensitive way to test cytochrome *c* maturation.An important finding of this study is that all mutants with a cytochrome *c*-negative phenotype mapped to periplasmic domains. New residues were shown to be functionally important: in domain I by mutants D47N and R55P, in domain II by mutant G111S, and in domain III by mutants S176Y, V177G and L183P. Other residues had previously been changed to alanine by site-directed mutagenesis (22). One of the important histidines, H184, and W119 within the W-rich motif were changed to Y and R, respectively, during the random mutagenesis. In addition, some highly conserved residues that had not been hit by the random mutagenesis were changed to alanine: W114A and R128A representing CcmC-specific extensions of the W-rich motif turned pink, whereas L82A close to the cytoplasmic side of the membrane and Y139A in the middle of transmembrane helix IV formed red colonies like the wild type. Truncation of the C-terminal, cytoplasmic domain led to low levels of CcmC' protein in the membrane. The truncated protein was able to transfer

some heme to CcmE and affected cytochrome *c* maturation even more. The physical interaction with CcmE was not detectable, perhaps due to the low level of protein. G111S and H184Y were the only mutants that lacked interaction with CcmE. An interaction between CcmC and CcmE, to which residues in the periplasmic domains II and III contribute, seems to be an obligatory step in heme delivery to CcmE. We have classified mutants according to their ability to transfer heme to apo-CcmE and to form holo-cytochrome *c* (Table III). Class I mutants had a full (in mutants G111S and H184Y) or partial (in mutants W119R, D126A, V177G and L183P) deficiency in holo-CcmE formation, a white colony phenotype and produced less than 10% of the *c*-type cytochromes found in the wild type. Class II mutants (D47N, R55P, W114A, W125I, R128A and S176Y) showed almost normal heme transfer to apo-CcmE, but were deficient in cytochrome *c* maturation. In this class, the most interesting mutant was S176Y. It produced wild-type levels of CcmC, CcmE and CcmC-CcmE co-precipitates and heme transfer was almost normal, but cytochrome *c* maturation failed. A similar but less prominent behavior was seen with mutants D47N and R55P, and to some extent even with W114A, W125I and R128A that produced intermediate pink colony phenotypes. Class III mutants (L82A, Y139A and W180A) were neither affected in holo-CcmE nor in holo-cytochrome *c* formation. We conclude that besides its role in heme delivery CcmC can affect cytochrome *c* maturation *after* heme is bound to CcmE. Prompted by this hypothesis, the idea that CcmC(D)E form a stable complex with CcmF was tested and rejected because co-precipitation between CcmC and CcmF was not observed even though CcmE precipitated with CcmC and CcmF individually. Our findings imply that CcmC does not interact with CcmF, neither directly nor indirectly via CcmE, and that CcmC and CcmF are involved in different, physically separable steps of cytochrome *c* maturation. Formally it is

also possible that CcmC interacts not only with CcmE but also with apo-cytochrome *c* directly. However, we could not find signals in co-precipitation experiments between CcmC and apo-cytochrome *c* (data not shown). From this work we have no evidence for a "maturase super complex" present in *E. coli* membranes as was hypothesized previously (1). As a more likely alternative explaining the cytochrome *c* deficiency of heme delivery-positive *ccmC* mutants, we consider CcmE to shuttle between CcmC and CcmF for heme transfer to apo-cytochrome *c* (Fig. 7). Subsequent dissociation of CcmC from CcmE may fail in these mutants, thereby inhibiting a proficient follow-up reaction of CcmE with CcmF. If a dynamic movement of CcmE between other components of the cytochrome *c* maturation machinery is essential, the irreversible binding of the two proteins in a CcmCE fusion polypeptide might stop the process. Therefore, a CcmCE fusion protein was constructed and functionally characterized. Heme delivery to the CcmE part of the fusion proteins was quite efficient, but a wild-type CcmE protein was the preferred heme acceptor. Unfortunately, the fusion protein produced a degradation product of approximately the size of wild-type CcmE, suggesting that a protease sensitive site close to the fusion site was present. Nevertheless it was obvious that the fusion protein was less efficient in cytochrome *c* formation than two separate molecules, again supporting the idea of CcmE shuttling between CcmC and CcmF. It had been noted previously that the small membrane protein CcmD is involved in heme transfer to CcmE by CcmC. It was not surprising to see that our mutants were all defective in heme delivery in the absence of CcmD. How CcmD influences the efficiency of heme transfer is unclear. We speculate that a direct interaction of CcmD with CcmC, CcmE or both takes place. The role of CcmD will be addressed in future work.

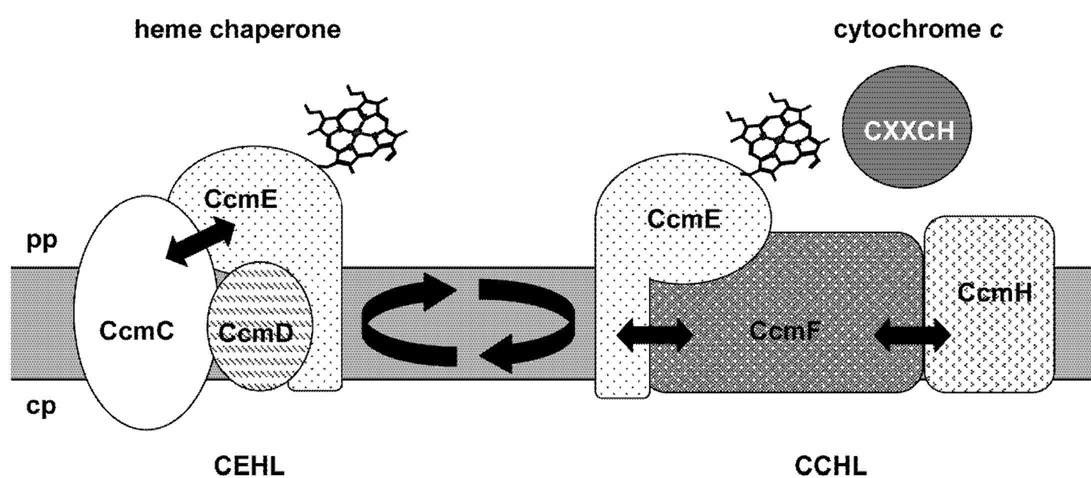

Fig. 7. Model for heme delivery and protein-protein interactions between subunits of the cytochrome *c* maturation apparatus. The putative functions of CcmC and CcmF are abbreviated as follows: CEHL, CcmE heme lyase; CCHL, cytochrome *c* heme lyase.

Acknowledgments—We thank R. Fabianek and H. Schulz for the construction of some plasmids and strains used in this work and D. Bischof for help with the screen. We gratefully acknowledge Joel H. Weiner for providing us with the strain DSS301. This work was supported by the Swiss National Foundation for Scientific Research.

REFERENCES

1. Thöny-Meyer, L. (1997) *Microbiol. Mol. Biol. Rev.* **61**, 337-376
2. Xie, Z., and Merchant, S. (1998) *Biochim. Biophys. Acta.* **1365**, 309-318
3. Kranz, R., Lill, R., Goldman, B., Bonnard, G., and Merchant, S. (1998) *Mol. Microbiol.* **29**, 383-396
4. Page, M. D., Sambongi, Y., and Ferguson, S. J. (1998) *Trends Biochem. Sci.* **23**, 103-108
5. Thöny-Meyer, L., Fischer, F., Künzler, P., Ritz, D., and Hennecke, H. (1995) *J. Bacteriol.* **177**, 4321-4326
6. Grove, J., Tanapongpipat, S., Thomas, G., Griffiths, L., Crooke, H., and Cole, J. (1996) *Mol. Microbiol.* **19**, 467-481
7. Choe, M., and Reznikoff, W. S. (1993) *J. Bacteriol.* **175**, 1165-1172
8. Rabin, R. S., and Stewart, V. (1993) *J. Bacteriol.* **175**, 3259-3268
9. Tanapongpipat, S., Reid, E., Cole, J. A., and Crooke, H. (1998) *Biochem. J.* **334**, 355-365
10. Schulz, H., Hennecke, H., and Thöny-Meyer, L. (1998) *Science* **281**, 1197-1200
11. Schulz, H., Fabianek, R. A., Pellicioli, E. C., Hennecke, H., and Thöny-Meyer, L. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 6462-6467
12. Goldman, B. S., Beckman, D. L., Monika, E. M., and Kranz, R. G. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 5003-5008
13. Gaballa, A., Baysse, C., Koedam, N., Muyldermans, S., and Cornelis, P. (1998) *Mol. Microbiol.* **30**, 547-555

14. Ren, Q., and Thöny-Meyer, L. (2001) *J. Biol. Chem.* **276**, 32591-32596

15. Throne-Holst, M., Thöny-Meyer, L., and Hederstedt, L. (1997) *FEBS Lett.* **410**, 351-355

16. Gaballa, A., Koedam, N., and Cornelis, P. (1996) *Mol. Microbiol.* **21**, 777-785

17. Page, M. D., and Ferguson, S. J. (1999) *Microbiology* **145**, 3047-3057

18. Viswanathan, V. K., Kurtz, S., Pedersen, L. L., Abu-Kwaik, Y., Krcmarik, K., Mody, S., and Cianciotto, N. P. (2002) *Infect. Immun.* **70**, 1842-1852

19. Thöny-Meyer, L., Ritz, D., and Hennecke, H. (1994) *Mol. Microbiol.* **12**, 1-9

20. Xie, Z., and Merchant, S. (1996) *J. Biol. Chem.* **271**, 4632-4639

21. Thöny-Meyer, L. (2000) *Biochim. Biophys. Acta* **1459**, 316-324

22. Schulz, H., Pellicioli, E. C., and Thöny-Meyer, L. (2000) *Mol. Microbiol.* **37**, 1379-1388

23. Goldman, B. S., Beckman, D. L., Bali, A., Monika, E. M., Gabbert, K. K., and Kranz, R. G. (1997) *J. Mol. Biol.* **268**, 724-738

24. Gon, S., Patte, J. C., Mejean, V., and Iobbi-Nivol, C. (2000) *J. Bacteriol.* **182**, 5779-5786

25. Iobbi-Nivol, C., Crooke, H., Griffiths, L., Grove, J., Hussain, H., Pommier, J., Mejean, V., and Cole, J. A. (1994) *FEMS Microbiol. Lett.* **119**, 89-94

26. Sambasivarao, D., and Weiner, J. H. (1991) *J. Bacteriol.* **173**, 5935-5943

27. Miller, J. H. (1992) *A short Course in Bacterial Genetics*, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY

28. Hanahan, D. (1983) *J. Mol. Biol.* **166**, 557-580

29. Bullock, W. O., Fernandez, J. M., and Short, J. M. (1987) *BioTechniques* **5**, 376-379

30. Thöny-Meyer, L., Künzler, P., and Hennecke, H. (1996) *Eur. J. Biochem.* **235**, 754-761

31. Pommier, J., Mejean, V., Giordano, G., and Iobbi-Nivol, C. (1998) *J. Biol. Chem.* **273**, 16615-16620

32. Oresnik, I. J., Ladner, C. L., and Turner, R. J. (2001) *Mol. Microbiol.* **40**, 323-331

33. Bonnefoy, V., and DeMoss, J. A. (1994) *Antonie van Leeuwenhoek* **66**, 47-56

34. Cole, J. (1996) *FEMS Microbiol. Lett.* **136**, 1-11

35. Gennis, R. B., and V. Stewart. (1996) *Respiration*, In: F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger, (eds) *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology, ASM Press, Washington, D. C., 217-261

36. Ren, Q., Ahuja, U., and Thöny-Meyer, L. (2002) *J. Biol. Chem.* **277**, 7657-7663

37. Meissner, P. S., Sisk, W. P., and Berman, M. L. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 4171-4175

38. Greener, A., and Callahan, M. (1994) *Strategies* **7**, 32-34

39. Fabianek, R. A. (1999) *New insights into the redox pathway of bacterial cytochrome c maturation: CycY/CcmG and CcmH,* Diss ETH Nr. 13324, Zürich

40. Tusnady, G. E., and Simon, I. (1998) *J. Mol. Biol.* **283**, 489-506

CHAPTER 3

CcmD is involved in complex formation between CcmC and the heme chaperone CcmE during cytochrome *c* maturation

Umesh Ahuja and Linda Thöny-Meyer

Institut für Mikrobiologie, Department Biologie

Eidgenössische Technische Hochschule,

Wolfgang Pauli Strasse 10, CH-8093 Zürich, Switzerland

Journal of Biological Chemistry 280 (1): 236-243 (2005)

SUMMARY

CcmD is a small membrane protein involved in heme delivery to the heme chaperone CcmE during cytochrome *c* maturation. Here we show that it physically interacts with CcmE and with CcmC, another essential component of the heme delivery system. We demonstrate the formation of a ternary complex consisting of CcmCDE. A deletion analysis of individual domains revealed that the central hydrophobic domain is essential for its function. Moreover, the C-terminal, cytoplasmic domain seems to require a net positive charge in order to be functional. Our topology analysis indicates that CcmD is an integral interfacial membrane protein with its N- and C-termini extruding into the cytoplasmic side of the membrane. Interactions of CcmD with either ferrochelatase, the last heme biosynthetic enzyme, or directly with heme were not detectable. We postulate a function for CcmD in protein-protein interaction or membrane protein assembly required for the heme delivery process.

INTRODUCTION

Cytochromes of the *c* type are a class of metal proteins that contain covalently bound heme as a prosthetic group to perform functions in respiratory and photosynthetic electron transport. Three distinct systems for the biogenesis of *c*-type cytochromes have evolved in nature, which are annotated as system I, II and III (reviewed in (1-6)). The hallmark of cytochrome *c* maturation in all three systems is the covalent attachment of heme via thioether bonds to cysteinyl residues of the conserved CXXCH signature motif of apo-cytochrome. The γ-proteobacterium *Escherichia coli* uses system I with eight membrane proteins encoded by the *ccmABCDEFGH* operon (7,8). A key intermediary step during this maturation pathway is the transfer and covalent attachment of heme to the cytochrome *c* maturation-specific heme chaperone CcmE (9); subsequently, heme is transferred from the holo-CcmE intermediate to apo-cytochrome *c*. It has been shown earlier that covalent binding of heme to an essential histidine (H130) of apo-CcmE requires the activity of the CcmC maturation factor. This activity as well as the levels of CcmE polypeptides in the membranes are significantly enhanced in the presence of the CcmD protein (10). CcmD is essential for cytochrome *c* maturation, but a *ccmD* mutant phenotype can be bypassed by overexpression of the CcmCE proteins (10). CcmD is a small polypeptide of 69 amino acid residues with a predicted N-terminal transmembrane helix and a hydrophilic C-terminal domain. The *ccmD* gene is present downstream of *ccmC* in most bacteria that use system I for cytochrome *c* maturation. Despite little sequence similarity between different CcmD homologues, the hydrophilic domains are rich in charged residues, predominantly arginines. The membrane topology of CcmD has been assumed to be like that of its *Rhodobacter capsulatus* homologue, HelD. In this case the C-terminus was proposed to reside in the cytoplasm based on the activity of a

LacZ fusion and on the inactivity of a corresponding PhoA fusion (11). In addition, the absence of positive charges N-terminal to the predicted transmembrane helix is in agreement with an N-terminus out-C-terminus-in topology. Here, we investigated the function of CcmD in the CcmC-mediated transfer of heme to CcmE by testing its involvement in the formation and/or stability of a CcmCDE membrane protein complex, assessing its topology in the membrane and defining functional domains and residues.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions—Bacterial strains and plasmids used in this study are listed in Table I. Bacteria were grown aerobically in LB medium or anaerobically in minimal salts medium (12) supplemented with 0.4% glycerol, 40 mM fumarate and 5 mM sodium nitrate as the terminal electron acceptor. Antibiotics were added at the following final concentrations: ampicillin, 200 μg/ml; chloramphenicol, 25 μg/ml. If necessary, cells were induced with 0.1% arabinose at mid-exponential growth phase.

Construction of plasmids and site-directed mutagenesis—*E. coli* strain DH5α (13) was used as host for different clonings. The expression plasmid pEC36 which codes for a N-terminal hexa histidine tagged (H_6-) CcmD was obtained by ligation of *Nde*I- and *Bam*HI-digested PCR-amplified *ccmD* fragment into the *Nde*I- and *Bam*HI-treated 5.4-kb pET28a (Novagen, USA). Plasmid pEC423 was then obtained by ligation of the 310 bp *Xba*I- and *Eco*RI-digested fragment from pEC36 into the 5.4-kb *Xba*I- and *Eco*RI-digested pEC411. Plasmid pEC474 coding for wild-type CcmD was constructed by re-ligation of *Nsi*I-treated plasmid pEC99, thereby deleting *ccmC*. The plasmids pEC834 and pEC835 expressing N-terminally H_6-tagged $CcmD^{1-37}$ and $CcmD^{1-53}$, respectively, were constructed by the QuikChange^{TM} site-directed mutagenesis using pEC423 as a template and the primers listed in Table II. The plasmids pEC858 and pEC859 expressing H_6-tagged $CcmD^{1-53}$R44A and $CcmD^{1-53}$R48A, respectively, were constructed by the QuikChange^{TM} site-directed mutagenesis using pEC835 as a template. Plasmid pEC860 expressing the double mutant H_6-$CcmD^{1-53}$R44AR48A was

obtained by QuikChange™ site-directed mutagenesis using pEC859 as a template. pEC842, expressing CcmD with a C-terminal strep-tag (-SAWSHPQFEK) was constructed by amplifying the *ccmD* gene from pEC86 with primers ccmDnNdeI and ccmDcstrepSalI-r. This fragment was digested with *Nde*I and *Sal*I and ligated into the 5.4-kb *Nde*I- and *Sal*I-digested pISC-2 (14). pEC856, expressing the N-terminally truncated $CcmD^{14\text{-}69}$-strep was constructed by amplifying a 200-bp fragment by PCR with the primers ccmDnM14NdeI and ccmDcSalI-2, using pEC86 as a template. The PCR product was digested with *Nde*I and *Sal*I and ligated into the 5.4-kb *Nde*I- and *Sal*I-digested pISC-2. For the construction of a plasmid expressing wild-type CcmD-strep, the *ccmD* gene was amplified by PCR using a forward primer, pACYC184:1613-1629, containing a *Bfm*I site and a reverse primer, ccmDcStrepSalI, with a *Sal*I site and pEC474 as a template. The PCR-amplified fragment was digested with *Bfm*I- and *Sal*I and ligated into the 3.7-kb *Bfm*I- and *Sal*I-digested pACYC184 (15). The resulting plasmid was pEC857. For the expression of the C-terminal domain of CcmD, plasmid pEC843 was constructed by cloning the 102-bp PCR product obtained after amplification with primers solccmDNdeI and solccmDEcoRI and pEC86 as a template, digesting with *Nde*I and *Eco*RI and ligating it into the 5.4-kb *Nde*I and *Eco*RI digested fragment of pISC-2. Plasmid pEC848 expressing a C-terminally H_6-tagged CcmH was constructed by amplifying the *ccmH* gene with primers ccmHNdeI and ccmHhis containing a *Xba*I site and using pEC86 as a template. The 1055-bp *Nde*I- and *Xba*I-digested fragment was then ligated into *Nde*I- and *Xba*I-digested 5.4-kb pISC-2. Plasmid pEC869 expressing N-terminally H_6-tagged and C-terminally strep-tagged CcmD was constructed by ligating the 340-bp *Xba*I and *Eco*RI digested PCR product obtained with primers pING1araB and ccmDcstrepEcoRI-r and pEC423 as the template into the 5383- bp *Xba*I and *Eco*RI-digested pEC423.

TABLE I: Bacterial strains and plasmids used in this study

Bacterial strains or plasmids	Relevant genotype	Reference
Escherichia coli		
DH5α	*SupE*44 Δ*lacU*169 (Φ80*lacZΔ*M15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	(13)
EC06	MC1061 Δ*ccmA-H*::*kan*	(7)
EC76	MC1061 Δ*ccmD*	(10)
Plasmids		
pRJ3290	*B. japonicum cycA*-H_6 cloned in pISC2, Ap^R	(24)
pEC36	H_6-*ccmD* cloned in pET28a, Kan^R	This study
pEC86	*ccmABCDEFGH* cloned into pACYC184, Cm^R	(25)
pEC99	*ccmCD* cloned into pACYC184, Cm^R	(17)
pEC406	*ccmCDE* cloned into pACYC184, Cm^R	(10)
pEC408	*ccmDE* cloned into pACYC184, Cm^R	(10)
pEC409	*ccmCE* cloned into pACYC184, Cm^R	(10)
pEC410	*ccmE* cloned in pACYC184, Cm^R	(10)
pEC411	H_6-*ccmC* cloned in pISC-3, Ap^R	(9)
pEC412	*ccmE* cloned into pISC-2, Ap^R	(9)
pEC422	H_6-*ccmC* cloned in pISC-2, Ap^R	(10)
pEC423	H_6-*ccmD* cloned in pISC-3, Ap^R	This study
pEC424	*ccmF*-H_6 cloned in pISC2, Ap^R	(18)
pEC474	*ccmD* cloned in pACYC184, Cm^R	This study
pEC486	*ccmC*-H_6 cloned in pACYC184, Cm^R	(17)
pEC701	*B. japonicum cycA*-H_6 cloned in pACYC184, Cm^R	(18)
pEC711	*ccmH*-H_6 cloned in pACYC184, Cm^R	(18)
pEC799	*ccmDE* cloned into pISC2, Ap^R	(24)
pEC834	H_6-*ccmD*$^{1\text{-}37}$ cloned in pISC-2, Ap^R	This study
pEC835	H_6-*ccmD*$^{1\text{-}53}$ cloned in pISC-2, Ap^R	This study
pEC841	*hemH*-strep cloned in pACYC184, Cm^R	This study
pEC842	*ccmD*-strep cloned in pISC-2, Ap^R	This study
pEC843	*ccmD*$^{38\text{-}69}$ cloned in pISC-2, Ap^R	This study

pEC848	*ccmH*-H_6 cloned in pISC-2, Ap^R	This study
pEC856	*ccmD*$^{14-69}$-strep cloned in pISC-2, Ap^R	This study
pEC857	*ccmD*-strep cloned in pACYC184, Cm^R	This study
pEC858	H_6-*ccmD*$^{1-53}$R44A cloned in pISC-2, Ap^R	This study
pEC859	H_6-*ccmD*$^{1-53}$R48A cloned in pISC-2, Ap^R	This study
pEC860	H_6-*ccmD*$^{1-53}$R44AR48A cloned in pISC-2, Ap^R	This study
pEC869	H_6-*ccmD*-strep cloned in pISC-3, Ap^R	This study

Plasmid pEC841 expressing *hemH* that encodes the *E. coli* ferrochelatase with a C-terminal strep-tag (-SAWSHPQFEK) was constructed by amplifying the *hemH* gene including its natural ribosome binding site from chromosomal DNA obtained from *E. coli* strain MC1061 with primers uphemHBamHI-f and hemHcstrepSalI-r. The 1041-bp fragment was digested with *Bam*HI and *Sal*I and ligated into the 4-kb *Bam*HI- and *Sal*I-digested pACYC184.

DNA sequencing—All plasmid constructs and mutants derived from PCR products were confirmed by DNA sequence analysis (Microsynth, Balgach, Switzerland). In plasmid pEC848, a spontaneous frame shift mutation was created by a T→C transition, extending the coding sequence for CcmH-H_6 by two additional codons for Q and L.

Co-purification with histidine-tagged proteins—10 mg of membrane proteins obtained from anaerobically grown cells were solubilized in 10 ml solubilization buffer containing 200 mM sodium phosphate, pH 8.0, 300 mM NaCl and 1% n-dodecyl-β-D-maltoside (GLYCON Biochemicals, Germany) for 1 hour at 4°C. The insoluble material was removed by

TABLE II: Oligonucleotide primers used for plasmid construction and sequencing

Primer	Bases	Nucleotide sequence (5´-3´)	Usage
ccmDnNdeI	26	cggatccatatgacccctgcatttgc	Forward primer for cloning pEC36 and pEC842
ccmDc	21	cgggatcctcatgcagcctcc	Reverse primer for cloning pEC36
ccmD37f	29	ggttttggtcgtgtgatcggtgatgcaac	QuikChange™ primer for cloning pEC832 and 834
ccmD37r	29	gttgcatcaccgatcacacgaccaaaacc	QuikChange™ primer for cloning pEC832 and 834
ccmD53f	23	ggcgcaacagtgagcgcgtgagg	QuikChange™ primer for cloning pEC833 and 835
ccmD53r	23	cctcacgcgctcactgttgcgcc	QuikChange™ primer for cloning pEC833 and 835
$ccmD_C$strepSalI-r	66	tgcgtcgactcatttttcgaactgcgggtggctcca agcgcttgcagcctcctgctgttgcgcagc	Reverse primer for cloning pEC842 and pEC857
solccmDNdeI	35	ggacgatccatatgcactcggtgatgcaacatcgc	Forward primer for cloning pEC843
solccmDEcoRI	25	cgggaattctcatgcagcctcctgc	Reverse primer for cloning pEC843
ccmHnNdeI	29	ggacgatccatatgaggtttttattgggc	Forward primer for cloning pEC848
ccmHHis	49	gctctagagcta$(gtg)_6$tttactctcctgcggcgac	Reverse primer for cloning pEC848 and contains *Xba*I site
ccmDnM14NdeI	36	cgggatcatatgggcggttacgcctttttttgtctgg	Forward primer for cloning pEC856
ccmDcsalI-2	27	tgcgtcgactcatttttcgaactgcgg	Reverse primer for cloning pEC856
pACYC184: 1613-1629	17	cggcaccgtcacctgg	Forward primer for cloning pEC857

ccmDR44A-f	22	gatgcaacatgccgc aattctg	QuikChange^{TM} primer for cloning pEC858 and pEC860
ccmDR44A-r	22	cagaattgcggcatgttgcat c	QuikChange^{TM} primer for cloning pEC858 and pEC860
ccmDR48A-f	23	cgcaattctggctggcgtggcgc	QuikChange^{TM} primer for cloning pEC859
ccmDR48A-r	23	gcgccacgccagccagaattgcg	QuikChange^{TM} primer for cloning pEC859
pING1araB	18	ggccgacgaaatcactcg	PCR primer for cloning pEC869
ccmDcstrepEcoRI-r	66	tgcgaattctcatttttcgaactgcgggtggctccaa gcgcttgcagcctcctgctgttgcgcagc	PCR primer for cloning pEC869
ccmH442-463	22	gttgtttatctgccgggtattg	Sequencing primer
uphemHBamHI-f	29	cgcggatccacttttccgctacaattatc	Forward primer for cloning pEC841
hemHcstrepSalI-r	66	tgcgtcgactcatttttcgaactgcgggtggctcca agcgctgcgatacgcggcaacaagattagc	Reverse primer for cloning pEC841
hemH461-479	19	cggggatatcgtttatacg	Sequencing primer

ultracentrifugation at 150,000 x g for 60 min at 4°C. The solubilized fraction (supernatant) was collected and directly loaded onto a 1 ml Ni-NTA column pre-equilibrated with 20 mM Tris-HCl buffer, pH 8.0, 300 mM NaCl and 0.1% n-dodecyl-β-D-maltoside. The column was washed with washing buffers (20 mM Tris-HCl buffer, pH 8.0, 300 mM NaCl and 0.1% n-dodecyl-β-D-maltoside) with increasing concentration of imidazole (5-30 mM). The proteins were eluted with equilibration buffer containing 300 mM imidazole. The eluted proteins were

separated by 15% SDS-PAGE, transferred onto a polyvinylidene difluoride (PVDF) membrane and immuno-stained as described below.

Co-purification with strep-tagged protein—10 mg membrane proteins obtained from anaerobically grown cells were solubilized as described above for 60 min at 4°C and directly loaded onto a 1-ml StrepTactin-Sepharose column pre-equilibrated with 100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% n-dodecyl-β-D-maltoside. The column was washed with equilibration buffer and the proteins were eluted with equilibration buffer containing 2.5 mM desthiobiotin. The eluted proteins were separated by 15% SDS-PAGE and transferred onto a PVDF membrane and immuno-stained as described below.

Cell fractionation—For the preparation of subcellular fractions bacterial cultures were grown anaerobically in the presence of nitrate. Periplasmic proteins obtained from 1200 ml cultures were treated with polymyxin B sulfate as described previously (16). For the preparation of membrane proteins 600 ml of anaerobically grown bacterial culture were harvested, and membrane proteins were extracted as described previously (16).

Biochemical methods—Protein concentrations were determined using the Bradford assay (Bio-Rad) and BSA as a standard. Holo-cytochrome c_{550} and holo-CcmE formation was analyzed qualitatively by heme staining and Immunoblot (17). Soluble cytochrome *c* (c_{550}) in the periplasmic fractions was quantified by absorption difference spectroscopy using a $\varepsilon_{550-536nm}$ of 23.2 $mM^{-1}cm^{-1}$ (14). Immunoblot analysis of H_6-tagged proteins was performed

using monoclonal tetra-His antibodies (Qiagen) at a dilution of 1:2000. Immunoblot analysis of the strep-tagged CcmD versions was performed using streptavidin-alkaline phosphatase conjugate (Amersham Pharmacia) at a dilution of 1:4000. Antibodies directed against the CcmE peptide (9) or CcmF peptides (18) were used at a dilution of 1:5000 and 1:10,000, respectively. Signals were detected using goat anti-mouse IgG (for H_6-tag detection) or goat anti-rabbit IgG (for detection of other antigens) conjugated to either alkaline phosphatase (Bio-Rad) or horse radish peroxidase (Sigma) as secondary antibody and CSPD (Roche Diagnostics) or ECL (Amersham Pharmacia) reagent as substrate, respectively.

Heme binding assays—150 µg total membrane proteins were solubilized at 4°C for 1 h in 1 ml of solubilization buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5 mM PMSF, 0.5% lauroyl sarcosine). After centrifugation, the supernatant was incubated with hemin agarose (Sigma) or aminododecyl agarose (Sigma) as described previously for CcmC (16). Agarose beads were washed three times with solubilization buffer and twice with PBS to remove unbound material, then treated with 2 x SDS loading dye (containing 50 mM DTT) and incubated at room temperature for 20 min. The mixture was subjected to 15% SDS-PAGE. Proteins in the gel were visualized by immunoblot analysis.

Tricine gels— 16% tricine gels with a 10% spacer gel were used to separate smaller truncated versions of CcmD as initially described by Schägger and Jagow (19).

Co-immunoprecipitation—1-2 mg membrane proteins were solubilized and precipitated with 5 µl undiluted anti-CcmE immunoglobulines, or 10 µl of anti-H_6-tag antibodies according to

the method described previously (16) except that solubilization was carried out in 200 mM phosphate buffer, pH8.0, containing 300 mM NaCl, 5mM EDTA and 1% n-dodecyl-β-D-maltoside. The proteins were subjected to 15% SDS-PAGE, transferred to a PVDF membrane and probed with avidine-alkaline phosphatase conjugate against the strep-tagged CcmD or the strep-tagged ferrochelatase.

Pulse chase experiment— The pulse chase experiment was performed as described previously (14) but with some modifications. Briefly, cells were grown aerobically at 37°C in M9 medium supplemented with glycerol and 18 amino acids except methionine and cysteine. Cells in their mid-exponential growth phase were induced with 0.1% arabinose for an hour prior to addition of 20 μCi/ml Trans ^{35}S-label™ (1175 Ci/mol, MP BIOMEDICALS). After 1 minute, 1 ml of the culture was withdrawn and immediately TCA-precipitated, and in parallel, the remaining culture was chased by adding unlabelled methionine and cysteine at 1 mg/ml each. For the immunoprecipitation of the samples 5 mg pre-swelled protein-A sepharose beads (Pharmacia) per sample were incubated with 10 μl anti-H_6 tag antibody (0.2 mg/ml, Qiagen) for 1 h at 4°C and then treated as described. Finally, the immuno-precipitated samples were incubated for 30 minutes at room temperature in 2X SDS loading dye containing 50 mM DTT and then loaded on a 15 % SDS polyacrylamide gel.

Formation of spheroplasts and inside out (ISO) vesicles and proteinase K treatment— Aerobically growing EC06 cells transformed with either plasmid pEC869 or pEC411 were induced with 0.1% arabinose at mid log growth phase for 3 h at 37°C. The spheroplasting and proteinase K treatment was performed as described by de Gier et al. (20). For ISO vesicles

cells were passed once through a French pressure cell (83 bars) and membrane vesicles were made as described previously (16). Proteinase K (Roche Diagnostics) was added at a final concentration of 0.33 mg/ml and incubated for 1 h on ice.

Treatment of membrane vesicles with various chaotropic agents— 50 μg membrane proteins obtained from bacterial cultures of strains EC06/pEC869 and EC06/pEC411 were treated with Na_2CO_3 (50 mM and 100 mM) pH 11.5, 500 mM $LiClO_4$ and 1M urea for 1 h on ice or by five freeze and thaw cycles, which involved quick freezing in liquid nitrogen and thawing at 37°C in a water bath. After the treatment, samples were centrifuged for 1 h at 150,000 g. Supernatant and pellet was collected from each sample. Proteins in the supernatant were TCA precipitated prior to separation by 15 % SDS-PAGE. The proteins were visualized by immunoblot analysis using anti-H_6-tag antibodies.

RESULTS

Functional analysis of truncated CcmD derivatives—CcmD has three distinct domains: an N-terminal domain (amino acids 1-10: MTPAFASWNE), a hydrophobic, putative transmembrane domain (amino acids 11-37: FFAMGGYAFFVWLAVVMTVIPLVVLVV) and a C-terminal highly charged domain (amino acids 38-69: HSVMQHRAILRGVAQQRAREARLRAAQQQEAA). To test whether each of these three domains is necessary for CcmD function, plasmids expressing derivatives lacking the N-terminal, C-terminal and N-terminal plus membrane domains were constructed as depicted in Fig. 1. In addition, an N-terminal H_6-tag and/or a C-terminal strep-tag were introduced to allow detection and/or purification of the proteins. In the *ccmD* in-frame deletion mutant EC76 (ΔM-A69) the entire *ccmD* gene is deleted (10). In our complementation experiments we overexpressed exogenous *Bradyrhizobium japonicum* cytochrome c_{550} to monitor cytochrome *c* maturation. The Δ*ccmD* mutant strain was co-transformed with a plasmid expressing a H_6-tagged *B. japonicum* cytochrome c_{550} plus a plasmid encoding either wild-type or mutant CcmD protein (Fig. 2A). Cytochrome *c* formation was assayed qualitatively by heme staining of periplasmic fractions (Fig. 2A, upper panel). Quantification of *c*-type cytochromes was done by absorption difference spectroscopy. The level of holo-cytochromes *c* produced by the *ccmD* mutant complemented with N-terminally H_6-tagged CcmD was set to 100%. The N-terminally truncated mutant $CcmD^{14-69}$ that in addition carried a C-terminal strep-tag (*lane 2*) was not inhibited in cytochrome *c* formation when compared to the wild type (*lane 1*), but even produced slightly higher levels of holo-cytochrome *c*. This showed

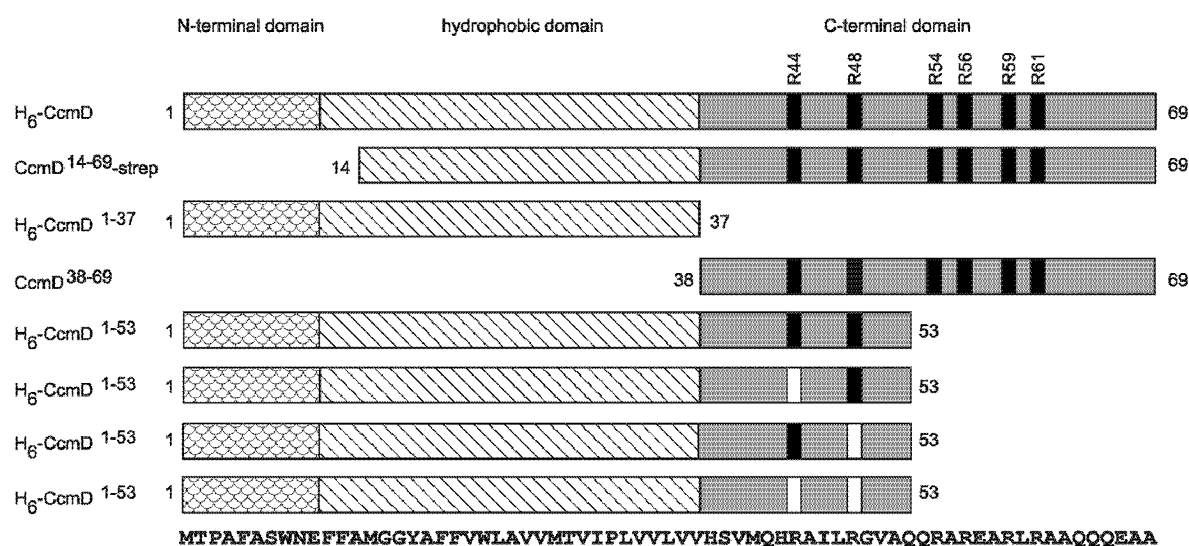

Fig. 1. Proposed topological domains of *E. coli* CcmD and constructs used in this study. The topology model was constructed by using the program HMMTOP (21). It predicts CcmD as a monotopic membrane protein. Full-length and truncated mutants of H_6-tagged CcmD were generated as represented schematically. The N-terminal H_6- and C-terminal strep-tags are indicated. The position of arginine residues in the C-terminal domain are designated in black, and the white boxes represent arginine to alanine point mutations. The entire amino acid sequence of CcmD corresponding to the respective domains is given at the bottom of the figure.

that neither lack of the first 13 N-terminal residues nor a C-terminal strep-tag in CcmD negatively affected cytochrome *c* maturation. Thus, the hydrophobic and the C-terminal domain are sufficient for CcmD function. When the C-terminal domain was deleted completely, cytochrome *c* formation was below the level of detection (*lane 4*), indicating that the membrane domain alone is not functional. Likewise, when the C-terminal domain was expressed in the absence of the hydrophobic domain, i.e. as a soluble cytoplasmic protein (*lane 8*), no cytochrome *c* was formed. This suggested that the hydrophobic and C-terminal domains together provide the CcmD function. Deletion of the last 16 amino acids removed

only half of the cytoplasmic domain and resulted in a ~50% level of cytochrome *c* formation. One of the prominent features of the C-terminal region among all the CcmD homologues is the extraordinary abundance of basic residues, particularly arginines. *E. coli* CcmD protein has six arginines at positions 44, 48, 54, 56, 59 and 61 in its C-terminal region. The mutant $CcmD^{1\text{-}53}$ retains two out of six arginines and two histidines, whereas mutant $CcmD^{1\text{-}37}$ lacks all of these basic residues. Our results show that the C-terminal region between the 37th and 53rd residue (HSVMQHRAILRGVAQQ) containing R44 and R48 is functionally important. In view of the abundance of positive charges in CcmD proteins, we constructed single point mutants R44A and R48A and a double mutant R44A/R48A of the H_6-$CcmD^{1\text{-}53}$ variant and tested them for cytochrome *c* maturation. The single mutants R44A (*lane 5*) and R48A (*lane 6*) produced similar levels of holo-cytochrome *c* as their parent CcmD derivative H_6-$CcmD^{1\text{-}53}$ (*lane 3*), whereas the double arginine mutant (*lane 7*) was drastically impaired in cytochrome *c* formation (20% residual cytochrome *c* level). We conclude that a C-terminal domain rich in positive charges is important for CcmD function.An intermediary, essential step during cytochrome *c* maturation is the delivery of heme to CcmE by CcmC. CcmD is known to aid this process by enhancing the levels of CcmE protein in the membrane (10). Thus, we tested whether the varied cytochrome *c* deficiencies observed in different CcmD mutants were due to the block in CcmC-E heme delivery and/or CcmE stability. Membranes were isolated from a strain lacking the entire *ccm* operon (EC06) and containing plasmid pEC409 encoding CcmC and CcmE plus a second plasmid expressing either the wild-type or a mutant *ccmD* allele.

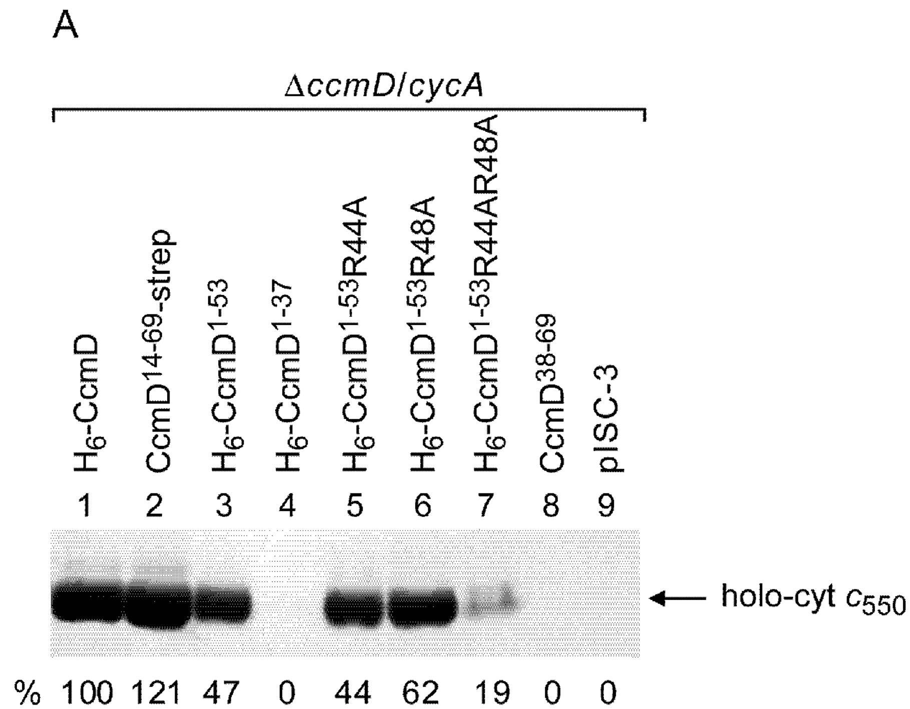

Fig. 2. Functional analysis of *ccmD* mutants. *A*, Cytochrome *c* formation. The Δ*ccmD* strain EC76 was co-transformed with plasmid pEC701 (expressing *B. japonicum* cytochrome c_{550}) and pEC423 (wild-type H_6-CcmD; *lane 1*), pEC856 ($CcmD^{14-69}$-strep; *lane 2*), pEC835 (H_6-$CcmD^{1-53}$; *lane 3*), pEC834 (H_6-$CcmD^{1-37}$; *lane 4*), pEC858 (H_6-$CcmD^{1-53}$R44A; *lane 5*), pEC859 (H_6-$CcmD^{1-53}$R48A; *lane 6*), pEC860 (H_6-$CcmD^{1-53}$R44AR48A; *lane 7*), pEC843 ($CcmD^{38-69}$; *lane 8*) or pISC-3 (empty vector; *lane 9*). Periplasmic proteins (100 µg per lane) from anaerobically grown cells were separated by 15% SDS-PAGE and stained for covalently bound heme. The percentage of holo-cytochrome *c* formed relative to the wild type as deduced from difference spectra is given below. The position of cytochrome c_{550} is indicated on the right.

Holo-CcmE formation was determined by heme stain (Fig. 2B, first panel) and was found to be enhanced in the N-terminally truncated mutant, $CcmD^{14-69}$-strep (*lane 2*) compared to the wild type (*lane 1*). The mutant H_6-$CcmD^{1-53}$ (*lane 3*) produced similar levels of holo-CcmE

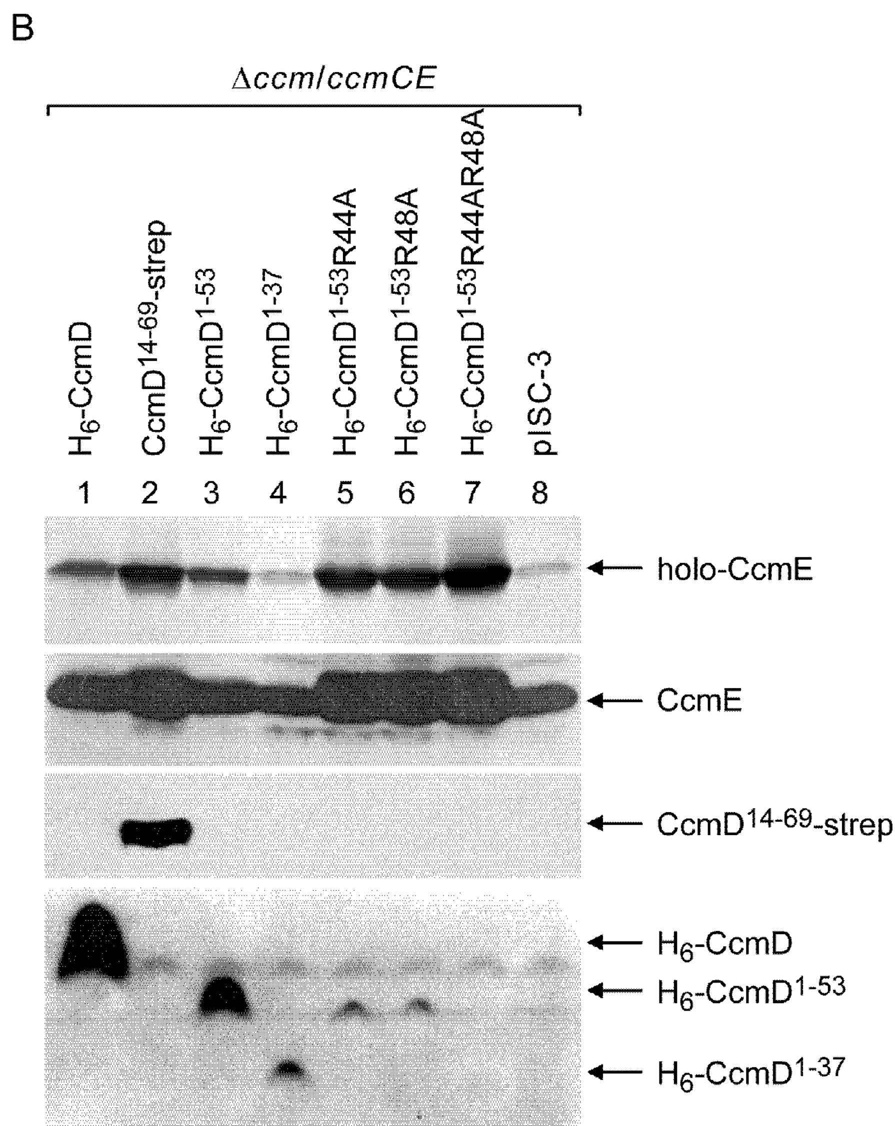

Fig. 2. Functional analysis of *ccmD* mutants. *B*, Heme transfer to CcmE. The Δ*ccmA-H* mutant strain EC06 was co-transformed with plasmids pEC409 (expressing CcmCE) and the same plasmids as in *A*. First panel: heme stain of membrane proteins (100 μg per lane) isolated from anaerobically grown cells after separation by 15% SDS-PAGE. Second panel: immunoblot of membrane proteins (100 μg per lane) probed with antiserum against CcmE. Third panel: immunoblot of membrane proteins (100 μg per lane) probed with streptavidin-alkaline phosphatase conjugate against the strep-tag. Fourth panel: immunoblot of membrane proteins (100 μg per lane) probed with antiserum against the H_6-tag after proteins were separated by 16% tricine-PAGE. Equal levels of membrane proteins in each lane were also visualized in a Coomassie stained gel (supplementary Figure 1). The positions of holo-CcmE, CcmE peptide and the various CcmD derivatives are indicated on the right.

like the wild type, whereas the mutant $CcmD^{1-37}$(*lane 4*) lacking the entire hydrophilic domain produced as little holo-CcmE as a strain lacking CcmD completely (*lane 8*). Surprisingly, the single and double arginine mutants of H_6-$CcmD^{1-53}$ (*lane 5-7*) produced slightly enhanced amounts of holo-CcmE. The levels of CcmE polypeptide (Fig. 2B, second panel) were probed by immunoblot analysis and found to correspond to those of holo-CcmE. The result of the double arginine mutant is particularly striking, because this mutant produces enhanced levels of apo- and holo-CcmE while it is inhibited in cytochrome *c* formation. To assess whether the various CcmD derivatives were localized to the membrane, we used immunodetection of their respective tags. The H_6- and strep-tagged CcmD versions were detected on immunoblots of tricine gels with the migration patterns and signal intensities corresponding to the molecular weights. However, only weak signals were obtained from the single and no signal from the double arginine mutants. We cannot exclude that the change of the charges within these small peptides has affected transfer efficiency during electroblotting. Alternatively, the removal of positive charges in CcmD alters the physical behavior of the protein.

Involvement of CcmD in heme delivery—No direct studies have been conducted on the protein-protein interaction of CcmD with other Ccm proteins. Because CcmD stimulates CcmC-mediated heme delivery to CcmE, we initially focused on these two proteins as possible interaction partners for CcmD. In addition, it is possible that CcmD is involved in mediating the further transfer of heme to cytochrome *c* via CcmF/CcmH. In this context we expected CcmD to interact with the membrane proteins CcmC, CcmE, CcmF and/or CcmH.

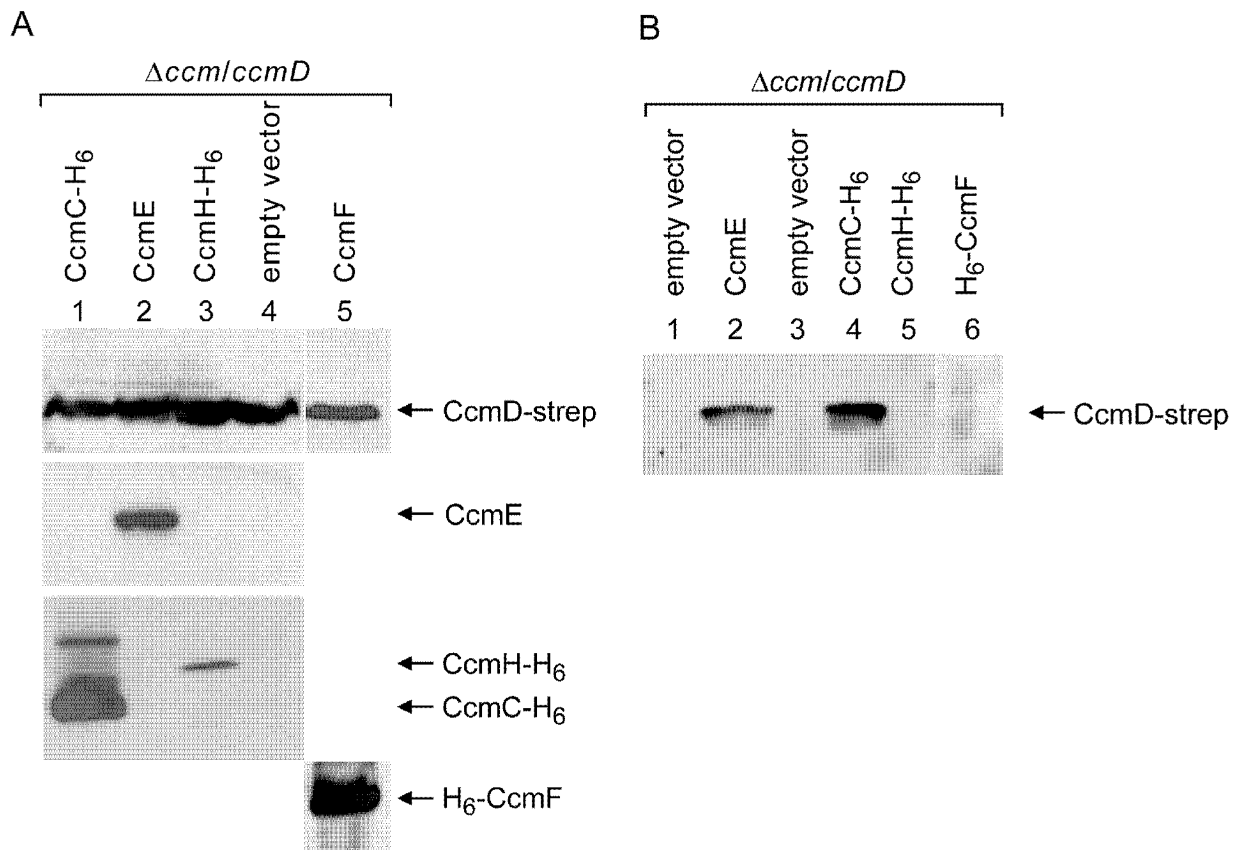

Fig. 3. Co-immunoprecipitation of CcmD with other Ccm proteins. The Δ*ccmA-H* mutant strain EC06 was co-transformed with plasmid pEC842 (*lanes 1-4*) or pEC857 (*lane 5*), expressing C-terminally strep-tagged CcmD, and pEC486 (CcmC-H_6; *lane 1*), pEC410 (CcmE; *lane 2*), pEC711 (CcmH-H_6; *lane 3*), pACYC184 (empty vector; *lane 4*), and pEC424 (H_6-CcmF; *lane 5*). Cells were grown anaerobically in the presence of nitrate. *A*, Detection of the membrane proteins (100 μg per lane) after separation by 15% SDS-PAGE and immuno detection with streptavidin for CcmD-strep (top panel), with antiserum against the CcmE (second panel), antiserum against the H_6-tag for CcmC and CcmH (third panel), and antiserum against the CcmF (fourth panel). The positions of CcmD-strep, CcmE, CcmC-H_6, CcmH-H_6 and CcmF are indicated on the right. *B*, Co-immunoprecipitation. The membrane proteins were precipitated with anti-H_6-tag antibodies (*lane 3-6*) and anti-CcmE-peptide antibodies (*lane 1-2*) and detected for CcmD-strep. The position of CcmD-strep is indicated on the right.

To test this hypothesis we performed pair wise co-immunoprecipitation experiments with these proteins, using strep-tagged CcmD that was co-expressed either with CcmC-H_6, CcmE, H_6-CcmF or CcmH-H_6, in the Δ*ccmA-H* strain EC06 (Fig. 3B). Membrane proteins were tested for the presence of the expressed polypeptides (Fig. 3A) and then precipitated either with anti-CcmE serum (Fig. 3B lanes *1 and 2*) or anti-H_6-tag antibody (Fig. 3B lanes *3-6*), and the precipitates were analyzed for the presence of CcmD-strep by immunoblot. CcmD was found to co-precipitate with CcmE (*lane2*) and CcmC (*lane 4*), but not with CcmF (*lane 6*) and CcmH (*lane 5*). Next we examined the interactions in a co-purification experiment to specifically evaluate the affinity of CcmD towards CcmC and CcmE. CcmD-strep was co-expressed with either CcmC-H_6 (Fig. 4A) or CcmE (Fig. 4B) in the Δ*ccmA-H* background strain EC06. After purification of solubilized membrane proteins on StrepTactin-sepharose CcmC co-eluted with CcmD-strep (Fig. 4A), while no traces of CcmE were found under identical conditions (Fig. 4B). Even TCA-precipitation of the eluted fractions leading to a 15-fold concentration of the proteins failed to reveal any detectable CcmE. These results indicate that CcmD binds to CcmC better than to CcmE under the conditions tested. We also tested the possibility of a direct interaction between CcmD and heme, but we failed to detect any CcmD-specific band in a hemin agarose binding experiment (data not shown). As CcmD is the only Ccm factor with a cytoplasmically oriented domain, we considered the possibility that it might provide a docking site for ferrochelatase, the last heme biosynthetic enzyme, thus facilitating heme delivery for cytochrome *c* maturation. To test this hypothesis, we constructed and used a plasmid overexpressing the gene for a strep-tagged ferrochelatase. Co-immunoprecipitation experiments were performed with membranes from cells expressing

strep-tagged ferrochelatase and H_6-CcmD. Precipitations were done with anti-H_6 antibodies, but detection of possible co-precipitating ferrochelatase with streptavidin repeatedly gave no signal (data not shown). Therefore, it is unlikely that ferrochelatase strongly interacts with CcmD.

Role of CcmD in ternary complex formation—After having shown that CcmD physically interacts with both CcmC and CcmE, but only co-purifies with CcmC, and that the double arginine mutant has an effect on heme delivery to cytochrome *c*, we came up with the following model: CcmD is involved (i) in docking apo-CcmE firmly onto CcmC and (ii) helping CcmC to release holo-CcmE after heme transfer to CcmE has taken place. In doing so CcmD should bind to CcmC and mediate the transient movement of CcmE. If our hypothesis is correct, then it is likely that the three proteins CcmC, CcmD and CcmE at some point form a ternary complex. This possibility was examined by expressing CcmC and CcmE in the presence and absence of CcmD. We co-expressed CcmC-H_6 with either CcmDE (Fig. 5A) or CcmE alone (Fig. 5B) in a Δ*ccm* background strain. When the solubilized membrane proteins were purified by nickel affinity chromatography, CcmE co-eluted with CcmC only in the presence of CcmD (Fig. 5A), but not in its absence (Fig. 5B). However, upon over-exposure of the immuno-blot in Fig. 5B, weak bands indicative of CcmE appeared (data not shown). Our results suggest that CcmD is involved in strengthening a transient interaction between CcmC and CcmE.

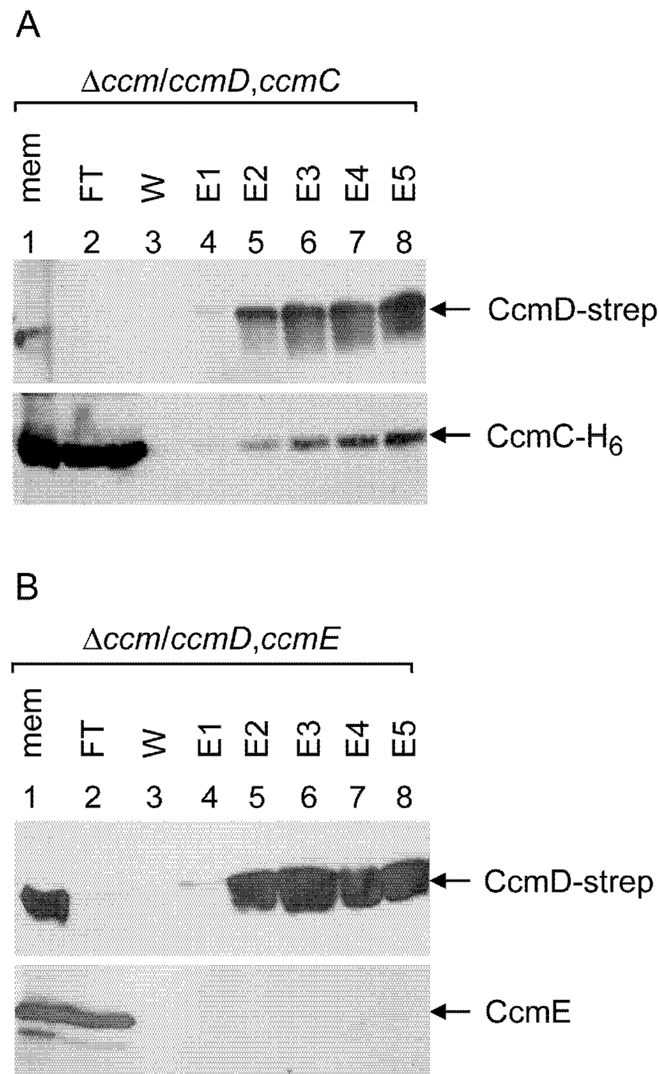

Fig. 4. Co-purification of CcmC and CcmE with CcmD. The solubilized membrane proteins from an anaerobically grown Δ*ccmA-H* mutant strain EC06 co-expressing CcmD-strep (from plasmid pEC842) and CcmC-H_6 (from plasmid pEC486; *A*) or CcmE (from plasmid pEC410; *B*) were subjected to StrepTactin-sepharose affinity chromatography as described in experimental procedures. Mem, 100 μg membrane protein (*lane 1),* FT, flow through (*lane 2*); W, wash fraction (*lane 3*); E1-E5, elution fractions (*lane 4-8*). *A*, purification of CcmD with co-expressed CcmC. Upper panel: immunoblot of proteins separated by 15% SDS-PAGE and probed with strepavidin conjugated to alkaline phosphatase. Lower panel: immunoblot of proteins probed with antiserum against the H_6-tag. *B*, purification of CcmD with co-expressed CcmE. Upper panel, as in *A*. Lower panel: immunoblot of proteins probed with antiserum against the CcmE peptide. Elution fractions were concentrated 15-fold by TCA precipitation (*lanes 4-8*). The position of CcmC, CcmD and CcmE is indicated on the right.

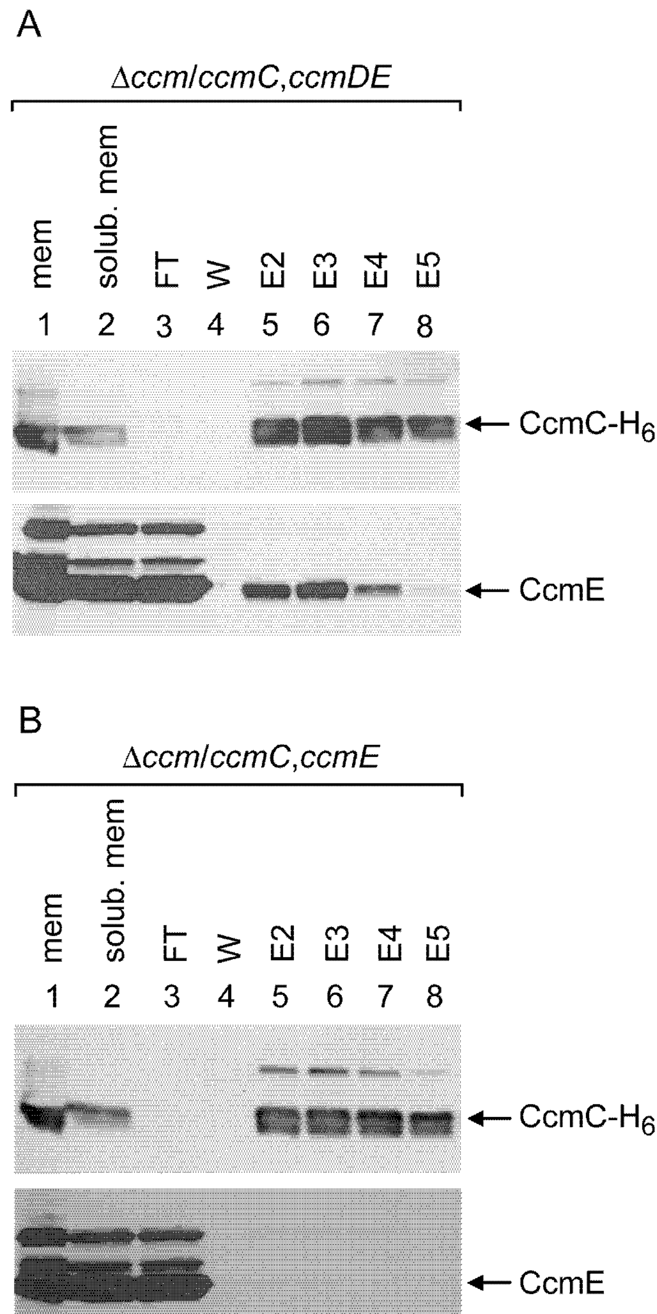

Fig. 5. Purification of ternary complex between CcmC, CcmD and CcmE. *A*, The solubilized membrane proteins from an anaerobically grown Δ*ccmA-H* mutant strain EC06 co-expressing CcmC-H_6 (from plasmid pEC486) and CcmD and CcmE (from plasmid pEC799; *A*) or CcmE only (from plasmid pEC412; *B*) were subjected to Ni-NTA affinity chromatography and analyzed as in Fig. 4. Upper panels: detection of CcmC-H_6 with antiserum against the H_6-tag. Lower panels: detection of CcmE with antiserum against CcmE. The position of CcmC-H_6 and CcmE is indicated on the right.

Influence of CcmD on CcmE stability—We have shown previously that the levels of CcmE in the membrane are increased in the presence of CcmD (10). The question therefore arose whether the stability of CcmE depends on CcmD. Pulse-chase experiments were conducted to investigate this problem. Cells expressing CcmE in the presence and absence of CcmD and CcmC (as a control) were pulse-labeled, and the fate of freshly labeled CcmE protein was followed over a period of 7 hours after a chase with an excess of non-labeled methionine and cysteine. There was no difference in the CcmE-specific signals in the presence or absence of neither CcmD, nor CcmC (data not shown). These results demonstrate that CcmD is not involved in the stability of CcmE in the membrane, but rather is a true membrane protein assembly factor organizing the proper interactions between two membrane proteins.

Topology of CcmD—A prediction of the CcmD topology using the HMMTOP program (21) envisages CcmD as a monotopic membrane protein and is in agreement with the experimentally determined cytoplasmic C-terminus of the *R. capsulatus* CcmD homologue HelD (11). Nevertheless, the periplasmic orientation of the N-terminal region as a result of a transmembrane orientation of the hydrophobic domain has only been tentative. Alternatively, CcmD may not traverse the membrane bilayer, but rather be peripherally associated with the membrane or insert in only one leaflet with its N- and C-termini on the cytoplasmic side of the membrane. It is difficult to experimentally assess this kind of membrane topology of monotopic membrane proteins. We addressed the CcmD topology by constructing a CcmD version with both an N-terminal H_6-tag and a C-terminal strep-tag. The doubly-tagged protein has full activity in cytochrome *c* maturation (not shown). This protein was then tested for

protection from proteinase K digestion in spheroplasts as well as in inverted vesicles. As a control we used N-terminally H_6-tagged CcmE, whose topology in the membrane is known: the H_6-tag resides on the cytoplasmic side of the membrane, whereas the bulk of the protein with the antigenic region is periplasmic. In spheroplasts CcmD was protected entirely from proteinase K digestion activity while CcmE was not (Fig. 6A, top panel). The presence of intact N- and C-termini in CcmD was confirmed by immunoblot before and after proteinase K treatment using anti-H_6 immunoglobulines and streptavidin, respectively (Fig. 6A, bottom left panels). In the CcmE control, the band of the full-length protein disappeared after proteinase K treatment and was not detected with anti-CcmE serum. However, a degradation product corresponding in size to the N-terminal H_6-CcmE membrane anchor could be detected with the H_6-tag-specific antibodies (Fig. 6A, bottom right panels). It was resistant to proteinase K digestion because it was not exposed to the periplasm. If the N-terminus of CcmD was cytoplasmically oriented, this part of CcmD should be susceptible to proteinase K digestion in inside out (ISO) vesicles to the same extent as the C-terminus. This was confirmed because the signals for both H_6- and strep-tag detection were strongly diminished after proteinase K treatment of the vesicles, a result that was also obtained for the N-terminal H_6-tag of CcmE (Fig. 6B,). Our results provide evidence that CcmD does not have a transmembrane helix and that its N- and C-termini both protrude into the cytoplasm. To test whether CcmD is a peripheral rather than an integral membrane protein, we tried to extract CcmD from the membrane fractions by either subjecting the membranes to five freeze-and-thaw cycles or by treating them with various chaotropic agents that are known to extract peripheral membrane proteins (22), as described in experimental procedures. As a control we treated the membranes obtained from EC06 cells over expressing the membrane-anchored CcmE in a similar manner.

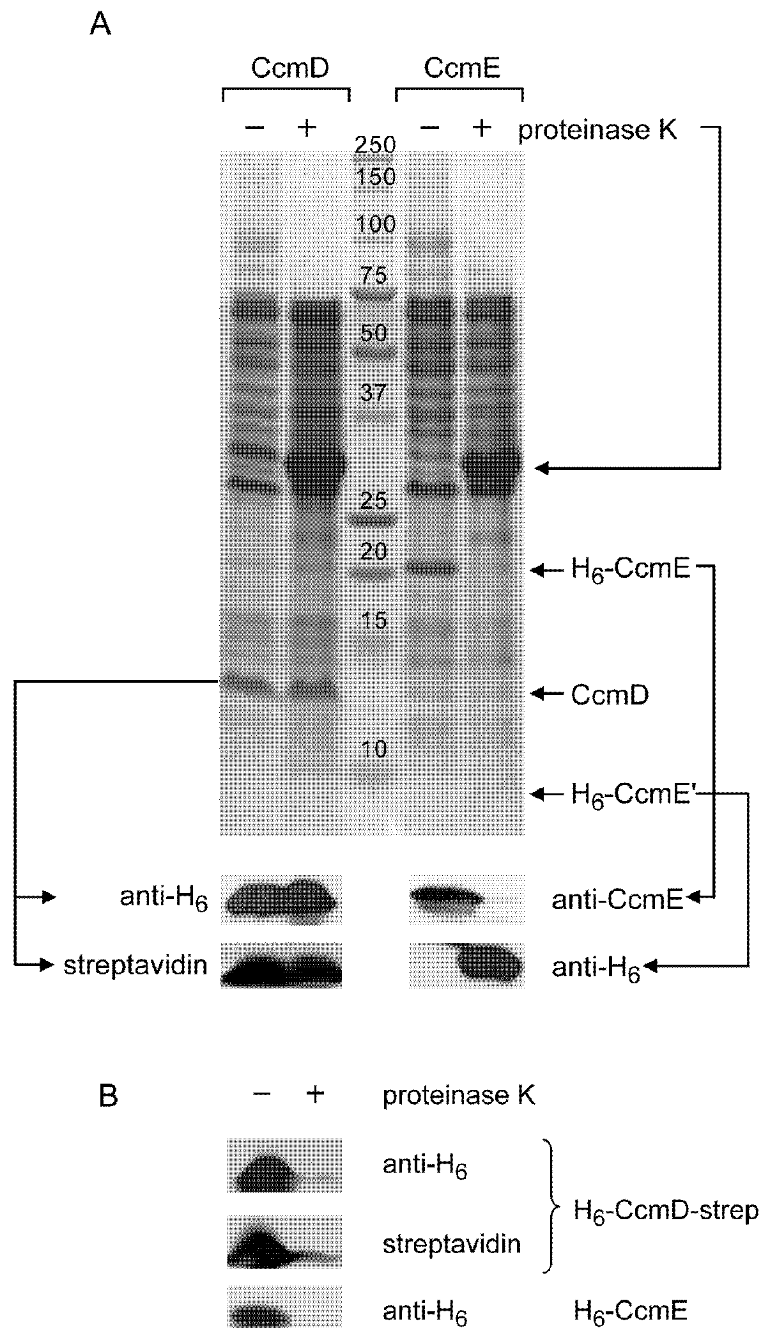

Fig. 6. Membrane topology of CcmD. *A*, Topology analysis in spheroplasts. Top panel, Coomassie stain of spheroplast proteins prepared from aerobically grown Δ*ccmA-H* mutant strain EC06 co-expressing H_6-CcmD-strep (from plasmid pEC869; left side) or H_6-CcmE (from plasmid pEC411, right side) untreated or treated with proteinase K. Proteins were separated in a 16% tricine polyacrylamide gel. The molecular weights of the protein size marker are indicated in kDa. In the bottom panels, the results from immunoblots of identical gels reveal the identity of the indicated protein bands. B, ISO vesicles from EC06/pEC869 without or with treatment with proteinase K were analyzed by immuno detection using anti-H_6-tag antibodies or streptavidin for strep-tag detection as indicated.

We were unable to extract any CcmD out of the membrane fraction (data not shown) under all mentioned conditions. Hence, we postulate that CcmD is firmly embedded in the lipid bilayer like an integral interfacial membrane protein.

DISCUSSION

The CcmD protein of *E. coli* is an essential factor for cytochrome *c* maturation; yet its precise role in this process is not known. The 69-amino-acid polypeptide has three domains: (i) an N-terminal domain consisting of residues 1-13 with partly polar side chains, (ii) a hydrophobic membrane anchor comprising residues 14-37 of entirely hydrophobic nature, and (iii) a C-terminal, soluble domain that is rich in charged residues with predominantly positive charges. This domain structure and charge distribution is very well conserved in CcmD homologues of other bacteria, whereas individual amino acid residues are not. However, all known CcmD homologues are encoded by a gene directly following the *ccmC* gene (23). In *E. coli ccmD* is part of the *ccmABCDEFGH* operon.

We have shown previously that CcmD assists the formation of the heme-binding form of the CcmE heme chaperone (10). The covalent attachment of heme to CcmE is catalyzed mainly by CcmC, but more holo-CcmE accumulates in membranes when CcmD is present. It has been excluded that CcmD influences the kinetics of heme attachment (10). Our study shows that CcmD is an integral membrane protein, and its hydrophobic domain is functionally essential. While it has been proposed from LacZ fusions of the *R. capsulatus* homologue HelX that the C-terminal soluble domain is exposed to the cytoplasm the location of the N-terminus had never been determined experimentally (11). Here, we have constructed an N-and

C-terminally tagged CcmD derivative and investigated the accessibility of the tags to proteolytic digestion in both spheroplasts and in-side-out vesicles. Both, N- and C-terminal tags had the same resistance or sensitivity towards proteinase K, implying that they are located on the same, i.e. the cytoplasmic, side of the membrane. Hence, we prefer the view that the hydrophobic domain of CcmD anchors the protein to the membrane with a loop structure rather than a transmembrane segment. While the small N-terminal domain is not required for function, the C-terminal charged domain is essential. Its partial truncation results in an equivalent 50% loss of function. Mutation of either of the two remaining arginines to alanines (R44A and R48A) did not inactivate the protein, but a drastic loss of activity was obtained in the double mutant (R44A/R48A), where the net positive charge was removed.

What is the function of the positively charged, cytoplasmic domain in cytochrome *c* maturation, a process which occurs predominantly in the periplasm? One idea we tested was an interaction of CcmD with the cytoplasmic enzyme ferrochelatase as part of metabolic channeling, linking biosynthesis and delivery of heme to *c*-type cytochromes. To investigate the possibility that CcmD could connect the two processes at the membrane by specifically docking the ferrochelatase to the Ccm heme delivery system, we tried to co-precipitate CcmD with ferrochelatase, however, without success. Alternatively, we can imagine a protein-protein interaction of CcmD with cytoplasmic domains of CcmC or CcmE, which in both cases are rather small. A common feature of CcmE homologues is that their N-terminal 7-9 amino acid residues, most likely extruding from the membrane into the cytoplasm, contain three to five arginines or lysines and thus are positively charged. It is possible that the positive net charges of the CcmE N-terminal end interferes with the positively charged C-terminal

domain of CcmD, causing some repulsion that might be necessary for the dynamic shuttling of CcmE between CcmC and CcmF during heme delivery (24).

A more classical role for CcmD is that of an assembly factor of membrane proteins. The enhanced levels of CcmE in the membrane in the presence of CcmD can be due to either increased stability or increased incorporation into the membrane. The former was excluded by a pulse chase analysis where CcmE was found to be stable over hours. Hence, CcmD is likely to function in assembly of CcmE with the membrane and/or with other membrane protein subunits of the Ccm system.

Acknowledgments—We thank Q. Ren, E. C. Pellicioli and H. Schulz for the construction of some plasmids and strains used in this work and D. Margadant for technical assistance. We also thank O. Christensen and M. Braun for helpful discussions. This work was supported by the Swiss National Foundation for Scientific Research.

REFERENCES

1. Thöny-Meyer, L. (1997) *Microbiol Mol Biol Rev* **61**, 337-376
2. Xie, Z., and Merchant, S. (1998) *Biochim Biophys Acta* **1365**, 309-318
3. Kranz, R., Lill, R., Goldman, B., Bonnard, G., and Merchant, S. (1998) *Mol Microbiol* **29**, 383-396
4. Page, M. D., Sambongi, Y., and Ferguson, S. J. (1998) *Trends Biochem Sci* **23**, 103-108
5. Thöny-Meyer, L. (2002) *Biochem Soc Trans* **30**, 633-638
6. Allen, J. W., Daltrop, O., Stevens, J. M., and Ferguson, S. J. (2003) *Philos Trans R Soc Lond B Biol Sci* **358**, 255-266
7. Thöny-Meyer, L., Fischer, F., Künzler, P., Ritz, D., and Hennecke, H. (1995) *J Bacteriol* **177**, 4321-4326
8. Grove, J., Tanapongpipat, S., Thomas, G., Griffiths, L., Crooke, H., and Cole, J. (1996) *Mol Microbiol* **19**, 467-481
9. Schulz, H., Hennecke, H., and Thöny-Meyer, L. (1998) *Science* **281**, 1197-1200
10. Schulz, H., Fabianek, R. A., Pellicioli, E. C., Hennecke, H., and Thöny-Meyer, L. (1999) *Proc Natl Acad Sci U S A* **96**, 6462-6467
11. Goldman, B. S., Beckman, D. L., Bali, A., Monika, E. M., Gabbert, K. K., and Kranz, R. G. (1997) *J Mol Biol* **268**, 724-738
12. Iobbi-Nivol, C., Crooke, H., Griffiths, L., Grove, J., Hussain, H., Pommier, J., Mejean, V., and Cole, J. A. (1994) *FEMS Microbiol Lett* **119**, 89-94
13. Hanahan, D. (1983) *J Mol Biol* **166**, 557-580

14. Thöny-Meyer, L., Künzler, P., and Hennecke, H. (1996) *Eur J Biochem* **235**, 754-761

15. Chang, A. C., and Cohen, S. N. (1978) *J Bacteriol* **134**, 1141-1156

16. Ren, Q., and Thöny-Meyer, L. (2001) *J Biol Chem* **276**, 32591-32596

17. Schulz, H., Pellicioli, E. C., and Thöny-Meyer, L. (2000) *Mol Microbiol* **37**, 1379-1388

18. Ren, Q., Ahuja, U., and Thöny-Meyer, L. (2002) *J Biol Chem* **277**, 7657-7663

19. Schägger, H., and von Jagow, G. (1987) *Anal Biochem* **166**, 368-379

20. de Gier, J. W., Scotti, P. A., Saaf, A., Valent, Q. A., Kuhn, A., Luirink, J., and von Heijne, G. (1998) *Proc Natl Acad Sci U S A* **95**, 14646-14651

21. Tusnady, G. E., and Simon, I. (1998) *J Mol Biol* **283**, 489-506

22. Fujiki, Y., Hubbard, A. L., Fowler, S., and Lazarow, P. B. (1982) *J Cell Biol* **93**, 97-102

23. Thöny-Meyer, L., and Künzler, P. (1997) *Eur J Biochem* **246**, 794-799

24. Ahuja, U., and Thöny-Meyer, L. (2003) *J Biol Chem* **278**, 52061-52070

25. Arslan, E., Schulz, H., Zufferey, R., Künzler, P., and Thöny-Meyer, L. (1998) *Biochem Biophys Res Commun* **251**, 744-747

CHAPTER 4

The membrane anchors of the heme chaperone CcmE and the periplasmic thioredoxin CcmG are functionally important

Umesh Ahuja and Linda Thöny-Meyer

Institut für Mikrobiologie, Department Biologie

Eidgenössische Technische Hochschule,

Wolfgang Pauli Strasse 10, CH-8093 Zürich, Switzerland

SUMMARY

The cytochrome *c* maturation system of *E. coli* contains two monotopic membrane proteins with periplasmic, functional domains, the heme chaperone CcmE and the thioredoxin CcmG. We show in a domain swap experiment that the membrane anchors of these proteins can be exchanged without drastic loss of function in cytochrome *c* maturation. By contrast, the soluble, periplasmic forms produced with a cleavable OmpA signal sequence have low biological activity. Both the chimerical CcmE (CcmG'-'E) and the soluble periplasmic CcmE produce low levels of holo-CcmE and thus are impaired in their heme receiving capacity. Also, both forms of CcmE can be co-precipitated with CcmC, thus restricting the site of interaction of CcmE with CcmC to the C-terminal periplasmic domain. However, the low level of holo-CcmE formed in the chimera is transferred efficiently to cytochrome *c*, indicating that heme delivery from CcmE does not involve the membrane anchor.

Keywords: cytochrome *c* maturation, domain swapping, heme chaperone, membrane anchor, periplasmic proteins, thioredoxin.

INTRODUCTION

Cytochromes of the *c*-type are an ubiquitous class of electron transfer proteins with a covalently bound heme as a cofactor. Heme is redox active and thus these proteins are typically found in respiratory or photosynthetic electron transport chains. The posttranslational process involving covalent attachment of heme, via thioether bonds to the cysteinyl residues of the conserved CXXCH signature motif of apo-cytochrome, is defined as cytochrome *c* biogenesis. Three distinct systems for the biogenesis of *c*-type cytochromes have evolved in nature, which are annotated as system I, II and III (reviewed in (1-6)). The γ-proteobacterium *Escherichia coli* uses system I which contains eight membrane proteins encoded by the *ccmABCDEFGH* operon (7,8). A key intermediary step during this maturation pathway is the transfer and covalent attachment of heme to the cytochrome *c* maturation-specific heme chaperone CcmE (9); subsequently, heme is transferred from the holo-CcmE intermediate to apo-cytochrome *c*. It has been shown previously that the covalent binding of heme to an essential histidine (H130) of apo-CcmE requires the activity of the CcmC maturation protein (10). The mechanism of heme delivery from the CcmC to apo-CcmE and the catalysis of the covalent attachment of heme to apo-CcmE are not known. Several CcmC point mutants obtained from an anaerobic genetic screen (11), as well as site directed mutagenesis of the best conserved residues within CcmE (12), failed to deliver any clue about the mechanism of heme delivery and covalent attachment. Recently, the structure of the soluble apo-CcmE from *E. coli* (13) and *Shewanella putrefaciens* (14) without its natural N-terminal membrane anchor was solved by NMR. Soluble CcmE poorly complemented a Δ*ccmE* mutant for holo-cytochrome *c* maturation (15), suggesting that the N-terminal transmembrane domain is important for function. In this work we addressed the question of

whether the N-terminal transmembrane domain serves to simply attach CcmE to the membrane, or whether it provides specificity related to the function of the heme chaperone. We chose the approach to use the membrane anchor of another monotopic membrane protein with periplasmic orientation of its functional domain, namely that of CcmG, to replace the CcmE membrane anchor. CcmG is also required for cytochrome *c* biogenesis in a redox pathway to ensure that the cysteines of the CXXCH motif in the apo-cytochrome remain reduced prior to heme attachment. Conversely, we also tested replacement of the CcmG membrane anchor with that of CcmE. In addition, we investigated heme transfer and protein-protein interactions between the CcmC with various CcmE derivatives.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions—Bacterial strains and plasmids used in this study are listed in Table I. Bacteria were grown aerobically in LB medium or anaerobically in minimal salts medium (16) supplemented with 0.4% glycerol, 40 mM fumarate and 5 mM sodium nitrate as the terminal electron acceptor. Antibiotics were added at the following final concentrations: ampicillin, 200 μg/ml; chloramphenicol, 25 μg/ml. If necessary, cells were induced with 0.5% arabinose at mid-exponential growth phase.

Construction of plasmids and site-directed mutagenesis—*E. coli* strain DH5α (17) was used as the host for cloning. The plasmids pEC865 and pEC866, which code for CcmGΔW22 and CcmEV26Q, respectively, were constructed by QuikChange^{TM} (Stratagene) site-directed mutagenesis using pEC210 (*ccmG*) and pEC410 (*ccmE*) respectively, as a template and the

primers listed in Table II. The QuikChange™ site-directed mutagenesis introduced a *Pst*I site at the required position in the *ccmG* and *ccmE* genes. To construct a plasmid expressing

TABLE I: Bacterial strains and plasmids used in this study

Bacterial strains or plasmids	Relevant genotype/phenotype	Reference
Escherichia coli		
DH5α	*supE*44 Δ*lacU*169 (Φ80*lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	(17)
EC06	MC1061 Δ*ccmA-H*::*kan*	(7)
EC29	MC1061 Δ*ccmG*	(10)
EC65	MC1061 Δ*ccmE*	(9)
Plasmids		
pRJ3290	*B. japonicum cycA*-H_6 cloned in pISC2, Ap^R	(11)
pEC210	*ccmG* cloned into pACYC184, Cm^R	(21)
pEC410	*ccmE* cloned in pACYC184, Cm^R	(10)
pEC412	*ccmE* cloned into pISC-2, Ap^R	(9)
pEC422	H_6-*ccmC* cloned in pISC-2, Ap^R	(10)
pEC486	*ccmC*-H_6 cloned in pACYC184, Cm^R	(19)
pEC701	*B. japonicum cycA*-H_6 cloned in pACYC184, Cm^R	(25)
pEC865	*ccmG*ΔW22 cloned into pACYC184, Cm^R	This study
pEC866	*ccmE*V26Q cloned into pACYC184, Cm^R	This study
pEC867	*ccmE*'-'*ccmG* cloned into pACYC184, Cm^R	This study
pEC868	*ccmG*'-'*ccmE* cloned into pACYC184, Cm^R	This study
pEC882	*ccmG* cloned into pISC2, Ap^R	This study
pEC884	*ompA*'-'*ccmG* cloned into pISC2, Ap^R	This study

chimerical CcmE'-'G (pEC867), the *Pst*I- and *Sal*I- digested 1157-bp fragment from pEC866 was ligated into the 5.2-kb *Pst*I- and *Sal*I- digested fragment from pEC865. To construct a

plasmid expressing chimerical CcmG'-'E (pEC868), the *Pst*I- and *Sal*I-digested 550-bp fragment from pEC865 was ligated into the 3.77-kb *Pst*I- and *Sal*I-digested fragment from pEC866. Plasmid pEC882 expressing CcmG was constructed by amplifying a 560-bp fragment by PCR with the primers *ccmG*n*Nde*I and *ccmG*c*EcoR*I, using pEC210 as a template. The PCR product was digested with *Nde*I and *EcoR*I and ligated into the 5.4-kb *Nde*I- and *EcoR*I- digested pISC-2. Plasmid pEC884 that codes for the soluble periplasmic domain of CcmG was constructed by amplifying a 489-bp fragment by PCR with the primers *ccmG*R26*Stu*I and *ccmG*c*EcoR*I, using pEC210 as a template. The PCR product was digested with *Stu*I and *EcoR*I and ligated into the 5.4-kb *Stu*I- and *EcoR*I- digested pEC415. In this plasmid, an *ompA* segment encoding the cleavable OmpA signal sequence is fused in-frame to the truncated *ccmG* gene.

DNA sequencing—All plasmid constructs and mutants derived from PCR products were confirmed by DNA sequence analysis (Microsynth, Balgach, Switzerland).

Cell fractionation—For the preparation of subcellular fractions bacterial cultures were grown anaerobically in the presence of nitrate. Preparation of whole cell lysates, periplasmic extracts and membranes was done as described previously (18).

TABLE II: Oligonucleotide primers used for plasmid construction and sequencing

Primer	Bases	Nucleotide sequence (5′-3′)	Usage
*ccmG*ΔW22-f	36	gcgattgcggcggcgctgcagctggcgcgtaatgcc	QuikChange™ primers for cloning pEC865
*ccmG*ΔW22-r	36	ggcattacgcgccagctgcagcgccgcggcaatcgc	
*ccmE*V26Q-f	33	ctgactatcggtctgcagctatatgcgctgcgc	QuikChange™ primers for cloning pEC866
*ccmE*V26Q-r	33	gcgcagcgcatatagctgcagaccgatagtcag	
*ccmG*n*Nde*I	37	cgggatccatatgaagcgcaaagtattgttaattccg	Forward primer for cloning pEC882
*ccmG*c*EcoR*I	31	cgggaattctcattgtgcggcctccttactg	Reverse primer for cloning pEC882
*ccmG*R26*Stu*I	24	aaggccttgcgtaatgccgaaggg	Forward primer for cloning pEC884
His'*EcoR*I	27	cgggaattctcagtggtggtggtggtg	Reverse primer for cloning pEC884
pING1*araB*	18	ggccgacgaaatcactcg	Sequencing primer

Biochemical methods—Protein concentrations were determined using the Bradford assay (Bio-Rad) and BSA as a standard. Holo-CcmE and holo-cytochrome c_{550} formation were analyzed qualitatively by heme staining and immunoblot (19). Soluble cytochrome *c* in the periplasmic fractions was quantified by absorption difference spectroscopy using an $\varepsilon_{550-536nm}$ of 23.2 $mM^{-1}cm^{-1}$(20). Immunoblot analysis of the N- or C-terminal hexa-histidine (H_6)-tagged CcmC (H_6-CcmC or CcmC-H_6, respectively) was performed using monoclonal tetra-His antibodies (Qiagen) at a dilution of 1:2000. Immunoblot analysis of the CcmE and CcmG polypeptide was performed using antibodies directed against specific peptides derived from them (9), (21) at a dilution of 1:5000 and 1:1000, respectively. Signals were detected using

goat anti-mouse IgG (for H_6-tag detection) or goat anti-rabbit IgG (for detection of other antigens) conjugated to alkaline phosphatase (Bio-Rad) as secondary antibody and CSPD (Roche Diagnostics) reagent as substrate.

Co-immunoprecipitation—1mg membrane proteins or whole cell lysates were solubilized and immunoprecipitated with 10 μl of anti-H_6-tag antibodies according to the method described previously (18). The proteins were subjected to 15% SDS-PAGE, transferred to a PVDF membrane and probed with the antibodies against the CcmE peptide as described above.

RESULTS

Functional analysis of chimerical CcmE—CcmE is a monotopic membrane protein with an N-terminal membrane anchor (residues 1-29) and a soluble C-terminal domain (residues 30-159) that protrudes into the periplasmic space (Fig. 1) (9). The periplasmic domain of CcmE was shown to complement cytochrome *c* maturation at an extremely low level (15) and thus indicated the importance of the membrane anchor. CcmG is similar to CcmE in terms of topology, i.e., a monotopic membrane protein with an N-terminal membrane anchor (residues 1-22) and a periplasmic domain (residues 23-185; Fig. 1)(21). Note that in both cases the precise extension of the transmembrane segment is not known and was predicted based only on hydrophobic and uncharged amino acid residues.

We first replaced the membrane spanning domain of CcmE with that of CcmG and named the product chimerical CcmE (CcmG'-'E). A plasmid encoding this chimera was used for complementation of the *ccmE* in frame deletion mutant EC65(ΔI3-S94), wherein the 92 N terminal codons are deleted from the chromosome (9). The mutant CcmEV26Q that resulted as an intermediate during the construction of the plasmid expressing the CcmG'-'E chimera was also tested. In our complementation experiments we overexpressed at the same time the soluble exogenous *Bradyrhizobium japonicum* cytochrome c_{550} to monitor cytochrome *c* maturation. The Δ*ccmE* mutant strain was co-transformed with a plasmid expressing a H_6-tagged *B. japonicum* cytochrome c_{550} plus a plasmid encoding wild-type, mutant or chimerical CcmE protein (Fig. 2A). We first assessed the stability and assembly of the mutant CcmEV26Q and the chimerical CcmE with the CcmG membrane anchor by immunodetection (Fig. 2A, upper panel).

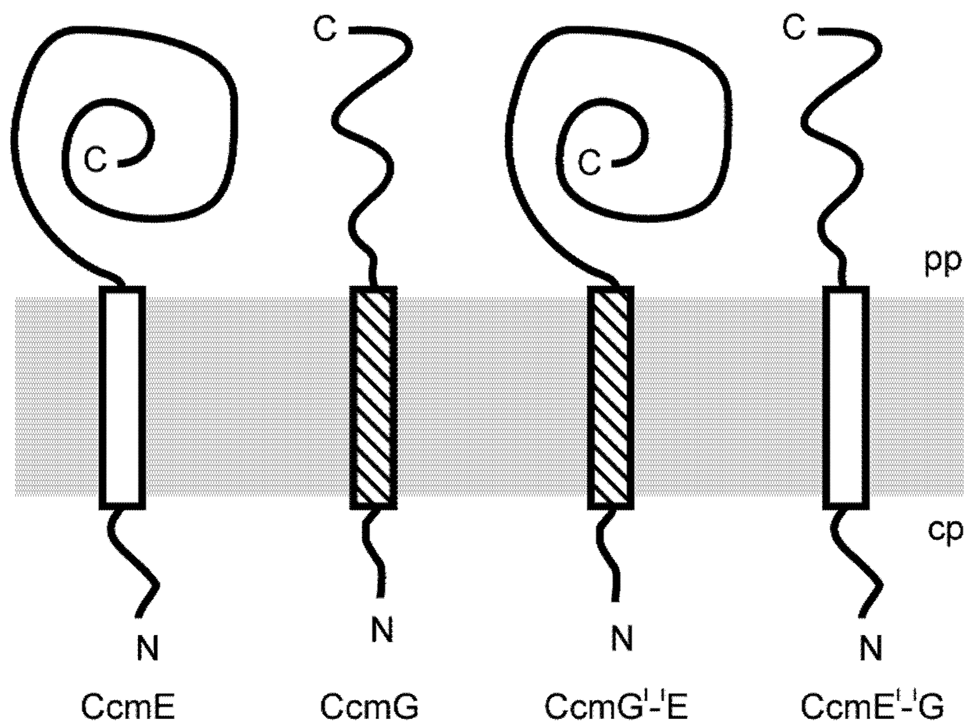

Fig. 1. Topology model for *E. coli* CcmE, CcmG and their chimerical versions. A schematic representation of the topology of the proteins predicted by the program HMMTOP (29) in the membrane (shaded) is depicted. The membrane anchor of CcmE is represented as a white and that of CcmG as a hatched rectangle. Details about the precise position of the amino acid residues constituting the different domains can be found in the text. N, amino terminus; C, carboxy terminus; pp, periplasm; cp, cytoplasm.

The point mutation (*lane 2*) and the replacement of the membrane anchor (*lane 3*) clearly affected protein expression when compared to the wild-type (*lane 1*). Heme binding of CcmE was strongly affected in the mutant as well as in the chimerical variant, as judged by heme staining of whole cells after SDS-PAGE (Fig. 2A, middle panel). Cytochrome *c* formation was assayed by heme staining of periplasmic fractions (Fig. 2A, lower panel). Quantification

of *c*-type cytochromes was done by absorption difference spectroscopy (Fig. 2B). The level of holo-cytochromes *c* produced by the *ccmE* mutant complemented with a plasmid encoding wild-type CcmE was taken as 100%. The mutant CcmEV26Q and the CcmE chimera both produced lower levels of holo-cytochrome *c* (>50%). This showed that neither the mutation nor the replacement of the membrane anchor of CcmE affected cytochrome *c* maturation substantially. Next we tested whether the reduction of heme attachment to apo-CcmE was due to loss of specific physical interactions between the membrane anchor and CcmC. The deficiency in heme transfer was investigated in a Δ*ccm* mutant strain background lacking all *ccm* genes, i.e., in a minimal system containing only CcmC and either wild-type or chimerical CcmE. In this system heme bound CcmE can accumulate as there is no further delivery of heme to *c*-type cytochromes. A heme stain of the membrane protein extracts obtained from cells expressing wild-type or chimerical CcmE confirmed our observation (Fig. 2 middle panel) of an extremely inefficient heme transfer to chimerical CcmE (Fig. 3 top panel). This phenomenon might in part be attributed to the lower levels of the CcmE polypeptide in the membranes (Fig. 3 second panel). The physical interaction of CcmC and CcmE was tested by co-immunoprecipitaion, and no significant difference was found for the chimerical CcmE when compared to wild-type CcmE (Fig.3 lowest panel).

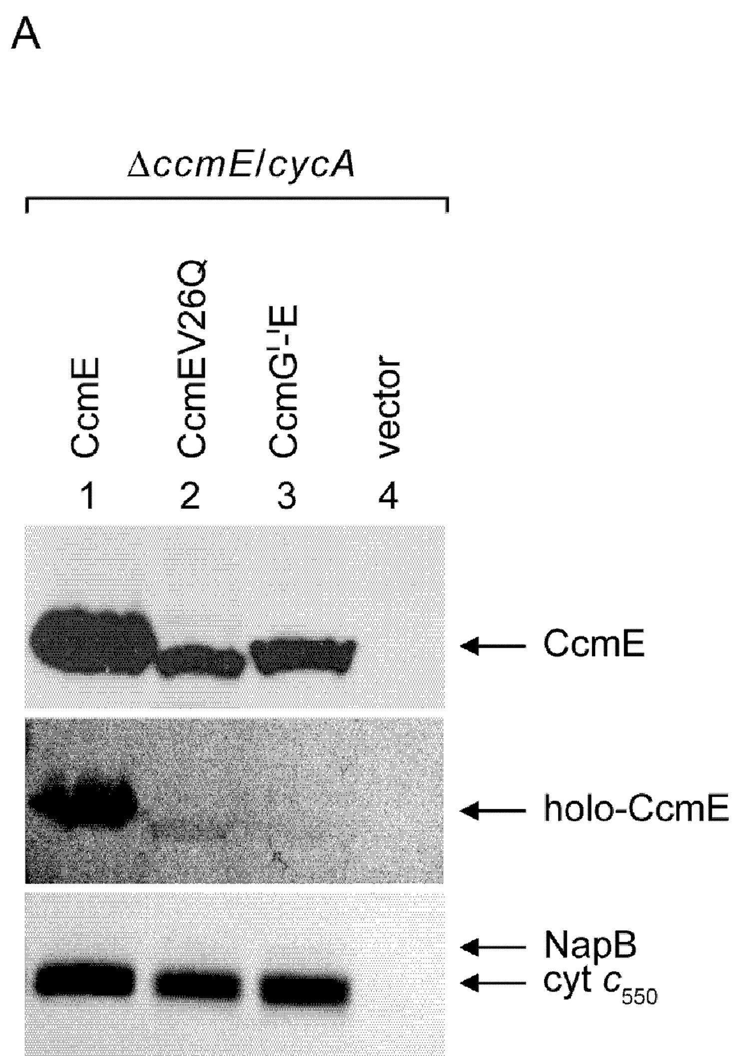

Fig.2. Functional analysis of the CcmG'-'E chimera. *A*, The Δ*ccmE* strain EC65 was co-transformed with the plasmid pRJ3290 (expressing C-terminal H_6-tagged *B. japonicum* cytochrome c_{550}) and pEC410 (wild-type CcmE; *lane 1*), pEC866 (CcmEV26Q; *lane 2*), pEC868 (CcmG'-'E; *lane 3*) or pACYC184 (empty vector; *lane 4*). Cells were grown anaerobically in the presence of 5 mM sodium nitrate. Upper panel: immunoblot of the total cell protein (after equalizing the cell density) probed with an antiserum against CcmE. Middle panel: trichloroacetate precipitates of the total cell protein (after equalizing samples to uniform cell density) separated by 15% SDS-PAGE and stained for covalently bound heme. Lower panel: periplasmic proteins (20 μg per lane) were separated by 15% SDS-PAGE and stained for covalently bound heme. The position of CcmE, endogenously expressed NapB and cytochrome c_{550} is indicated on the right. *B*, difference spectra of the periplasmic extracts as described in *A*. The percentage of the holo-cytochrome *c* formed relative to the wild-type is indicated on the right.

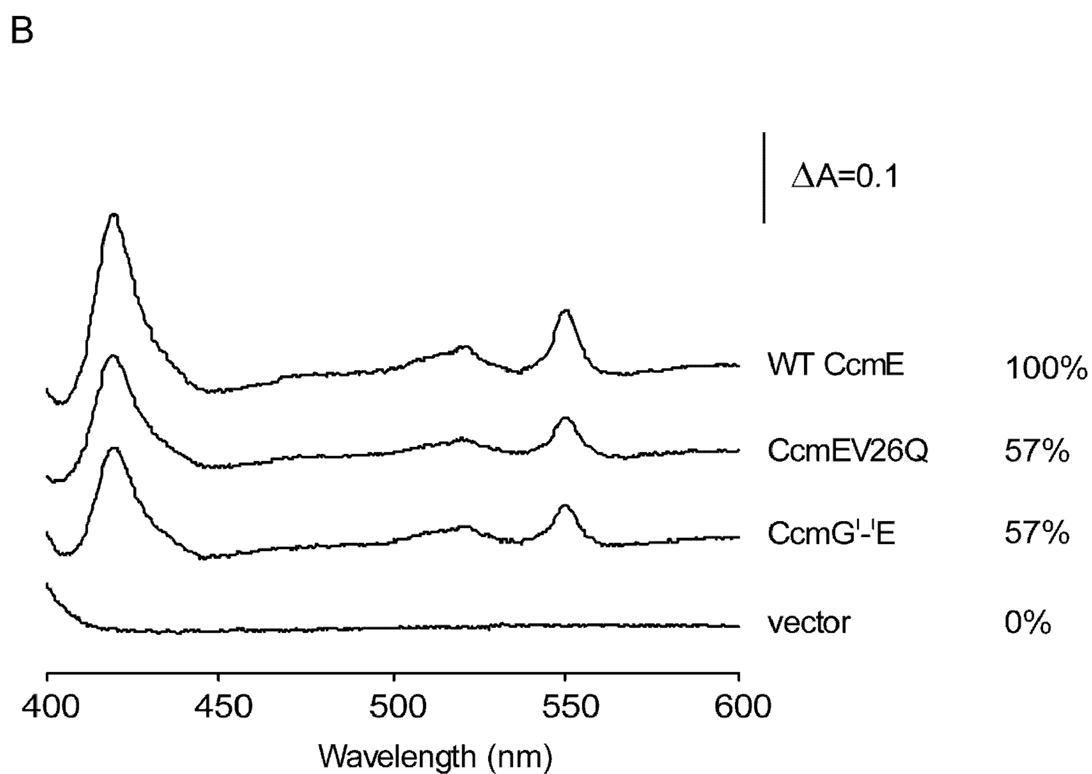

Fig. 2. Functional analysis of the CcmG'-'E chimera. *B*, difference spectra of the periplasmic extracts as described in *A*. The percentage of the holo-cytochrome *c* formed relative to the wild-type is indicated on the right.

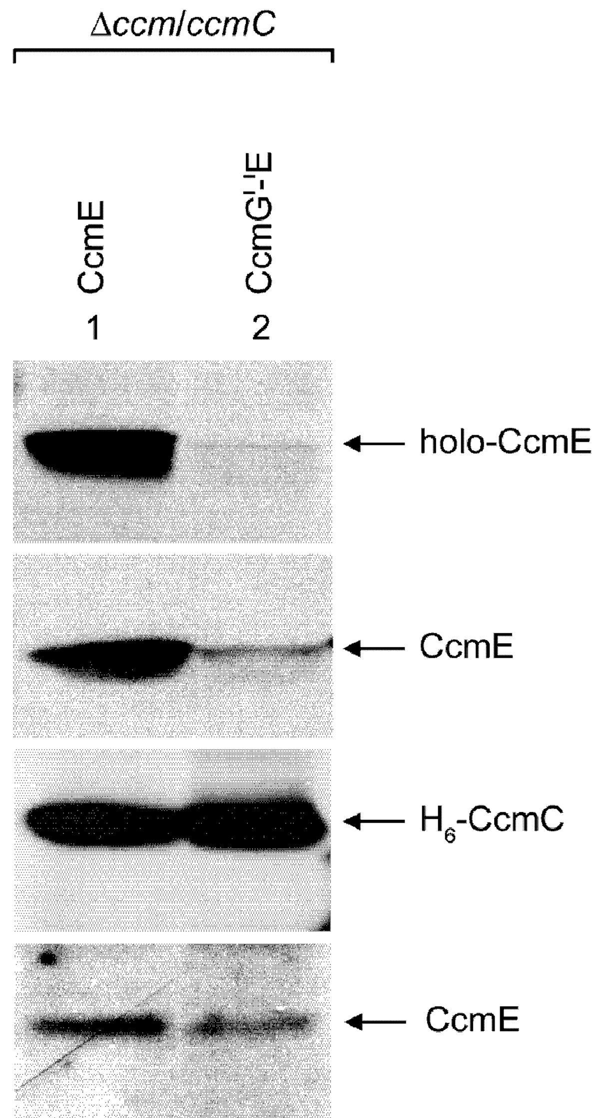

Fig. 3. Physical interaction of the CcmG'-'E chimera with CcmC for heme transfer. The Δ*ccmA-H* mutant strain EC06 was co-transformed with plasmid pEC422 expressing N-terminally H_6-tagged CcmC and either pEC410 (CcmE; *lane 1*) or pEC868 (CcmG'-'E; *lane 2*). Cells were grown anaerobically in the presence of 5 mM sodium nitrate. Upper panel: membrane proteins (50 μg per lane) were separated by 15% SDS-PAGE and stained for covalently bound heme. Second panel: immunoblot of membrane proteins (100 μg per lane) probed with antiserum against the CcmE polypeptide. Third panel: immunoblot of membrane proteins (100 μg per lane) probed with antiserum against the H_6 tag. Fourth panel: membrane proteins were immunoprecipitated with anti-H_6 tag antibodies and detected for CcmE by immunoblot. The positions of holo-CcmE, CcmE, and H_6-CcmC are indicated on the right.

Functional analysis of chimerical CcmG —In a parallel approach we performed the domain swapping experiment in the opposite direction by replacing the CcmG membrane anchor with that of CcmE and complementing a Δ*ccmG* mutant for holo-cytochrome *c* formation. For the construction of the corresponding CcmG chimera (CcmE'-'G) an intermediate with a ΔW22 deletion within CcmG was obtained that was included in the tests. Immunodetection of CcmG in membranes of the Δ*ccmG* mutant complemented with wild-type or mutant CcmG versions resulted in similar bands, indicating a stable expression and assembly of these proteins (Fig. 4A, upper panel). Cytochrome *c* formation was assayed by heme staining of the periplasmic fractions of the corresponding strains that also overexpressed the *B. japonicum* cytochrome c_{550}. Quantification of the *c*-type cytochromes was done by absorption difference spectroscopy (Fig. 4B). The level of the holo-cytochrome *c* produced by the Δ*ccmG* mutant complemented with wild-type was taken as 100%. Quite surprisingly, the CcmGΔW22 mutation had a drastic effect on holo-cytochrome *c* formation (Fig. 4A, lower panel, *lane 2*) and produced only 5% of the wild-type levels (Fig. 4B). By contrast, the CcmG chimera with the CcmE membrane anchor still produced about 50% of the holo-cytochrome *c* levels obtained with wild-type CcmG.

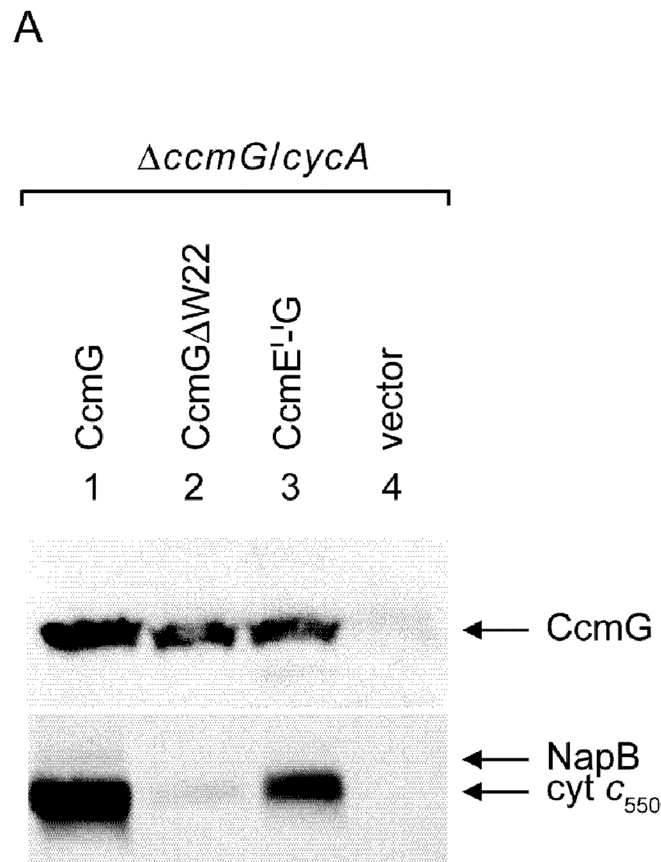

Fig. 4. Functional analysis of CcmE'-'G chimera. *A*, The Δ*ccmG* strain EC29 was co-transformed with the plasmid pRJ3290 (expressing C-terminal H_6-tagged *B. japonicum* cytochrome c_{550}) and pEC210 (wild-type CcmG; *lane 1*), pEC865 (CcmGΔW22; *lane 2*), pEC867 (CcmE'-'G; *lane 3*) or pACYC184 (empty vector; *lane 4*). Cells were grown anaerobically in the presence of 5 mM sodium nitrate. Upper panel: immunoblot of the total cell protein (equalized to cell density) probed with antiserum against CcmG. Lower panel: periplasmic proteins (20 µg per lane) were separated by 15% SDS-PAGE and stained for covalently bound heme. The position of CcmG, the endogenous *c*-type cytochrome NapB and cytochrome c_{550} is indicated on the right. *B*, difference spectra of periplasmic extracts of the samples as in *A*. The percentage of the holo-cytochrome *c* formed relative to the wild type is indicated on the right.

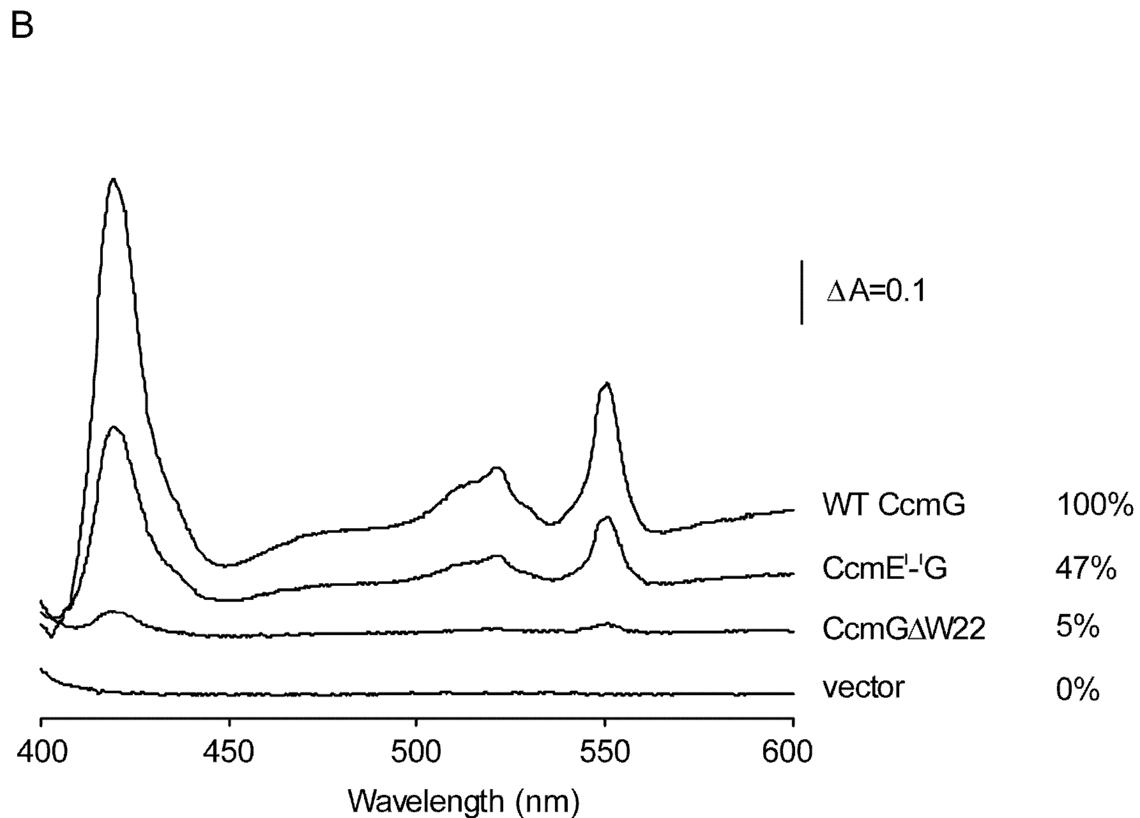

Fig. 4. Functional analysis of CcmE'-'G chimera. *B*, difference spectra of periplasmic extracts of the samples as in *A*. The percentage of the holo-cytochrome *c* formed relative to the wild type is indicated on the right.

Is the membrane anchor essential for the function of CcmE and CcmG?—We have previously addressed the question of whether soluble CcmE can complement a Δ*ccmE* mutant for cytochrome *c* maturation (15). Only for the membrane-bound *c*-type cytochrome NapC we detected a weak heme staining band, whereas for the soluble cytochrome *c* NapB complementation was not detectable. We now wanted to confirm this ambiguous result by a more sensitive approach, i.e. by overproducing the soluble *B. japonicum* cytochrome c_{550} in a Δ*ccmE* strain together with soluble CcmE. The problem with this experiment is that soluble CcmE and *B. japonicum* cytochrome c_{550} have a very similar running behaviour in SDS

polyacrylamide gels (Fig. 5). When the membrane-bound wild-type CcmE was used, NapB was clearly visible in the periplasmic fractions as the upper, and holo-cytochrome c_{550} as the lower, much stronger band (*lane 1*). When soluble CcmE was used for the complementation in cytochrome *c* maturation, a single heme staining band migrating in the range of holo-cytochrome c_{550} was detected in the periplasmic fractions. This protein band according to its size and heme staining characteristic could correspond to both soluble CcmE and cytochrome c_{550} or to soluble CcmE only, depending on whether or not soluble CcmE is functional in cytochrome *c* maturation. The absence of NapB and the lower levels of heme-staining protein in the range expected for soluble CcmE and cytochrome c_{550} suggest that the complementing activity may indeed be very low. In lane 3 the control experiment is presented, where only soluble CcmE, but no *c*-type cytochrome was overproduced. Here, the visible band must correspond to soluble CcmE. Since soluble CcmE and cytochrome c_{550} cannot be distinguished clearly by SDS-PAGE analysis, absorption difference spectra of the three periplasmic fractions were recorded (Fig. 5B). This method enables the distinction between cytochrome c_{550} with an α-band at 550 nm and the soluble CcmE with an α-band at 554 nm (9,22). The top trace in the inset of Fig. 5B shows the spectrum of the periplasm from a strain overexpressing soluble CcmE and cytochrome c_{550} and shows a maximum at 551 nm and a slightly asymmetric shape. The middle trace was obtained from periplasm of the strain expressing only soluble CcmE, showing a small peak at 554 nm. The arithmetic difference of the two spectra shown at the bottom reveals a peak at 550 nm, thus demonstrating that the two periplasmic fractions differ in their content of cytochrome c_{550}. Therefore, soluble CcmE indeed functions at very low levels (less than 1% by quantification) in cytochrome *c* maturation.

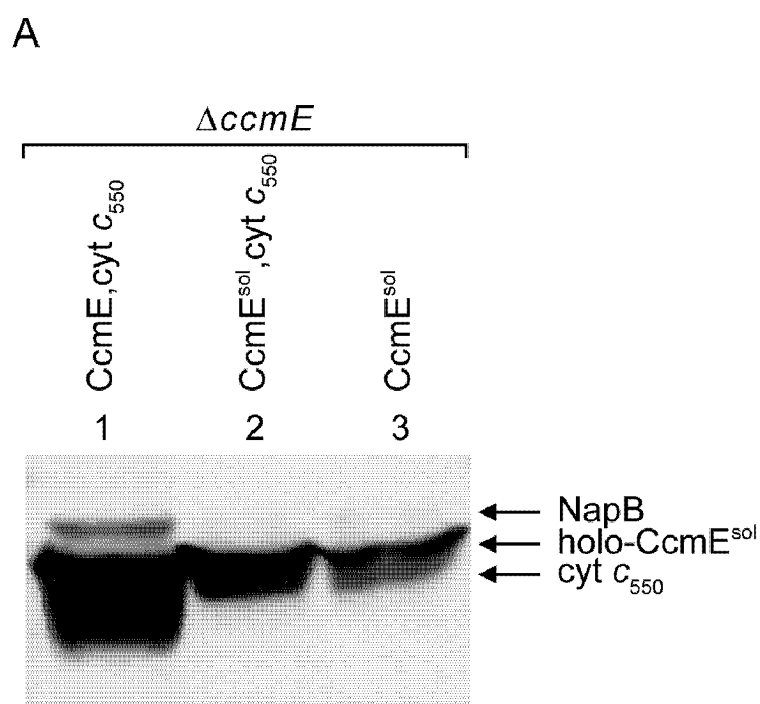

Fig. 5. Functional analysis of soluble CcmE. The Δ*ccmE* strain EC65 was co-transformed with the plasmid pEC701 (expressing *B. japonicum* cytochrome c_{550}; *lane 1* and *2*) or pACYC184 (empty vector; *lane 3*) and pEC412 (wild-type CcmE; *lane 1*) or pEC301 ($CcmE^{sol}$; *lane 2* and *3*). Cells were grown anaerobically in the presence of 5 mM sodium nitrate. *A*, trichloroacetate precipitates of the periplasmic extracts (100 µg protein per lane) separated by 15% SDS-PAGE and stained for covalently bound heme. The positions of holo-NapB, holo-$CcmE^{sol}$, and holo-cytochrome c_{550} are indicated on the right. *B*, difference spectra of periplasmic extracts of the samples as in *A*. The inset shows the spectrum at higher magnification of the middle (+ c_{550}) and lower (-c_{550}) traces of the parent figure. The lower trace (difference) in the inset represents the arithmetic difference of the upper two traces.

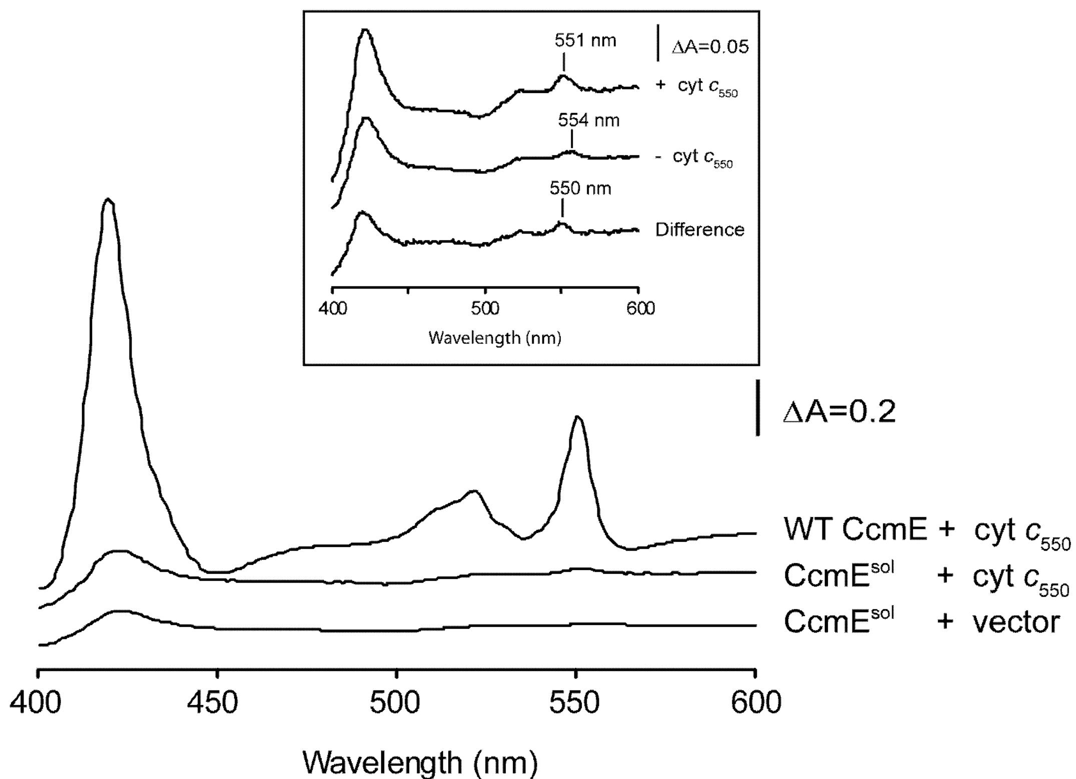

Fig. 5. Functional analysis of soluble CcmE. *B*, difference spectra of periplasmic extracts of the samples as in *A*. The inset shows the spectrum at higher magnification of the middle (+ c_{550}) and lower (-c_{550}) traces of the parent figure. The lower trace (difference) in the inset represents the arithmetic difference of the upper two traces.

The question of whether or not soluble CcmG can complement cytochrome *c* formation in a Δ*ccmG* mutant strain had not been addressed previously. We have now tested such complementation by co-transforming the *ccmG* mutant strain EC29 with a plasmid expressing a *B. japonicum* cytochrome c_{550} plus a plasmid encoding either wild-type or soluble CcmG

(OmpA'-'CcmG; Fig. 6A). The soluble CcmG complemented poorly for cytochrome *c* maturation (Fig. 6 top panel, *lane 2*). This was not due to the lack of stability of soluble CcmG, because the protein was readily detectable by immunoblot of whole cells (middle panel) and of the periplasmic fractions (bottom panel). The results from the heme stain were confirmed and quantified by comparing the absorption difference spectra of the holo-cytochrome *c* formed in the presence of either wild-type or soluble CcmG (Fig.6B *inset*).

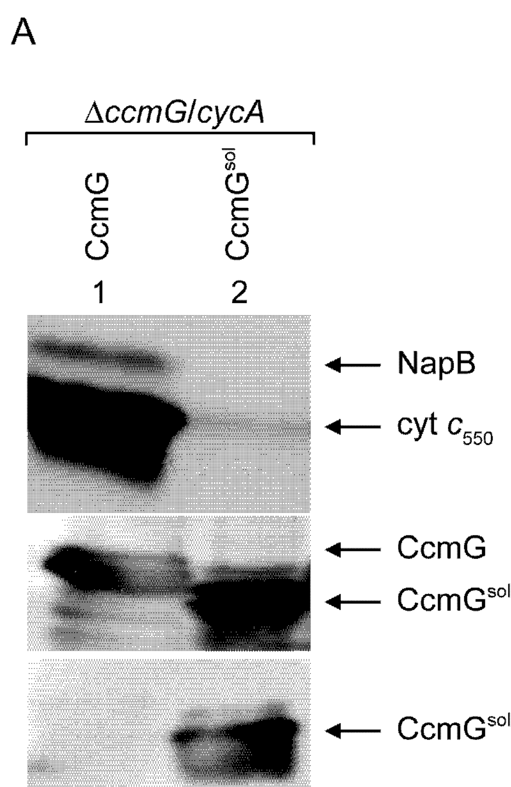

Fig. 6. Functional analysis of soluble CcmG. The Δ*ccmG* strain EC29 was co-transformed with the plasmids pEC701 (expressing *B. japonicum* cytochrome c_{550}) and pEC882 (wild-type CcmG; *lane 1*) or pEC884 ($CcmG^{sol}$-H_6; *lane2*). Cells were grown anaerobically in the presence of 5 mM sodium nitrate. *A*, Upper panel: trichloroacetate precipitates of the periplasmic extracts (100 μg per lane) separated by 15% SDS-PAGE and stained for covalently bound heme. Middle panel: immunoblot of total cell protein (after equalizing to uniform cell density) probed with antiserum against CcmG. Lower panel: immunoblot of trichloroacetate-precipitated periplasmic fractions. Holo-NapB, holo-cytochrome c_{550}, CcmG and $CcmG^{sol}$ are indicated on the right.

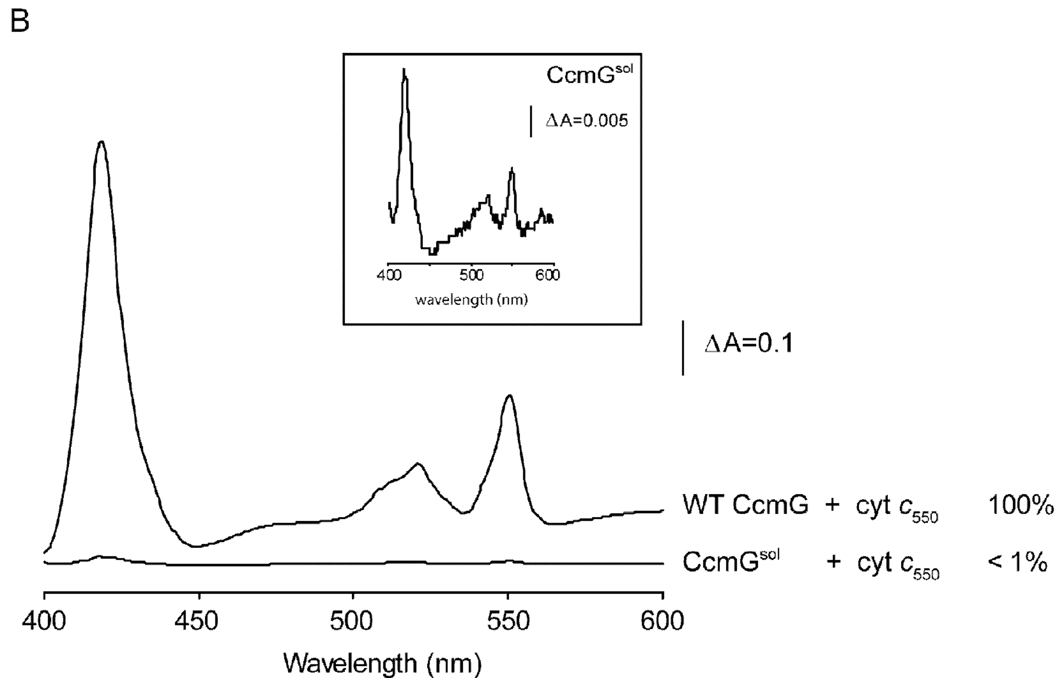

Fig. 6. Functional analysis of soluble CcmG. *B*, difference spectra of periplasmic extracts of the samples as in *A*. The inset shows the lower trace of the parent figure at a higher magnification. The percentage of holo-cytochrome *c* formed relative to the wild type is indicated on the right.

Heme transfer and co-immunoprecipitation of soluble CcmE with CcmC—In the absence of the membrane anchor of CcmE less than 1% of holo-cytochrome *c* was formed. It was important to find out whether this deficiency was due to (i) loss of membrane attachment, (ii) lack of specific protein-protein interactions with CcmC or (iii) impairment in heme delivery to CcmE. These possibilities were examined by co-expressing CcmC-H_6 and either wild-type or soluble CcmE in a Δ*ccm* background strain lacking all *ccm* genes followed by co-immunoprecipitation (Fig. 7). It is known that the CcmC-CcmE pair is sufficient for heme

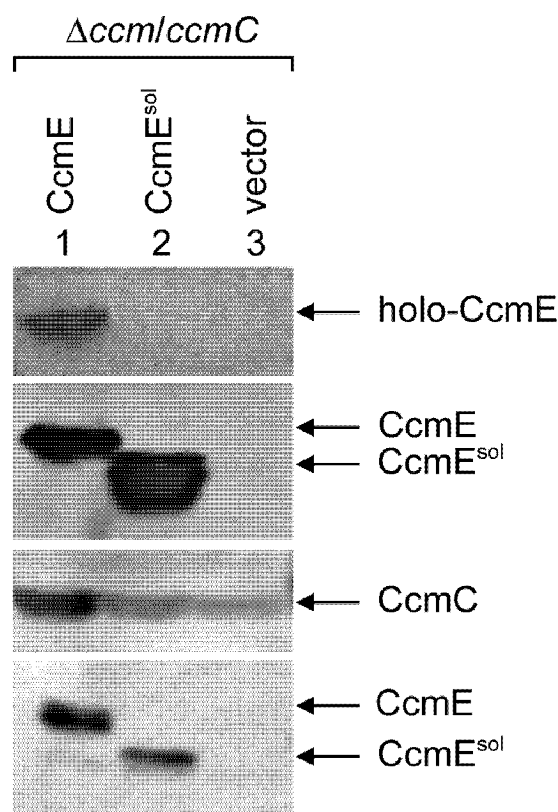

Fig. 7. Physical interactions of soluble CcmE with CcmC for heme transfer. The Δ*ccmA-H* mutant strain EC06 was co-transformed with the plasmid pEC486 expressing C-terminally H_6-tagged CcmC and pEC412 (CcmE; *lane 1*), pEC301 (CcmE^{sol}; *lane 2*) or pISC2 (empty vector; *lane 3*). Cells were grown anaerobically in the presence of 5 mM sodium nitrate. Upper panel: whole cell lysates (300 μg per lane) were separated by 15% SDS-PAGE and stained for covalently bound heme. Second panel: immunoblot of the whole cell lysate (100 μg per lane) probed with antiserum against CcmE. Third panel: immunoblot of the whole cell lysates (100 μg per lane) probed with antiserum against the H_6 tag. Fourth panel: immunoprecipitation of whole cell lysates with anti-H_6 tag antibodies and detection of CcmE by immunoblot. The positions of holo-CcmE, CcmE, CcmE^{sol} and CcmC-H_6 are indicated on the right.

transfer to CcmE (9), which was tested first (Fig.7 *upper panel*). To our surprise, heme delivery to soluble CcmE was severely affected (*lane 2*) when compared to the wild-type CcmE (*lane 1*), which is in contrast to the result shown in Fig. 5 and earlier experiments (15). However, in those experiments the CcmAB and CcmD had been co-expressed, in addition to

CcmC and CcmE. We already know that CcmD increases the stable assembly of CcmE in the membrane (19,23). The presence of CcmC-H_6, CcmE and soluble CcmE was confirmed by immunoblot (second and third panel). A normal interaction of soluble CcmE with CcmC-H_6 was found in the co-immunoprecipitation experiment (lower panel).

DISCUSSION

The heme chaperone CcmE serves as a periplasmic sink for heme during cytochrome *c* maturation by binding heme transiently on the periplasmic side of the membrane. This binding is covalent and can be measured experimentally. Heme is delivered subsequently to cytochrome *c*. We can therefore look at the function of CcmE in two steps: first, formation of holo-CcmE and second, formation of holo-cytochrome *c*. CcmE is attached to the membrane by an N-terminal transmembrane helix. Except for its length and hydrophobicity, this domain is the least conserved within CcmE homologues. A periplasmic soluble form of CcmE has been shown previously to be extremely inefficient as a heme chaperone for cytochrome *c* formation (15), suggesting an important role of the transmembrane domain. In this study we asked if it was possible to replace the membrane anchor of CcmE with that of another monotopic membrane protein of the cytochrome *c* maturation system, CcmG, without loss of function. This would be expected if the function of the membrane anchor was simply to attach the periplasmic domain of these proteins to the membrane in the correct position and orientation. Alternatively, a more specific role would have to be postulated for the membrane anchor.

Soluble CcmE can be produced as a heme binding protein, which has been used to produce large amounts of the protein for biochemical and biophysical characterization (9,15,22,24).

We have noticed that soluble holo-CcmE is formed at reasonable levels when the genes *ccmABCD*, and most efficiently when the entire *ccm* gene cluster was co-expressed (M. Braun, unpublished observations; (22)). The minimal requirement for heme transfer to CcmE is the presence of CcmC (10). Our finding that co-immunoprecipitation of CcmC with either chimerical or soluble CcmE was not substantially affected suggests that a physical interaction between the two molecules occurs efficiently in the absence of the authentic membrane anchor, and thus most likely involves the periplasmic domain of CcmE. Nevertheless, the heme transfer step for the formation of holo-CcmE is affected for both soluble CcmE and the CcmG'-'E chimera. However, once holo-CcmE is formed, the delivery of heme to *c*-type cytochromes does not seem to depend on the authentic membrane anchor, because the CcmG'-'E chimera was rather active in cytochrome *c* formation at low cellular levels. Our results are compatible with a view of a stimulatory role of the CcmE membrane anchor, which might be to signal the correct conformation and assembly of the protein for heme uptake and release. We have previously postulated a model where CcmE dynamically interacts with either CcmC or CcmF, the putative heme lyase (25), and the control of this process may lie in specific intermolecular recognition of transmembrane helices.

In addition to the heme chaperone CcmE, the cytochrome *c* maturation system contains a second monotopic membrane protein with an N-terminal membrane anchor and a C-terminal periplasmic domain of a similar size: the thioredoxin-like protein CcmG, also known as DsbE. CcmG is known to receive electrons from DsbD and provide them to the cytochrome *c* maturation machinery to keep the cysteines in apo-cytochrome *c* reduced (21,26). Previously, it had been shown that mutants with alterations in the active site cysteines of CcmG were deficient in cytochrome *c* maturation and this could be restored by the addition of a reducing

agent such as cysteine. By contrast, a Δ*ccmG* in frame deletion strain could not be restored by adding reducing agents (21), suggesting that CcmG has yet a different function, perhaps associated with another protein domain. One candidate domain was the transmembrane segment that attaches CcmG to the membrane and perhaps directs interaction with another factor of the cytochrome *c* maturation system. The results of the present study confirm that the membrane anchor of this protein has an important function: while the soluble CcmG version, despite massive overproduction compared to the other Ccm proteins in the system, was unable to efficiently support cytochrome *c* maturation; the chimera with a CcmE transmembrane domain was partially active. We conclude that the membrane attachment, even when provided by the "wrong" transmembrane helix, is an essential prerequisite for CcmG function.

An unexpected finding of this study is the strong decrease of CcmE polypeptide in the membrane in the CcmEV26Q mutant and the drastic reduction of cytochrome *c* formation in the CcmGΔW22 mutant. Both mutants were obtained as intermediates during the construction of the chimera polypeptides, and they both carry point mutations at the periplasmic end of the transmembrane segment. Even though the transmembrane segments represent the least conserved parts of these monotopic membrane proteins, changes of individual amino acids within them can have drastic consequences. This suggests that these domains must have evolved in a functional context. Thus far, structural data are available only for the periplasmic domains of CcmE (13,14) and CcmG (27,28) produced in a soluble form. Our findings indicate that the membrane anchor might also have a structural role, perhaps by positioning the periplasmic domain precisely and in a certain orientation relative to the membrane surface and to other interacting partner molecules. It would be interesting to obtain structures of the

entire membrane proteins including their membrane anchors to get better insight into this question.

Acknowledgments—We thank E. Enggist, Q. Ren and H. Schulz for the construction of some plasmids and strains used in this work. We also thank M. Braun and J. Stevens for helpful comments on the manuscript. This work was supported by the Swiss National Foundation for Scientific Research.

REFERENCES

1. Thöny-Meyer, L. (1997) *Microbiol. Mol. Biol. Rev.* **61**(3), 337-376
2. Xie, Z., and Merchant, S. (1998) *Biochim. Biophys. Acta.* **1365**(1-2), 309-318
3. Kranz, R., Lill, R., Goldman, B., Bonnard, G., and Merchant, S. (1998) *Mol. Microbiol.* **29**(2), 383-396
4. Page, M. D., Sambongi, Y., and Ferguson, S. J. (1998) *Trends. Biochem. Sci.* **23**(3), 103-108
5. Thöny-Meyer, L. (2002) *Biochem. Soc. Trans.* **30**(4), 633-638
6. Allen, J. W., Daltrop, O., Stevens, J. M., and Ferguson, S. J. (2003) *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **358**(1429), 255-266
7. Thöny-Meyer, L., Fischer, F., Künzler, P., Ritz, D., and Hennecke, H. (1995) *J. Bacteriol.* **177**(15), 4321-4326
8. Grove, J., Tanapongpipat, S., Thomas, G., Griffiths, L., Crooke, H., and Cole, J. (1996) *Mol. Microbiol.* **19**(3), 467-481
9. Schulz, H., Hennecke, H., and Thöny-Meyer, L. (1998) *Science* **281**(5380), 1197-1200
10. Schulz, H., Fabianek, R. A., Pellicioli, E. C., Hennecke, H., and Thöny-Meyer, L. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**(11), 6462-6467
11. Ahuja, U., and Thöny-Meyer, L. (2003) *J. Biol. Chem.* **278**(52), 52061-52070
12. Enggist, E., Schneider, M. J., Schulz, H., and Thöny-Meyer, L. (2003) *J. Bacteriol.* **185**(1), 175-183
13. Enggist, E., Thöny-Meyer, L., Güntert, P., and Pervushin, K. (2002) *Structure (Camb)* **10**(11), 1551-1557

14. Arnesano, F., Banci, L., Barker, P. D., Bertini, I., Rosato, A., Su, X. C., and Viezzoli, M. S. (2002) *Biochemistry* **41**(46), 13587-13594

15. Enggist, E., and Thöny-Meyer, L. (2003) *J. Bacteriol.* **185**(13), 3821-3827

16. Iobbi-Nivol, C., Crooke, H., Griffiths, L., Grove, J., Hussain, H., Pommier, J., Mejean, V., and Cole, J. A. (1994) *FEMS Microbiol. Lett.* **119**(1-2), 89-94

17. Hanahan, D. (1983) *J. Mol. Biol.* **166**(4), 557-580

18. Ren, Q., and Thöny-Meyer, L. (2001) *J. Biol. Chem.* **276**(35), 32591-32596

19. Schulz, H., Pellicioli, E. C., and Thöny-Meyer, L. (2000) *Mol. Microbiol.* **37**(6), 1379-1388

20. Thöny-Meyer, L., Künzler, P., and Hennecke, H. (1996) *Eur. J. Biochem.* **235**(3), 754-761

21. Fabianek, R. A., Hennecke, H., and Thöny-Meyer, L. (1998) *J. Bacteriol.* **180**(7), 1947-1950

22. Daltrop, O., Stevens, J. M., Higham, C. W., and Ferguson, S. J. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**(15), 9703-9708

23. Ahuja, U., and Thöny-Meyer, L. (2005) *J. Biol. Chem.* **280**(1), 236-243

24. Uchida, T., Stevens, J. M., Daltrop, O., Harvat, E. M., Hong, L., Ferguson, S. J., and Kitagawa, T. (2004) *J. Biol. Chem.* **279**(50), 51981-51988

25. Ren, Q., Ahuja, U., and Thöny-Meyer, L. (2002) *J. Biol. Chem.* **277**(10), 7657-7663

26. Rietsch, A., Belin, D., Martin, N., and Beckwith, J. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**(23), 13048-13053

27. Edeling, M. A., Guddat, L. W., Fabianek, R. A., Thöny-Meyer, L., and Martin, J. L. (2002) *Structure (Camb)* **10**(7), 973-979

28. Li, Q., Hu, H. Y., Wang, W. Q., and Xu, G. J. (2001) *Biol. Chem.* **382**(12), 1679-1686

29. Tusnady, G. E., and Simon, I. (1998) *J. Mol. Biol.* **283**(2), 489-506

PUBLICATIONS

Ahuja, U. and L. Thöny-Meyer (2005). "CcmD is involved in complex formation between CcmC and the heme chaperone CcmE during cytochrome *c* maturation." *J. Biol. Chem.* **280** (1): 236-43.

Edeling, M. A., U. Ahuja, B. Heras, L. Thöny-Meyer, and J. L. Martin (2004). "The acidic nature of the CcmG redox-active center is important for cytochrome *c* maturation in *Escherichia coli*." *J. Bacteriol.* **186** (12): 4030-3.

Ahuja, U. and L. Thöny-Meyer (2003). "Dynamic features of a heme delivery system for cytochrome *c* maturation." *J. Biol. Chem.* **278** (52): 52061-70.

Ren, Q., U. Ahuja, and L. Thöny-Meyer. (2002). "A bacterial cytochrome *c* heme lyase. CcmF forms a complex with the heme chaperone CcmE and CcmH but not with apocytochrome *c*." *J. Biol. Chem.* **277** (10): 7657-63.

CURRICULUM VITAE

Umesh Ahuja

Born on February 10th, 1976 in New Delhi

Citizen of India

2001-2005	Ph.D thesis at the Institute of Microbiology, Swiss Federal Institute of Technology, Zürich, Switzerland
1999-2001	Assistant researcher at the National Institute of Immunology, New Delhi, India
1996-1998	Masters degree with honors in Microbiology from the Punjab University, Chandigarh, India
1993-1996	Bachelors degree with honors in Microbiology from the University of Delhi, Delhi, India
1991-1993	High school in Delhi, India
1990-1991	Secondary school in Delhi, India
1981-1990	Primary school in Delhi, India