Doctoral Thesis

CcmA and CcmB, an ABC transporter involved in cytochrome c maturation

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CcmA and CcmB, an ABC transporter involved in cytochrome c maturation

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Abstract

C-type cytochromes are electron transfer proteins that carry heme as a prosthetic group. In Gram-negative bacteria, the precursor forms of cytochrome c as well as heme are synthesized in the cytoplasm. Heme and the apo-cytochrome c are translocated separately across the membrane to the periplasm, the site of assembly. It is known that apo-cytochrome c is translocated by the Sec machinery, but it is still an open question how heme is transported.

In Escherichia coli, eight proteins, CcmA-H, are encoded by the ccm operon and are essential for cytochrome c maturation. Among them, the proteins CcmA, CcmB and/or CcmC are believed to constitute an ABC transporter. Until now, it was unclear, whether the ABC transporter consists of the two proteins CcmA and CcmB or of the three proteins CcmA, CcmB and CcmC.

I present a major breakthrough to answer the question of assembly. This was achieved by interaction studies, which clearly showed that CcmA and CcmB form an ABC transporter, most likely of the composition CcmA2B2 with CcmC as an accessory protein. The active CcmAB complex was purified and its intrinsic ATPase activity was determined. This activity could be stimulated slightly by the addition of the CcmE protein, but not by heme or other redox-active substances. A mutation in the Walker A motif of CcmA abolished not only ATP hydrolysis, but also cytochrome c formation. This demonstrates that ATP hydrolysis by CcmA is an essential prerequisite of cytochrome c maturation. Complementation of a ccmA mutant with extracellular heme failed. Thus, it is very unlikely that heme is transported by CcmA and CcmB.

Determination of essential residues for CcmB activity was also tried, which involved expression of ccmB from a plasmid in a strain lacking the chromosomal gene. This led to a surprising finding: overexpression of ccmB caused a dominant negative phenotype, i.e. ccmB expressed from a plasmid was unable to complement the ccmB deletion strain. The dominant negative phenotype could be relieved when ccmB was overexpressed together with ccmA and ccmC, or with certain point mutations in some of the conserved residues of CcmB. Mutations of other conserved amino acid residues within CcmB had no detectable phenotype, neither regarding the dominant negative effect, nor with respect to cytochrome c maturation.

Another unexpected observation was the formation of stable CcmE dimers visible in denaturing SDS polyacrylamide gels. This dimer formation was further investigated, because strains mutated in ccmA or ccmB were not able to form CcmE dimer. This dimer formation was not observed, when heme was not incorporated into CcmE, which was dependent on i) CcmC, ii) heme
biosynthesis and iii) an intact heme binding site of CcmE, i.e. the presence of histidine 130. The hypothesis that CcmE dimers have a biological role in cytochrome c formation was further supported by the finding that CcmE dimer formation was always observed in strains able to produce holo-cytochrome c. Separation of the dimer from the monomer followed by mass spectroscopy showed that the dimer consists of two holo-CcmE polypeptides. Moreover, attempts to dissociate these dimers failed. Thus, a strong interaction of two molecules, probably by a covalent bond between the two CcmE polypeptides and/or heme is hypothesized. Although the actual substrate of CcmAB still remains unknown, this work revealed important characteristics of CcmAB that need to be considered in future research.
Zusammenfassung


Man weiß zwar, dass das Apo-Cytochrom c mit Hilfe des Sec Apparates transloziert wird, es ist jedoch nach wie vor unbekannt, wie Häm über die Membran transportiert wird.

Im Bakterium *Escherichia coli* sind acht Proteine (CcmA-H) essentiell, um reifes Cytochrom c zu bilden. Die Proteine CcmA-H werden durch das *ccm* Operon kodiert. Von diesen acht Proteinen bilden CcmA, CcmB und/oder CcmC einen ABC-Transporter. Bis jetzt war es jedoch unklar, ob der ABC-Transporter lediglich aus den zwei Proteinen CcmA und CcmB besteht oder aus den Proteinen CcmA, CcmB und CcmC.

Bei der Fragestellung nach dem Aufbau des ABC-Transporters wurde mittels Interaktionsstudien ein grosser Durchbruch erreicht. In dieser Arbeit konnte klar gezeigt werden, dass der ABC-Transporter aus CcmA und CcmB mit der Anordnung CcmA₂B₂ besteht, wobei CcmC als Zusatzprotein mit dem ABC-Transporter interagiert.

Der CcmAB Komplex wurde gereinigt, und es wurde eine intrinsische ATPase Aktivität festgestellt. Diese Aktivität wurde durch das CcmE Protein leicht stimuliert, jedoch weder durch Häm noch durch andere redox-aktive Substanzen. Weiter hemmte eine Mutation im Walker A Motif von CcmA nicht nur die ATP Hydrolyse, sondern ebenfalls die Cytochrom c Reifung.


Es wurde versucht, essentielle Aminosäuren in CcmB zu bestimmen. Dies sollte erreicht werden, indem das *ccmB* Gen in einer *ccmB* Mutante exprimiert wurde. Es zeigte sich jedoch ein interessantes Ergebnis: Überexpression des *ccmB* Gens verursachte einen dominant-negativen Phänotyp. Das bedeutet, dass das Gen *ccmB*, welches von einem Plasmid exprimiert wurde, nicht in der Lage war, eine *ccmB* Deletionsmutante zu komplementieren. Dieser dominant-negative Phänotyp konnte jedoch aufgehoben werden, wenn *ccmB* zusammen mit *ccmA* und *ccmC*

Eine andere unerwartete Beobachtung war die Bildung von stabilen CcmE Dimeren in denaturierenden SDS Polyacrylamidgelen. Untersuchungen zeigten, dass diese Dimere in ccmA oder ccmB Mutanten nicht vorhanden waren. Es konnte weiter gezeigt werden, dass keine CcmE Dimere gefunden werden, wenn Häm nicht in das CcmE eingebaut wird. Dieser Einbau ist abhängig von i) CcmC ii) der Hämbiosynthese und iii) einer intakten Hämbindung am CcmE, die nur in Gegenwart von Histidin 130 erfolgen kann. Die Hypothese, dass die CcmE Dimere eine biologische Rolle in der Cytochrom c Bildung haben, wurde weiter unterstützt durch die Erkenntnis, dass die Dimere immer nur dann gefunden wurden, wenn ebenfalls reifes Cytochrom c vorhanden war.

In dieser Arbeit konnten die Dimere von den Monomeren getrennt werden. Ein Massenspektrum des isolierten Dimers ergab, dass die Dimere aus zwei Holo-CcmE Molekülen bestehen. Es schlugen alle Versuche fehl, die Dimere in Monomere zu zerteilen. Dies deutet auf eine starke Interaktion zwischen den beiden CcmE Monomeren hin, und eine kovalente Binding zwischen den beiden Häm Molekülen kann nicht ausgeschlossen werden.

Obwohl das eigentliche Substrat von CcmAB immer noch nicht gefunden werden konnte, beinhaltet diese Arbeit dennoch wichtige Erkenntnisse über CcmAB, welche für die zukünftige Forschung berücksichtigt werden müssen.
Chapter I

Introduction
Introduction

As the name indicates, cytochromes (cellular pigments) are cellular proteins that absorb light at specific wavelengths. Their color originates from heme, which they carry as their prosthetic group. Heme is usually understood as any tetrapyrrolic chelate of iron (Moss, 1988). The name cytochrome was introduced by Keilin in 1925 to describe a chemical group involved in oxidation-reduction processes. Upon reduction, this group exhibits strong absorption between 510 and 615 nm (α-bands) (Keilin, 1925). Keilin divided the cytochromes according to their spectral type into cytochromes a, b and c. The group of cytochrome d was introduced by the IUB Enzyme Commission in 1961. The classical definition of a cytochrome is as follows: a cytochrome is a hemoprotein whose characteristic mode of action involves transfer of reducing equivalents associated with a reversible change in oxidation state of the prosthetic group (NC-IUB, 1992).

Because heme can easily be detected by absorption spectroscopy, cytochromes are ideal proteins to study the posttranscriptional maturation, including the insertion of the cofactor into the polypeptide (Fig. 1).

**Figure 1. Holo-enzyme maturation.** General overview of the steps required for maturation of holo-enzyme complexes. Cytochrome c maturation as an example requires the translocation of heme and apo-cytochrome from the cytoplasm to the periplasm, followed by the incorporation of heme into the apo-cytochrome to form mature holo-cytochrome c. Modified from (Thöny-Meyer, 1997).
In general, a protein is matured by the conversion of a gene product (apo-protein) into its mature form (holo-protein) by insertion of its cofactor. The maturation process becomes complicated when the synthesis of apo-protein and cofactor takes place in a different compartment from where the assembly occurs. Therefore, translocation of both the polypeptide and cofactor into or through membranes is needed. Last, proteolytic cleavage and modification of the polypeptide may occur before or after cofactor insertion.

General features of cytochromes

Cytochromes show specific spectral characteristics, depending on the type of heme and its binding to the protein. The reduced form of a cytochrome gives rise to three main absorption maxima in the visible to UV spectrum which are designated as α, β and γ (Soret) peaks. The α-peak can be used to classify cytochromes: c-type cytochromes have a maximal absorption between 550 and 557 nm, b- and o-type cytochromes have an absorption peak between 555 and 565 nm, a-type cytochromes have a maximum around 600 nm, and d-type cytochromes have a maximum around 630 nm (NC-IUB., 1992). In addition, the cytochromes are defined by the attachment of a specific heme: while the cytochromes a, b, d and o have the heme non-covalently attached, the heme in cytochrome c is covalently attached via its vinyl side chains to the reduced cysteines within a CXXCH motif of the cytochrome polypeptide. The heme cofactor itself is redox active and can either be reduced (ferrous, Fe^{2+}) or oxidized (ferric, Fe^{3+}), and therefore, cytochromes are one-electron carriers.

Most bacterial cytochromes have a function in aerobic or anaerobic respiration, whereas the ATP formation is coupled to the oxidation of reduced substrates such as organic compounds, hydrogen, reduced sulfur, or even metals. In addition, they are involved in electron transport during photosynthesis. The transport of electrons and protons across the cytoplasmic membrane leads to a proton gradient that can be used by the F_{1}F_{0} ATPase to generate ATP. During aerobic respiration, the electrons are shuttled through a number of electron carriers to oxygen. During anaerobic respiration, the electrons are transported to alternative terminal electron acceptors like nitrite or fumarate (Fig. 2).
Introduction

Figure 2. Bacterial respiratory chains containing cytochrome c. Q/QH₂ represent the quinone pool in the membrane. On the left, b-type cytochromes are shown which oxidize the substrates and deliver the electrons to the quinol pool (quinone-reducing branch). On the right, terminal oxidases are shown, which use oxygen or alternative electron acceptor for reduction (quinone-oxidizing branch). Oxidoreductases are shown in boxes, with the corresponding type of cytochrome shown below. The substrates of the alternative reductases are shown next to the type and name of cytochrome.

Cytochrome c maturation

C-type cytochromes can be found in the cytoplasmic membrane or in the periplasm of prokaryotes, in the intermembrane space or in the inner membrane of mitochondria and in the lumen or thylakoid membrane of chloroplasts (Fig. 3). Cytochrome c maturation is generally defined as a biogenesis pathway, where an unfolded polypeptide (the apo-cytochrome c) is assembled into its three-dimensional structure by insertion of one or more covalently bound heme cofactors, resulting in the holo-cytochrome c.
Cytochrome c maturation

This covalent attachment distinguishes them from b-type cytochromes, where the heme is non-covalently attached. The main question of cytochrome c maturation is how heme is attached covalently to the heme binding motif CXXCH of the apo-cytochrome (Fig. 4). As heme and the apo-cytochrome are not synthesized at the location where holo-cytochrome c formation takes place, heme and the apo-cytochrome must be translocated to these compartments. Within these compartments, heme must find the two cysteines of the apo-cytochrome, and the vinyl groups of...
heme must be attached to the thiol groups of apo-cytochrome in a stereospecific manner. This reaction requires that the cysteines are reduced. In addition, Nicholson and Neupert have demonstrated that the heme iron must be in a reduced state for attachment of heme to the cysteines (Nicholson and Neupert, 1989).

Fig. 4. Attachment of heme to a c-type cytochrome. Heme is covalently attached to the reduced cysteines of apo-cytochrome. The stereospecific attachment of the vinyl groups of heme is catalyzed by a heme lyase and other proteins involved in cytochrome c maturation (see systems I to III).

Recently, for the first time it was possible to achieve the covalent attachment of heme to a cytochrome c polypeptide in vitro (Daltrop et al., 2002). For cytochrome c maturation in vivo, cells have evolved at least three different types of maturation systems to assemble c-type cytochromes. These systems have different levels of complexity and have been named system I, II and III (Allen et al., 2003; Kranz et al., 1998; Thöny-Meyer, 1997, 2002). Furthermore, sequence analysis has shown that the system II organisms *Bordetella bronchiseptica* and *Bordetella parapertussis* have elements (CcmE, CcmF) of system I and II. Interestingly, *Anopheles gambiae* contains elements of all three systems (CcmE, CcmG, CcmH, ResB, ResC and CCHL) (Thöny-Meyer, 2003; Stevens et al., 2004). The most complex system (system I) is found in Gram-negative bacteria and in mitochondria of plant and protozoa. System II can be found in thylakoids and as well as in Gram-positive and Gram-negative bacteria whereas the least complex system (system III) is found in mitochondria of fungi, invertebrates and vertebrates.
Cytochrome c maturation

System I

The genes of system I, which are essential for cytochrome c maturation have been discovered in α- and γ- proteobacteria, but also in plant and protozoal mitochondria. In E. coli, these genes are clustered and have been named \textit{ccm}ABCDEFGH, where \textit{ccm} stands for cytochrome \textit{c} maturation (Fig. 5). The gene products of this system can be divided into several parts: the ABC transporter (Goldman and Kranz, 2001), the heme delivery system (Thöny-Meyer, 2000; Ren and Thöny-Meyer, 2001; Ahuja and Thöny-Meyer, 2003), the heme chaperone (; Schulz et al., 1998; Enggist et al., 2002), the heme lyase (Ren et al., 2002) and the redox control (Fabianek et al., 1998; Fabianek et al., 1999; Edeling et al., 2004). The well studied organism E. coli will be used as a representative of system I to discuss the main features of this system. The ABC transporter predicted to be involved in cytochrome c maturation is the main topic of this work and will be discussed in detail in a separate chapter.

\begin{center}
\includegraphics[width=\textwidth]{cytochrome_c_maturation.png}
\end{center}

\textit{Fig. 5 Model of the cytochrome c maturation and gene products in \textit{Escherichia coli}.} The cytochrome c maturation machinery of system I can be divided into 5 subclasses: i) an ABC transporter of unknown function, ii) the heme delivery system, which attaches heme to the iii) heme chaperone, which might protect other molecules against the attack of heme or serves as intermediated heme storage, before attachment of heme to the apocytochrome, iv) heme lyase is needed for the heme attachment to the apocytochrome, v) the redox control is needed for the reduction of the cysteines prior of the attachment of heme.
Introduction

The heme delivery system CcmCD

In *E. coli*, heme is transferred to the heme chaperone CcmE with the help of CcmC and CcmD. Overproduced CcmC alone is able to transfer heme in the absence of CcmD, whereas CcmD was shown to enhance the efficiency of heme attachment (Schulz et al., 1999). CcmC is a 27.9 kDa membrane protein that spans the cytoplasmic membrane six times. It is conserved in over 40 different organisms including α- and γ-proteobacteria, archaea and in mitochondria of plant and protozoa. CcmC contains a conserved tryptophan-rich signature motif (WGΦWXWDXRLT, Φ indicates an aromatic amino acid residue) in the second periplasmic domain, which is flanked by two conserved histidines located in the first and second periplasmic loops (Thöny-Meyer, 1997; Kranz et al., 1998). This motif shares a high sequence homology with the tryptophan-rich motifs of CcmF, NrfE and even with the system II homologue CcsA (Thöny-Meyer, 2000). Previously, it has been speculated that the tryptophan rich motif serves as a platform for the interaction of heme with CcmE, with the two histidines as axial heme ligands (Goldman et al., 1997; Schulz et al., 2000). However, the tryptophan-rich motif and the conserved histidines have been shown to be important for the interaction of heme with CcmE (Ren and Thöny-Meyer, 2001).

CcmD is a small protein of 69 amino acid residues, essential for cytochrome c maturation and involved in heme delivery to CcmE. CcmD was shown to interact with both CcmC and CcmE (Ren and Thöny-Meyer, 2001). It was earlier proposed for the *R. capsulatus* homologue HelX that its C-terminus is located in the cytoplasm and its N-terminus in the periplasm (Goldman et al., 1997). Umesh Ahuja could show by a proteolytic digestion assay that the *E. coli* CcmD is anchored to the membrane via its hydrophobic domain, which is essential for its function, whereas both the N-terminus and the C-terminus are located in the cytoplasm (Ahuja and Thöny-Meyer, 2005). This clearly shows that CcmD is not a membrane-spanning protein and is oriented towards the cytoplasm.
The term “molecular chaperone” is used to describe a group of proteins that recognize and bind misfolded proteins and assist them in folding into their correct three dimensional structures. The definition of a “chaperone” has been expanded and is now used also in the context of metal cofactor delivery (Finney and O'Halloran, 2003). Initially, when the Ccm proteins were overproduced in a ccm deletion strain, analysis of the membrane fraction by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) showed an 18 kDa protein which exhibited a peroxidase activity typical for cytochromes with covalently bound heme. Further analysis showed that this protein corresponds to CcmE (Schulz et al., 1998), which binds heme covalently at the histidine at position 130 in the conserved signature motif LAKHDENY. This feature is unique among the three cytochrome c maturation systems, and the discovery was surprising because at that time, only proteins with a CXXCH motif had been reported to bind heme covalently. When CcmE was expressed as a soluble protein in the periplasm, heme binding occurred, but it was absent when CcmE was expressed in the cytoplasm (Schulz et al., 1998), indicating that heme attachment occurred in the periplasm. Elisabeth Enggist solved the structure of the apo-CcmE by NMR (Fig. 6, left panel) and predicted that the α-helical domain positioned in the vicinity of the heme binding site might protect the heme molecule from interaction with other molecules and, because of the flexibility of its C-terminal domain, aids the release and delivery of heme to the apo-cytochrome (Enggist et al., 2002). Furthermore, CcmE was shown to have an affinity for oxidized (ferric) heme rather than reduced (ferrous) heme in vitro, but only reduced heme was bound covalently (Daltrop et al., 2002). Recently, the chemical structure of the covalent bond between H130 of CcmE and heme was solved by NMR. It was found that the β-carbon of one of the vinyl groups of heme was covalently bound to the Nε1 of the histidine, representing a novel type of heme-histidine complex (Fig. 6, right panel) (Lee et al., 2005).
From structural predictions as well as from Raman resonance spectroscopy (Enggist et al., 2002; Stevens et al., 2004), it is most likely the vinyl 2 that forms the covalent bond. The bond at the β-carbon is unexpected because covalent heme binding in c-type cytochromes always occurs at the α-carbon, according to the Markovnikov rule, by an electrophilic addition to the vinyl group. For a covalent bond with the β-carbon, radicals might be involved in the reaction mechanism, thus following the anti-Markovnikov rule (Loundon, 2002; Lee et al., 2005). However, it is still not clear how heme is transferred to CcmE, how this heme-histidine linkage is catalyzed in vivo and how heme is released from CcmE and attached to the apo-cytochrome. Another interesting feature of CcmE is its formation of dimers that can be detected in denaturing polyacrylamide gels, where a small fraction of heme-containing CcmE runs at the double molecular weight of CcmE, indicating a covalent binding between two CcmE proteins. This formation is not only observed with the E. coli CcmE, but also with the Bradyrhizobium japonicum CycJ (Schulz and Thöny-Meyer, 2000). However, whether this dimer formation occurs in other organisms of system I needs to be shown. Also, the nature of the CcmE dimer and its potential function in cytochrome c formation have not yet been addressed.
The heme lyase CcmF

CcmF is a large membrane protein with eleven transmembrane helices. It shares the strictly conserved tryptophan-rich motif with CcmC, whereby the motif is slightly different, with the consensus sequence WGGXWFWDVPVEN. CcmF is predicted to catalyze the thioether bond formation between heme and apo-cytochrome c (Grove et al., 1996; Xie and Merchant, 1998; Thöny-Meyer, 2000; Ren et al., 2002). This is in agreement with the finding that CcmF is not required for heme delivery to CcmE, but for transfer of heme from CcmE to the apo-cytochrome c (Schulz et al., 1999; Schulz et al., 2000). This hypothesis was further supported by the fact that CcmF co-immunoprecipitates with CcmE, indicating a complex formation between CcmE and CcmF and with CcmH, which is believed to be involved in reducing the cysteines in the CXXCH motif of apo-cytochrome c (Fabianek et al., 1999; Ren et al., 2002).

Redox control

Heme attachment to cytochrome c requires the covalent binding of the two vinyl groups of heme to the thiol groups of the two cysteines of the apo-cytochrome c. As cytochrome c formation takes place in the periplasm, the cysteine residues are thought to be oxidized after translocation by the strong oxidase DsbA (Sambongi and Ferguson, 1996). Another essential oxidoreductase is DsbB, which reoxidizes the reduced DsbA and transfers the electrons to the quinone pool of the respiratory chain (Collet and Bardwell, 2002). Thus, the disulfide-bonds of the apo-cytochrome c have to be re-reduced prior to heme attachment. This is thought to occur via CcmG and CcmH, as these two proteins contain a CXXC motif typical for thioredoxin-like proteins (Fabianek et al., 1998; Fabianek et al., 1999). In addition, the protein DsbD was reported to interact with CcmG (Katzen and Beckwith, 2000). DsbD is an integral membrane protein and is believed to shuttle electrons from the cytoplasmic protein thioredoxin (TrxA) via its hydrophobic membrane domain (β-domain) and the periplasmic γ and α domain to CcmG or DsbC (Goldstone et al., 2001; Gordon et al., 2000). This is in agreement with the finding that mutations in either ccmG, ccmH, dsbD or trxA affect cytochrome c maturation (Crooke and Cole, 1995; Sambongi and Ferguson,
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This complex redox system of E. coli ensures that the cysteine residues of the apo-cytochrome c are reduced prior to heme attachment.

System II

In addition to system I, a second system has emerged from studies of the Gram-positive bacterium Bacillus subtilis and of the chloroplasts of Chlamydomonas reinhardtii. The key proteins involved in the formation of c-type cytochromes are ResA, ResB (Ccs1), ResC (CcsA) and CcdA (Le Brun et al., 2000; Crow et al., 2004; Schiött et al., 1997; Sun et al., 1996; Page et al., 2004). CcdA shares sequence similarity with the β-domain of DsbD and was proposed to function in delivering electrons to disulfide isomerases on the outer side of the cytoplasmic membrane (Schiött et al., 1997). ResC (CcsA) has a tryptophan-rich motif which is also found in the system I proteins CcmC and CcmF (Sun et al., 1996). The most interesting finding is that neither homologues of the heme chaperone CcmE nor of the ABC transporter CcmA and CcmB was found in system II organisms, indicating a different pathway for attachment of heme to the apo-cytochrome c.

System III

In fungal, vertebrate and invertebrate mitochondria, the biogenesis of c-type cytochromes appears to be much less complicated than in systems I and II. Genetic studies on Saccharomyces cerevisiae and Neurospora crassa more than a decade ago showed the presence of two cytochrome c heme lyases (Dumont et al., 1987; Zollner et al., 1992). The cytochrome c heme lyase (CCHL) of yeast and N. crassa is required for the covalent heme attachment to cytochrome c (Dumont et al., 1987; Drygas et al., 1989), whereas the cytochrome c₁ lyase (CC₁HL) is essential for the heme attachment to cytochrome c₁ (Zollner et al., 1992), an integral part of the bc₁ complex of the respiratory electron transport chain. There is evidence that the heme lyase
Cytochrome c maturation

interacts with apo-cytochrome c and heme (Mayer et al., 1995; Steiner et al., 1996). This indicates that either the heme lyase acts as a catalyst for the attachment of heme to the thiol groups of the apo-cytochrome c, or the lyases present the heme to the apo-cytochromes, thereby activating a spontaneous heme ligation reaction. Recently, it was shown that overexpressed CCHL could mature the substrate of CCHL₁. In addition, the protein Cyc2p was proposed to control the redox chemistry of the heme lyase reaction (Bernard et al., 2003).
Heme metabolism

Iron is an essential element for most organisms, including bacteria. The oxidized form (Fe$^{3+}$) is insoluble and the reduced form is highly toxic because of the damage it can cause to macromolecules. Thus, in biological systems, iron is sequestered by iron- and heme-carrier proteins. Heme, the iron protoporphyrin IX molecule, is a prosthetic group found in many enzymes. Because of its high toxicity (it is a hydrophobic molecule that accumulates in membranes and promotes nonenzymatic redox reactions), it is rarely found free. To have a source of heme, bacteria have evolved two different mechanisms: either the synthesis of heme or the acquisition of heme from the environment with specialized import systems.

Heme biosynthesis

Bacteria which are capable of synthesizing heme have evolved a synthesis pathway as depicted in Fig. 7. E. coli synthesizes heme in the cytoplasm in a process involving the condensation of eight δ-aminolevulinc acid (δ-ALA) precursors in the tetrapyrrole synthesis pathway (O'Brian and Thöny-Meyer, 2002). δ-ALA is either synthesized from glutamate or from glycine and succinyl-CoA. The ALA dehydratase (HemB) catalyses the condensation of two δ-ALA molecules to porphobilinogen (PBG), and PBG is further processed by the PBG deaminase HemC and the synthase HemD to produce uroporphyrinogen III. This intermediate in heme biosynthesis is then further modified by the decarboxylase HemE and the oxidases HemF or HemN, and HemG or HemY to produce the heme precursor molecule protoporphyrin IX. The last step in heme biosynthesis is the insertion of the iron (Fe$^{3+}$), which is carried out by ferrochelatase (HemH). Ferrochelatase apparently is a cytoplasmic protein that can associate with the membrane (Frustaci and O'Brian, 1993).
Heme metabolism

Heme transformers

In Gram-negative bacteria, uptake of heme by diffusion is prevented by the outer membrane, which forms a permeability barrier for substrates of more than 600 Da. The import of heme across the membranes and its transport routes are poorly characterized. Most of the knowledge comes from pathogenic bacteria, which use heme as iron source and therefore have to be able to take up heme. Gram-negative bacteria have developed specialized, high affinity outer membrane
proteins called receptors to capture heme (Klebba et al., 1993; Wandersman and Stojiljkovic, 2000; Wandersman and Delepelaire, 2004). The receptors bind heme and import it across the outer membrane by a system that requires the TonB/ExbBD proteins and a proton motive force across the cytoplasmic membrane (Braun and Killmann, 1999; Wandersman and Delepelaire, 2004). Within this TonB/ExbBD-dependent system, the heme acquisition of bacteria can be divided into two general mechanisms (Fig. 8): the first involves the close contact between the bacterium and the extracellular heme source. The second mechanism relies on compounds produced and excreted by the cell to scavenge heme from various sources. These hemophores chelate the heme with high affinity and return it to specific outer membrane receptors (Létoffé et al., 1994). Hemophores have been found only in Gram-negative bacteria.

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**Figure 8. Heme import in pathogenic bacteria.** Heme is used by pathogenic bacteria as a source of iron. Two systems have evolved to capture heme from the extracellular matrix; both are TonB/ExbBD dependent. The first system uses an outer membrane heme receptor which directly interacts with the heme source. The second system secretes hemophores which capture heme and deliver it to the outer membrane heme receptor.

HemR of *Yersinia enterocolitica* is the best studied member of the first heme acquisition system (Bracken et al., 1999). The HemR receptor recognizes heme-containing molecules, extracts heme and transfers it from the external site to the internal site of the outer membrane, such that heme is
transported through the TonB-dependent receptor channel (Bracken et al., 1999; Wandersman and Stojiljkovic, 2000). As heme is buried within the different hemoproteins, it remains unclear how it becomes accessible for interaction with HemR. Thus, it was proposed that HemR acts as a surface chaperone that unfolds hemoproteins, but so far, no HemR-hemoprotein interaction has been shown (Lazdunski et al., 1998).

The second mechanism to take up heme uses the secretion of hemophores. This group of proteins is best studied in the organism Serratia marcescens and Yersinia enterocolitica, where the specific outer membrane receptor HasR and its hemophore HasA are present (Ghigo et al., 1997; Létoffé et al., 1994). The hemophore HasA is secreted upon iron starvation by the ABC transporter HasDE (in S. marcescens), a process involving the C-terminal secretion signal (Wandersman, 1998). After secretion into the extracellular medium, HasA binds heme-proteins and delivers them to the specific outer membrane receptor HasR. The mechanism by which HasA extracts heme from hemoproteins is unknown. Clearly, heme transfer does not involve a stable complex between HasA and the heme-bound protein (Létoffé et al., 1999). Furthermore, the mechanism of how heme is translocated across the outer membrane by the heme receptor is not known. Stojiljkovic et al predicted a model in Y. enterocolitica where heme is bound subsequently to the periplasmic heme binding protein HemT, followed by association with the ABC transporter HemUV. How heme is transferred into the cytoplasm by HemUV remains to be shown (Stojiljkovic and Hantke, 1994).

Non-pathogenic E. coli is not able to take up heme as it lacks the genes for a heme receptor and uptake system.

**Heme export into the periplasm**

C-type and b-type cytochromes are assembled with their heme cofactor in the periplasm. As heme is synthesized in the cytoplasm, it needs to be transported across the cytoplasmic membrane into the periplasm. However, how heme is transported across the membrane after the ferrochelatase has inserted the iron into the protoporphyrin IX molecule is still an enigma. One hypothesis of transport across the membrane assumes that the ferrochelatase releases heme...
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directly into the membrane and then heme diffuses passively through the lipid bilayers (Cook and Poole, 2000; Rose et al., 1985). Phospholipid membranes have a high affinity for heme and thus, the removal of heme from the membrane is difficult. Moreover, it was shown that in the presence of a protein with a high affinity for heme, heme can be extracted from the membrane (Cannon et al., 1984; Rose et al., 1985).

As diffusion is slow (Cook and Poole, 2000), transport of heme across the membrane would be expected to be more efficient, especially when heme-binding proteins are overproduced in the periplasm and need high amounts of heme within a short time.

Thus, it was postulated that transport of heme requires an energy-dependent translocation. Several proteins predicted to assemble in a heme transporter can be found in the database, i.e. the CydDC and CcmABC proteins. CydD, CydC and CcmA contain the conserved motifs Walker A, Walker B and the C-motif, thus belonging to the ABC transporter family. The putative heme transporter CcmABC is involved in cytochrome c maturation and thus, these proteins were suggested to transport heme across the membrane and to deliver heme to the cytochrome c (Goldman et al., 1997). The second putative heme transporter CydDC plays a role in the biogenesis of bd-type terminal oxidase as well as in the biosynthesis of c-type cytochromes (Poole et al., 1993; Poole et al., 1994). Recently, it was shown that cysteine is transported by CydDC in an ATP-dependent manner, thus excluding CydDC as a heme transporter (Pittman et al., 2002). Last, it cannot be excluded that there is a yet unknown transporter responsible for the translocation of heme across the membrane.
ABC transporter

ABC transporters couple ATP hydrolysis to the uptake and efflux of solutes across the cell membrane in bacteria and eukaryotic cells. ABC (ATP binding cassette) transporters comprise one of the largest protein families. Almost 5% of the entire E. coli genome encodes components of ABC transporters (Linton and Higgins, 1998). Higgins et al. predicted 57 protein complexes in E. coli to be ABC transporters. Among them, the substrate specificity of 28 transporters is known. In contrast, other organisms contain far fewer ABC transporters, perhaps reflecting their restrictive environment. 28 components of ABC transporters have been identified in yeast, around 50 in humans and up to 153 in Agrobacterium tumefaciens. In humans many diseases such as cystic fibrosis, hyperinsulinemia and macular dystrophy are associated with defects in ABC transporters (Gottesman and Ambudkar, 2001; Grimberg et al., 2001; Stefkova et al., 2004; Sun and Nathans, 2001). In general, each ABC transporter is specific for its own particular substrate or group of chemically related substrates. Most remarkably, there is an ABC transporter for essentially every class of molecule: for small molecules, large molecules, highly charged molecules, highly hydrophobic molecules; for inorganic ions, sugars, amino acids, proteins and complex polysaccharides. Furthermore, even though most ABC transporters are highly substrate specific, some are multispecific like the oligopeptide transporter OppA (Tame et al., 1994), while others like the LmrA protein have an extremely broad specificity for hydrophobic compounds (van Veen et al., 1998). The diversity of substrate specificity is reflected in the diversity of physiological roles played by ABC transporters in the cell. Many of them play a role in elimination of waste products or toxins from the cell, while some ABC transporters in fungi confer resistance to antifungal agents (Wolffger et al., 2001). Other ABC transporters are essential for the export of cellular components which function outside the plasma membrane, for example for the export of cell wall polysaccharides (Silver et al., 2001) or the ABC transporter HasDE for export of the HasA hemophore (Létoffé et al., 1994). In addition, some ABC transporters appear to have diversified in function. The cystic fibrosis protein has a structure typical for an ABC transporter, but is a chloride channel (Higgins, 1995). In addition, some transporters serve a regulatory rather than a transport role. The best characterized example is the Sap/Trk system of S. thyphimurium which regulates a potassium channel independently of any transport activity (Parra-Lopez et al., 1994).
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Importers and exporters

An ABC transporter is defined via its ABC domain, where the characteristic Walker A (GXXGXGKXT) and Walker B (XWXLDE) motifs can be found. Besides the Walker motifs, which can be found in all common ATPases, an additional motif, the C motif (LSGGQ), is restricted to the ABC transporter family. A typical ABC transporter has four domains or subunits, two of which are hydrophobic and are predicted to span the membrane multiple times in an alpha-helical conformation, and two are exposed to the cytoplasm, which bind and hydrolyze ATP. For many ABC transporters, the transmembrane domain (TMD) or permease is fused to the ATP or nucleotide binding domain (NBD), producing a single TMD-NBD unit, which forms a dimer (Davidson and Chen, 2004; Higgins, 2001). However, for many import and export systems, particularly in bacteria, the membrane spanning domain and the ATP binding domain are on two or more polypeptides. Importantly, the TMD is the primary determinant of substrate specificity, through a specific substrate-binding site, while the ATP-binding domain interacts with the intracellular loops of the TMD on the cytoplasmic site of the membrane and delivers the energy for transport.

ABC transporters generally can be divided into importers and exporters. The main difference between importers and exporters is the presence of a periplasmic binding protein (PBP) associated with importers. The first crystal structure of an ABC transporter was that of the vitamin B₁₂ importer BtuCD of E. coli (Fig. 9) (Locher and Borths, 2004), where it interacts with BtuF, the periplasmic binding protein for vitamin B₁₂. High-affinity solute binding proteins are located in the periplasm of Gram-negative bacteria (Boos and Shuman, 1998) or bound to the cell surface as lipoproteins in Gram-positive bacteria. In contrast to efflux systems, where the substrate specificity is located in the transmembrane domain, import systems are generally assumed to determine their substrate specificity via their periplasmic binding proteins.
Figure 9. Example of a classical ABC importer. Shown is the ribbon structure of the vitamin B$_{12}$ importer BtuCD with its periplasmic binding protein BtuF, solved by x-ray diffraction of crystals (Locher and Borths, 2004). The transporter consists of the two transmembrane subunits BtuC and two nucleotide binding subunits BtuD, which form a heterodimer. BtuF has the vitamin B$_{12}$ bound and exposes the substrate to the TMD.

Mechanism of transport

The detailed mechanisms by which ABC transporters move solutes across the membrane is still far from clear. However, an overall picture can be drawn from the studies of both prokaryotic and mammalian systems. For exporters, the transport cycle is initiated by the interaction of the substrate with the TMD from the intracellular site of the membrane. For importers, the substrate can be considered as the PBP-substrate complex interacting at the extracellular side of the membrane. The substrate is then released from the PBP to interact with the TMD (Fig 10).
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Figure 10. Model of ABC-transporter-dependent translocation across the membrane. The maltose transport is shown, which uses a periplasmic binding protein for translocation and specificity. a) The maltose binding protein MBP, in a closed conformation with the bound maltose, interacts with the transporter MalFGK2 to initiate transport and ATP hydrolysis. b) In the transition state, MBP is bound to the transporter in an open conformation that has a lower affinity for maltose, and the transmembrane helices have reoriented their internal sugar-binding site to the periplasm. c) Following ATP hydrolysis, maltose is transported, and the MBP is released as the transporter returns to its original confirmation. MBP activates the ATPase activity of the transporter by bringing the two nucleotide binding domains into close proximity (Davidson and Chen, 2004).

Substrate binding induces a conformational change in the TMD which is transmitted to the NBD to initiate ATP hydrolysis. It is clear that both NBD’s are required and both must hydrolyze ATP, in a mechanism where ATP is "sandwiched" between the two NBD’s (Fig. 11) (Loo et al., 2002; Smith et al., 2002). As yet, it remains unclear whether one or two ATP molecules are hydrolyzed per transported substrate. ATP hydrolysis induces further conformational changes which are transduced to the TMD (Hunke et al., 2000), thus releasing the substrate into either the cytoplasm, the periplasm or the extracellular medium.

Figure 11. “Sandwich” model of the nucleotide binding subunits. Left panel, Ribbon-structure of the NBD MalK of the maltose transporter. WA is the Walker A motif, which interacts via the bound ATP (represented as ball-stick model) with the LSGGQ, the C-motif, of the other MalK. The Walker B motif (WB) is located in the outer site of the NBD. Note that two ATP per dimer are bound. Right panel, Schematic diagram of the interaction between one of the two ATP molecules bound to the homodimer MalK2. The lysine at position 42 (K42) corresponds to the lysine at position 40 in the CcmA protein (Davidson and Chen, 2004).

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CcmAB, a transporter involved in cytochrome c maturation

Cytochrome c maturation requires the translocation of apo-cytochrome c and heme across the membrane from the place of synthesis in the cytoplasm to the periplasm, where the covalent attachment of heme to the cytochrome c occurs. While the translocation of apo-cytochrome c was shown to require the sec translocation machinery (Thöny-Meyer and Künzler, 1997), the transport of heme from the cytoplasm into the periplasm is still unknown. CcmA was identified as the ATPase subunit of an ABC transporter with CcmB and CcmC as its transmembrane subunits. CcmB and CcmC are encoded by genes directly downstream of ccmA, and both containing six transmembrane helices. Thus, it was predicted that CcmA together with CcmB and CcmC constitute an ABC transporter involved in translocation of heme across the membrane (Goldman et al., 1997). Cook and Poole tested this hypothesis by using inverted membrane vesicles of a wild-type and a ccmA deficient strain in heme uptake but could not detect a significant difference between the membrane vesicles of these two strains (Cook and Poole, 2000). Throne-Holst showed that in frame deletion mutants of ccmA and ccmC can produce cytochrome b, but not cytochrome c (Throne-Holst et al., 1997), indicating a ccm independent pathway for heme transport. However, this does not exclude the possibility of a need for a heme transporter exclusively for cytochrome c maturation, as b-type cytochromes may have different requirements for their assembly. Schulz et al. showed that overexpressed CcmC attaches heme to the heme chaperone CcmE independently of CcmA and CcmB (Schulz et al., 1999). In addition, the mutant strain of the ccmC homologue helC in Pseudomonas fluorescens was severely affected in pyoverdine production and cytochrome c formation, whereas the mutant strain of the ccmAB homologues helAB were only affected in cytochrome c maturation (Gaballa et al., 1996). These data indicate that ccmC has a role in cytochrome c maturation independent of ccmAB and heme is translocated across the membrane independently of CcmA and CcmB. The situation gets even more complicated as CcmA and CcmB are needed in heme incorporation into CcmE, when CcmC is not overproduced (Schulz et al., 1999). Another question of dispute has been the subunit composition of the ABC transporter (Goldman and Kranz, 2001). A subunit composition of the ABC transporter containing CcmA and CcmB only is further supported by the order of the structural genes in the genomes of system I-containing organisms and archaea (Fig. 12). The genes ccmA and ccmB are always found next to each other except for Rhodobacter prokazekii, whereas ccmC does not seem to be strictly linked to the other two genes. In E. coli, ccmC is
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separated by 41 nucleotides from the end of \textit{ccmB}, whereas the end of \textit{ccmA} and the start of \textit{ccmB} are overlapping.

**Bacterial ccm genes**

\begin{itemize}
  \item \textit{E. coli}
  \item \textit{H. influenzae}
  \item \textit{X. campestris}
  \item \textit{D. radiodurans}
  \item \textit{S. meliloti}
  \item \textit{M. loti}
  \item \textit{R. capsulatus}
  \item \textit{R. prowazekii}
\end{itemize}

**Archaeal ccm genes**

\begin{itemize}
  \item \textit{P. aerophilum}
  \item \textit{M. mazei}
  \item \textit{A. flegidis}
  \item \textit{A. pernix}
\end{itemize}

Figure 12. Location of \textit{ccmABC} in the genomes of bacteria and archaea. While \textit{ccmC} is located at different positions, \textit{ccmA} and \textit{ccmB} are always found close to each other, with one exception, in \textit{R. prowazekii}, where all \textit{ccm} genes are located separately from each other. In archaea, only \textit{ccmC} and \textit{ccmF} can always be found. The gene \textit{ccmE} is not found in archaea.

Moreover, in the two organisms \textit{Xanthomonas campestris} and \textit{Deinococcus radiodurans}, the genes \textit{ccmA} and \textit{ccmB} are linked to each other but are separately located to the other \textit{ccm} genes.

An experimental approach to determine the subunits of the ABC transporter was done by Goldman \textit{et al.} (Goldman \textit{et al.}, 1997). In this experiment the CcmA homologue HelA of \textit{Rhodobacter capsulatus} was found to co-immunoprecipitate together with either HelB (CcmB
homologue) or HelC (CcmC homologue) and even with the small membrane protein HelD (CcmD homologue). Furthermore, HelA was not obtained in membranes containing overexpressed HelB or HelC, indicating that assembly of HelA with the membrane is dependent on both, HelB and HelC. Therefore, the ABC transporter was proposed to consist of HelA\textsubscript{2}BC. Due to the ambiguous suggestions concerning the subunit composition and the substrate involved in cytochrome c maturation, an ambitious goal of this thesis was twofold: First, does the ABC transporter consist of CcmA\textsubscript{2}B\textsubscript{2} or CcmA\textsubscript{2}BC and second: what is the substrate of the ABC transporter, which is involved in cytochrome c maturation? These questions were to be addressed in the model system of *Escherichia coli*, where chromosomal in frame deletions of all *ccm* genes existed and various plasmid constructs and antibodies were available. Genetical and biochemical methods were to be used to get more insight into the role of CcmAB during cytochrome c maturation.
Chapter II

Material and Methods
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Bacterial strains and growth conditions

Bacterial strains used in this work are listed in Table I. The *E. coli* strain DH5α (Hanahan, 1983) was used as host for clonings. The *E. coli* strain C43 (DE3) (Miroux and Walker, 1996) was used for overexpression of membrane proteins. The strain EC06 is a derivative of MC1061 (Meissner et al., 1987), which carries a deletion of *ccmA*-*H* with the deleted DNA being replaced by a kanamycin resistance cassette. The strain C600 *Δ*hemA (Ghigo et al., 1997) was used for complementation experiments with heme. The strain DSS301 was used for P1 transduction of the *dmsABC::kan* locus into the *ΔccmA* and *ΔccmB* mutants.

Bacteria were grown aerobically in Luria Bertani (LB) medium (Sambrook et al., 1989) or anaerobically in minimal salts (MS) medium (Iobbi-Nivol et al., 1994) supplemented with 0.4% glycerol, 40 mM fumarate and 5 mM sodium nitrite or 10 mM sodium nitrate as the terminal electron acceptor. Anaerobic growth on plates containing TMAO was done using modified MS medium containing 33 mM KH₂PO₄, 60 mM K₂HPO₄, 7.5 mM (NH)₂SO₄, 1.7 mM Tri-Na-citrate, 0.4 mM MgSO₄, 67.6 μM Na₂SeO₃, 100 μM Na₂MoO₄, 10% LB medium, 0.4% glycerol and 10 mM TMAO as the terminal electron acceptor (Ahuja and Thöny-Meyer, 2003). The plates were incubated for 72 hours at 37°C inside an anaerobic jar under argon.

Antibiotics were added at the following final concentrations: ampicillin, 100 μg/ml; chloramphenicol, 10 μg/ml; kanamycin, 50 μg/ml. If necessary, cells were induced with 0.1% arabinose or 1 mM IPTG at mid-exponential growth phase. Hemin was dissolved in 20 mM NaOH to a final concentration of 10 mM.
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**P1 phage transduction**

The ΔdmsABC::kan marker of strain DSS301 (Sambasivarao and Weiner, 1991) was transduced into the ΔccmA mutant EC21 (Schulz et al., 1999) and into the ΔccmB mutant EC75 (Schulz et al., 1999) by P1 phage transduction (Miller, 1992), resulting in the strains ΔccmAΔdms and ΔccmBΔdms, respectively.

**Construction of plasmids and site-directed mutagenesis**

Plasmids used in this work are listed in Table I and are presented in a schematic overview in Fig. 13. The primers used for plasmid constructions are shown in Table II. The plasmid pEC508 expressing ccmB-strep was constructed by blunt-end ligation of the PCR product into the EcoRV digested pUCBM20. The PCR product was obtained by inverse PCR, using the pEC86 as a template and the primer CcmBN and ccmB_strep_rev. The 5’ primer contained the coding sequence for the 3’ end of ccmB and for the strep-tag (AWRHPQFGG) upstream of the stop codon and a BspHI site. The 3’ primer contained an NdeI and a BamHI site and the 5’ coding sequence of ccmB. The plasmid pEC510 expressing ccmC-His6 was constructed by blunt-end ligation of the inverse PCR product into the EcoRV digested pUCBM20. The PCR product was obtained using pEC86 as the template and using the primers ccmC_BspHI_for and ccmC_his_rev. The 5’ primer contained the coding sequence for the 3’ end of ccmC and for 6 histidines upstream of the stop codon and a SalI site. The 3’ end contained a BspHI site and a ribosomal binding site upstream of the ccmC gene. The plasmid pEC511 expressing ccmAB-strep was constructed by ligation of the CpoI/BspHI-digested pEC508 into the CpoI/BspHI-digested pEC86. The plasmid pEC512 expressing His6-ccmAB-strep was constructed by ligation of the CpoI/BspHI-digested pEC508 fragment into CpoI/BspHI-digested plasmid pEC23. The plasmid pEC513 expressing ccmAB-strep and ccmC-His6 was constructed by ligation of the BspHI/SalI-digested pEC510 into BspHI/SalI-digested pEC511. The plasmid pEC514 expressing ccmAB-strep was constructed by ligation of XbaI/SalI-digested and blunt-ended pEC511 into EcoRV-digested pBR322. The plasmid pEC515 ccmAB-
strepccmC-His$_6$ was constructed by ligation of XbaI/SalI-digested and blunt-ended pEC513 into EcoRV-digested pBR322. The plasmid pEC516 expressing His$_6$-ccmA(K40D)B-strep was constructed by ligation of the BglII/CpoI-digested pEC98 into the BglII/CpoI-digested pEC512. The plasmid pEC517 expressing ccmB-strep was constructed by ligation of the BamHI/SalI-digested pEC511 into the BamHI/SalI-digested pACYC184. The plasmid pEC519 expressing ccmAB-His$_6$ and ccmC was constructed by QuickChange™ site-directed mutagenesis (Stratagene, Amsterdam, NL) using pEC101 as a template and the primers CcmB_His_for and CcmB_His_rev. The plasmid pEC520 expressing ccmABC was constructed by QuickChange™ site-directed mutagenesis using pEC101 as a template and the primers ccmCNo254Yes_for and ccmCNo254Yes_rev. The plasmids pEC521, pEC522, pEC523, pEC524, pEC525, pEC526, pEC527, pEC528, pEC529, pEC530 and pEC531 expressing ccmAB-His$_6$, ccmC carrying in ccmB the codons for the mutant CcmB protein F28A, W55A, R68A, F70A, D73A, D76A, E80A, K96A, H100A, W101A and F175A, respectively, were constructed by QuickChange™ site-directed mutagenesis using pEC519 as a template and the primers listed in Table I. The plasmids pEC532, pEC533, pEC534, pEC535, pEC536, pEC537, pEC538 and pEC539 expressing ccmB-strep carrying in ccmB the codons for the mutant CcmB protein F28A, R68A, F70A, D73A, D76A, H100A, W101A and F175A, respectively, were constructed by QuickChange™ site-directed mutagenesis using pEC517 as a template and the same primers used for the quick change mutagenesis of pEC519 (Table II).

DNA sequencing

All constructs and mutants derived from PCR products were confirmed by DNA sequence analysis (Microsynth, Balgach, Switzerland). The sequences established for confirmation of the ccmB mutants covered the entire insert including ccmA and ccmC.
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Cell fractionation

Periplasmic proteins were obtained from 1200 ml cultures grown anaerobically in MS medium or aerobically in LB. Harvested cells were treated with polymyxin B sulfate as described previously (Ren and Thöny-Meyer, 2001). For the preparation of membrane proteins, cells grown anaerobically in MS medium or grown aerobically in LB were harvested, and membrane proteins were extracted as described previously (Ren and Thöny-Meyer, 2001).

Biochemical methods

Protein concentrations were determined using the Bradford assay (Bio-Rad) and BSA as a standard. Holo-cytochrome c\textsubscript{550} and holo-CcmE formation was analyzed qualitatively by SDS-PAGE and staining for covalently bound heme (Schulz et al., 2000). Optical spectra were recorded from samples reduced with 10 mM Na-dithionite or oxidized with 10 mM ammonium persulfate (APS) on a Hitachi Model U-3300 spectrophotometer and exported to a Microsoft EXCEL table for calculations of difference spectra and determination of absorption maxima (Enggist and Thöny-Meyer, 2003). Soluble cytochrome c (c\textsubscript{550}) in the periplasmic fractions was quantified by absorption difference spectroscopy using a $\varepsilon_{550-536}\text{nm}$ of 23.2 mM$^{-1}$cm$^{-1}$ (Thöny-Meyer et al., 1996). Immunoblot analysis of the His\textsubscript{6}-tagged proteins (CcmA, CcmB or CcmC) was performed using monoclonal tetra-His antibodies (Qiagen) at a dilution of 1:2000. Immunoblot analysis of CcmE and CcmA polypeptides were done using antibodies directed against the CcmE peptide (Schulz et al., 1998) or directed against the CcmA peptide (DVNGVDRLTQRMAQHTE) at a dilution of 1:5000 and 1:3000, respectively. Immunoblot of the streptavidin-tagged (AWRHPQFGG or WSHPQFEK) CcmB was performed using streptavidin-alkaline conjugate (Amersham Biosciences) in a dilution of 1:4000. Signals were detected using goat anti-mouse IgG (for His\textsubscript{6}-tag detection) or goat anti-rabbit IgG (for detection of other antigens) conjugated to alkaline phosphatase (Bio-
Rad) as secondary antibodies in a dilution of 1:3000 and CSPD (Roche Diagnostics) as substrate in a dilution of 1:100.

Pull down assay

1 mg membrane proteins were solubilized in 1 ml solubilization buffer containing 50 mM Tris-HCl, pH 8.0 and 1% n-dodecyl-β-D-maltoside for 1 h at 4°C. The insoluble material was removed by ultracentrifugation at 150,000 x g for 60 min at 4°C. The solubilized fraction (supernatant) was incubated with either 5 mg Ni-NTA agarose beads or 5 mg StrepTactin beads for 1 h at 4°C. The protein-bead mix was centrifuged at 15,000 g for 15 minutes. The beads in the pellet were resuspended in 2 x SDS loading dye containing 20 mM DTT and subjected to SDS-15% PAGE, followed by immunoblot probed with antibodies against the His<sub>6</sub> tag of CcmC or against the strep-tag of CcmB.

Purification of CcmA and CcmB

The *E. coli* C43 (DE3) cells expressing either His<sub>6</sub>-CcmACcmB-strep or His<sub>6</sub>-CcmA(K40D)CcmB-strep were grown aerobically in 6 liters LB at 30°C. After OD<sub>600</sub> = 0.8 was reached, cells were induced with 1 mM IPTG and further grown over night at 30°C. The membrane proteins were isolated as described (Ren and Thöny-Meyer, 2001). 50 mg of membrane proteins were solubilized in 50 ml buffer A (50 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 300 mM NaCl, 20% glycerol) containing 1% n-dodecyl-β-D-maltoside and 5 mM imidazole for 1 h at 4°C. The insoluble material was removed by ultracentrifugation at 150,000 x g for 60 min at 4°C. The solubilized fraction (supernatant) was collected and loaded onto a Ni<sup>2+</sup>-chelating column (nickel-nitrilotriacetic acid (NTA)-agarose, Qiagen, Basel, Switzerland) of 3 ml bed volume,
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which was equilibrated with buffer A containing 0.1% \( n \)-dodecyl-\( \beta \)-D-maltoside and 5 mM imidazole. The column was washed with washing buffer (buffer A containing 0.1% \( n \)-dodecyl-\( \beta \)-D-maltoside and 20 mM imidazole). The proteins were eluted with buffer A containing 0.1% \( n \)-dodecyl-\( \beta \)-D-maltoside and 300 mM imidazole, and 1 ml fractions were collected.

ATP hydrolysis assay

The activity of the ATPase CcmA was determined by a coupled spectrophotometrical assay using pyruvate kinase and lactate dehydrogenase (Laubinger and Dimroth, 1987). The cuvette contained 1 ml of 50 mM Tris-HCl buffer, pH 8.0, 5 mM \( \text{MgCl}_2 \), 5 mM \( \text{NaCl} \), 0.25 mM NADH, 3 mM phosphoenolpyruvate, 15 U lactate dehydrogenase (Roche, Basel, Switzerland), 10 U pyruvate kinase (Roche, Basel, Switzerland), 1% \( n \)-dodecyl-\( \beta \)-D-maltoside and purified CcmA protein (1 to 80 \( \mu \)g). The reaction was initiated by adding 2.5 mM ATP, and NADH oxidation was recorded at 340 nm on a Hitachi model U-3300 spectrophotometer. To measure multiple samples, measurements were done in microtiter plates with an end volume of 200 \( \mu \)l on a SPECTRAmx plus (Molecular Devices). The formula below was used to calculate the specific ATPase activity.

\[
\frac{A \Delta \times 1000 \mu l \times 1 \text{cm}^{-1}}{10 \text{Min.} \times 6.22 \text{mM}^{-1} \times 1 \text{cm}^{-1} \times 10 \mu l} = x \text{ \mu mol} \times \text{min}^{-1}/\text{ml} \text{ or } x \text{ U/ml}
\]
Purification of His$_6$-tagged CcmE

Periplasmic extract containing soluble CcmE-His$_6$ was applied to a NTA-agarose column of 3 ml bed volume, which was equilibrated with 5 mM imidazole, 300 mM NaCl, 50 mM TrisHCl, pH 8.0. The column was washed with 10 bed volumes of the same buffer (300 mM NaCl, 50 mM TrisHCl, pH 8.0) containing 20 mM imidazole, and the protein was eluted from the column with 5 bed volumes of 250 mM imidazole, 300 mM NaCl, 50 mM TrisHCl, pH 8.0. Fractions of 1 ml were collected. Fractions containing soluble CcmE-His$_6$ were concentrated to a final volume of 1 ml by using the Ultrafree®-4 Centrifugal Filter Units (nominal molecular weight limit (NMWL) at 5000 Da, purchased from Millipore (Volketswil, Switzerland)). Proteins were concentrated by centrifugation at 4500 rpm at 4°C at an angle of 45° and washed with 50 mM TrisHCl, pH 8.0.

Separation of CcmE dimer from monomers

Gel filtration chromatography was performed on a fast protein liquid chromatography (FPLC) system (AEKTA, Amersham Pharmacia, Sweden) using a size-exclusion gel filtration column (16/60 Superdex-75 prepgrade, Amersham Pharmacia, Sweden). The column was equilibrated overnight with 200 ml vacuum-filtered 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, after which 500 μl (50 mg) of His$_6$-tagged purified soluble CcmE-His$_6$ were applied to the column using a 1 ml loop at 1 ml/min flow rate. Twenty fractions were collected in 5 ml volumes and tested for the separation of monomers and dimers. The fraction containing the highest proportion of dimer (A12) was applied a second time onto the same size-exclusion gel filtration column. The column was equilibrated overnight with 200 ml vacuum-filtered 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, after which 100 μl (2.5 mg) of fraction A12 were applied using a 200 μl loop at 0.25 ml/min flow rate. 105 fractions of 1 ml were collected, and main peaks were tested for the content of monomer and dimer. Fractions were stored at -20 °C until further analysis.
Mass spectroscopy

Molecular masses of purified polypeptides were determined by matrix-assisted laser desorption ionization/time-of-flight MS on an Applied Biosystems Voyager-DE Elite mass spectrometer. Measurements were done by the Protein Service-Lab, ETH Zürich.
Table I. Bacterial strains and plasmids used in this work

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### Material and Methods

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### Material and Methods

#### Table II. Oligonucleotide primers

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Figure 13. Schematic representation of some strains and plasmids

**E. coli** strains  

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<td>EC75</td>
<td>/nap → ccmA → ccmF</td>
</tr>
</tbody>
</table>

**pACYC184** plasmid derivatives

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant genotype</th>
</tr>
</thead>
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<tr>
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</tr>
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<td>pEC517</td>
<td>/nap → ccmA → ccmB</td>
</tr>
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<td>pEC86</td>
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<td>pEC94</td>
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<tr>
<td>pEC97</td>
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</table>

**Relevant genotype**

- nap: Naphthalene  
- ccmA, ccmB, ccmC: Toluene  
- ccmF: Xylenes  
- ccmG, ccmH: Benzene
Material and Methods

expression plasmids | Relevant genotype
---|---
pEC23 (pET28c) | [ccmA] B
pEC98 (pET28c) | [ccmA]
pEC512 (pET28c) | [ccmA] ccmB
pEC516 (pET28c) | [ccmA] ccmB
pEC514 (pBR322) | [ccmA] ccmB
pEC515 (pBR322) | [ccmA] ccmB ccmC
pEC412 (pISC2) | [ccmB]
pEC415 (pISC2) | [ccmB]
prJ3290 (pISC2) | [cycA]
Chapter III

Results
Results

Characterization of ccmA

The genes ccmA-H are essential for cytochrome c maturation (Thöny-Meyer et al., 1995). Analysis of in frame deletion mutants in any of the eight genes of the ccm operon showed that each of them is essential for cytochrome c maturation.

Within the ccmA-H operon, ccmA is the first gene. It encodes a 205 amino acid protein that shows strong similarity to members of the ATP binding cassette (ABC) transporter family, in particular to the ATP binding domains. In contrast to the other Ccm proteins, which span the membrane one to several times, and based on hydrophobicity analysis, CcmA is predicted to be soluble without a transmembrane helix. Nevertheless, it was shown to be membrane associated (Goldman et al., 1997).

The CcmA homologues belong to the putative heme exporter (HemeE) subfamily of the ABC transporter family (transport classification database, http://tcdb.ucsd.edu/index.php). In addition, Elie Dassa classified by phylogenetic analysis the CcmA protein to the subclass 1 of the ABC transporter family, which comprises essentially all known exporters with fused ABC and inner membrane domains. However, in the case of CcmA, these domains are not fused (Dassa and Bouige, 2001). CcmA contains the Walker A, Walker B and C motif, which are conserved characteristics of the ATP binding subunit of the ABC transporter family (Fig. 14). It has been postulated that CcmA together with CcmB and CcmC, perhaps including CcmD as an accessory protein, assemble to an ABC transporter involved in heme export to the periplasm (Goldman et al., 1997; Page et al., 1997). However, it has also been shown that the protein CcmC has a role independent of CcmA and CcmB in cytochrome c maturation (Schulz et al., 1999). Therefore, it was of interest to characterize the role of CcmA in cytochrome c maturation.
Characterization of *ccmA*

**Figure 14.** Amino acid sequence alignment of CcmA homologues from representative organisms. Strictly conserved residues are shaded in black and strongly conserved residues are shaded in grey. The underlined I indicates the Walker A motif, the underlined II indicates the C motif and the underlined III indicates the Walker B motif. The asterisk indicates the mutated K40 in the *E. coli* CcmA protein.
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Characterization of a ccmA in frame deletion mutant

First, an in vivo experiment showing bacterial growth of a ΔccmA strain (Throne-Holst et al., 1997) under anaerobic respiratory conditions with trimethylamine N-oxide (TMAO) as electron acceptor was investigated. This growth test was established previously to detect cytochrome c negative mutants in E. coli (Ahuja and Thöny-Meyer, 2003). It has been shown that a ccmC mutant in combination with a mutant in the dmsABC operon cannot grow anaerobically under respiratory conditions with TMAO as the terminal electron acceptor. The reason for this growth defect is shown in Fig. 15.

![Scheme of the reduction pathway during TMAO respiration.](image)

**Figure 15. Scheme of the reduction pathway during TMAO respiration.** DmsABC constitutes a dimethyl sulfoxide (DMSO) reductase that catalyzes the reduction of DMSO and trimethylamine N-oxide (TMAO) under anaerobic conditions. The dms mutant cannot grow under anaerobic conditions on minimal medium with glycerol as the sole carbon source and DMSO as terminal electron acceptor. However, it can grow in the presence of TMAO, because the TMAO reductase is still active. The TMAO reductase is a heterodimeric enzyme composed of TorA, a periplasmic reductase and TorC, a membrane bound pentaheme c-type cytochrome. During anaerobic growth, electrons (e–) are transferred from the menaquinone pool (Q / QH2) to TorC and then delivered to TorA, which reduces TMAO to trimethylamine (TMA). TorC requires the cytochrome c maturation (ccm) system composed of the CcmA-H proteins to insert the five 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.

In a strain lacking c-type cytochrome, i.e. a ccm mutant, the TorC c-type cytochromes subunit of the TMAO reductase cannot bind its heme cofactors and thus cannot transfer electrons to the TMAO reductase TorA. However, the DMSO reductase complex DmsABC is still active, as it does not contain a c-type cytochrome, and it will reduce not only DMSO, but also TMAO. In a strain mutated in the dms operon, there is no DMSO reductase, but the TMAO reductase is
Components of the ABC transporter

active. In the double mutant dms⁻ ccm⁻, a growth defect in TMAO respiration is expected due to a defective TorC protein. To test, whether this is also true for a ΔccmA Δdms mutant, the dms::kan locus (Sambasivarao and Weiner, 1991) was transduced by the P1 phage into a ΔccmA strain. The ccmA mutant had previously been created by removing 114 internal codons (coding for S35 to W148) (Throne-Holst et al., 1997).

The Δdms strain formed red colonies, when grown under anaerobic conditions on minimal salt agar plates containing TMAO as the sole electron acceptor, while the strain with the genotype ΔccmA Δdms formed small white colonies (Fig. 16).

However, both strains grew normally under aerobic conditions on LB plates (data not shown). Thus, the colony phenotype under anaerobic respiratory conditions with TMAO as the terminal electron acceptor can be used as a qualitative measure for lack of cytochrome c maturation of an ABC transporter mutant.
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Complementation of the ΔccmA mutant

To assess the question of whether the ΔccmA Δdms mutant can be complemented for cytochrome c maturation by providing the ccmA gene in trans, a plasmid was constructed expressing ccmA on the low copy vector pACYC184 and it was named pEC71. When the ΔccmA Δdms strain was transformed with pEC71 and streaked out on minimal salt agar plates containing TMAO, red colonies were observed after three days of anaerobic growth, indicating complementation for cytochrome c maturation (Fig. 16C). This shows that the in frame deletion of the ccmA gene does not have a polar effect on downstream genes.

ATP hydrolysis is important for cytochrome c formation

As described above, CcmA is predicted to be the ATP binding subunit of an ABC transporter involved in cytochrome c maturation. This hypothesis was tested by site-directed mutagenesis of the Walker A motif in CcmA. Plasmid pEC97 producing CcmA(K40D) and CcmB-H was constructed, where the amino acid residue lysine at position 40, located within the Walker A motif of CcmA, was changed to aspartic acid. It has been proposed on the basis of structural studies that the conserved lysine in the ATP binding site (Walker A) interacts with either the α- or γ-phosphate of ATP (Fry et al., 1985). It was therefore expected that the negatively charged aspartic acid introduced in the CcmA K40D mutant prevents such interaction and thus the mutant is unable to use the energy of ATP hydrolysis to transport the substrate needed for cytochrome c maturation.

The ΔccmA-H strain EC06 carrying the B. japonicum cytochrome c₅₅₀ was transformed either with pEC86 encoding CcmA-H or pEC97 encoding CcmA(K40D)-H. Cells were grown in minimal salt medium supplemented with nitrate as the terminal electron acceptor. Periplasmic fractions were prepared and analyzed by optical difference spectroscopy (Fig. 17). While the strain EC06 strain expressing ccmA-H was able to form c-type cytochromes as indicated by the peak at 550 nm, no cytochrome c₅₅₀ could be detected in the periplasm of strain EC06 expressing the ccm genes with the point mutation K40D in ccmA.
Components of the ABC transporter

![Absorption spectrum](image)

**Figure 17. Functional CcmA is required for cytochrome c formation.** Dithionite-reduced minus ammonium persulfate oxidized difference spectra of periplasmic extracts are shown. The $\Delta ccma-H$ strain EC06 was co-transformed with pRJ3290 (cytochrome c550) and either the plasmid pEC97 (CcmA(K40D)-H) or pEC86 (CcmA-H). Periplasmic proteins were isolated from anaerobically grown cells and protein levels were equalized to 500 μg/ml.

**CcmA activity is not needed for holo-CcmE formation**

Membranes were analyzed for the presence of CcmA to rule out the possibility that CcmA(K40D) was unstable due to its mutation. Membrane proteins were separated by SDS-15% polyacrylamide gel electrophoresis (SDS-15% PAGE) and an immunoblot was probed with antibodies against a CcmA-specific peptide (D158-E174). The $\Delta ccma-H$ strain EC06 expressing the genes $ccma-H$ (pEC86) revealed a band running just below the 25 kDa marker, corresponding to the predicted size of CcmA of 22.9 kDa (Fig. 18, upper panel, lane 2). This signal was not detected in a wild-type strain expressing the Ccm proteins from the chromosomal genes, most likely because only a few CcmA molecules are made per cell, thus escaping detection (data not shown). The plasmid pEC94 encodes $ccmB-H$ at which the coding sequence for S35 to W148 of $ccma$ was deleted. The CcmA antibody was raised against the peptide D158 to E174, which is
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still present on the truncated CcmA encoded on the plasmid pEC94. The CcmA deletion peptide has an expected size of 92 Da and immunoblot of proteins expressing this gene do not give any specific bands in the expected range. Thus a CcmA-specific band was absent (Fig. 18, upper panel, lane 3). However, when the genes ccmA-H were expressed with ccmA carrying the point mutation K40D, the CcmA-specific band was present. This showed that the inactivated CcmA protein was still assembled in the membrane. By contrast, when CcmA alone was expressed from the pACYC184 vector in the absence of the other ccm genes (pEC71), no CcmA protein was found in the membrane (Fig. 18, upper panel, lane 1).

These results indicated that CcmA associates with the membrane by interacting with another Ccm protein, most likely with the permease subunit of the ABC transporter. The heme chaperone
CcmE binds heme transiently and then delivers it to apocytochrome c. Therefore cytochrome c maturation can be divided into two steps: (i) formation of holo-CcmE and (ii) transfer of heme from CcmE to cytochrome c. It was therefore possible to analyze whether CcmA is required for holo-CcmE formation. Cells expressing either ccmB-H (pEC94) or ccmA(K40D)-H (pEC97) were compared to cells expressing the wild-type ccmA-H operon (pEC86) with respect to heme incorporation into the CcmE protein. Membrane proteins isolated from the ΔccmA-H strain overproducing either CcmA (pEC71), CcmA-H (pEC86), CcmB-H (pEC94) or CcmA(K40D)-H (pEC97) were separated by SDS-15% PAGE and stained for covalently bound heme (Fig. 18, middle panel). No heme stainable band was observed in membrane proteins from ΔccmA-H cells producing CcmA only, as CcmE was not present (Fig 18, middle panel, lane 1). As expected, the ΔccmA-H cells expressing ccmA-H (pEC86) were able to transfer heme to CcmE (Fig 18, middle panel, lane 2). Although deficient in cytochrome c formation, the cells expressing ccmB-H or ccmA(K40D)-H were still able to incorporate heme into CcmE, indicating that the ATPase activity of CcmA is a prerequisite for cytochrome c formation, but not for heme incorporation into CcmE (Fig 18, middle panel, lane 3 and 4). In addition, apo-CcmE was observed in all lanes expressing ccmE from a pACYC184 vector, indicating that CcmA is not needed for CcmE incorporation into the membrane (Fig.18, lower panel, lane 2 to 4). Note, that the amount of CcmE protein in the membranes of cells lacking CcmA is somewhat reduced.
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Characterization of \textit{ccmB}

A classical ABC transporter consists of four domains or subunits, two transmembrane domains each typically containing six transmembrane \(\alpha\) helices and two ABC or ATPase domains (Higgins, 2001; Davidson, 2002; Nikaido, 2002). In some cases, these two domains are encoded by a single polypeptide, whereas in other cases, two adjacent genes encode two separate polypeptides. In the case of the \textit{E. coli} Ccm system, the ATPase or nucleotide binding subunit of the ABC transporter is encoded by \textit{ccmA}. The next gene in the operon is \textit{ccmB}. It encodes a 220 amino acid hydrophobic protein. Using the program TMpredict (http://www.ch.embnet.org/software/TMPRED_form.html), the protein CcmB is predicted to span the membrane six times. A C-terminal LacZ fusion to the \textit{R. capsulatus} CcmB homologue HelB had previously been used to show that the protein indeed is an integral membrane protein (Goldman et al., 1997). Therefore, it was of interest to characterize the CcmB protein as the most likely partner of CcmA, i.e. the permease subunit of the ABC transporter.

Characterization of a \textit{ccmB} in frame deletion mutant

An in-frame deletion of 147 internal codons (G44 to D190) of \textit{ccmB} has been created (Throne-Holst et al., 1997; P. Künzler and L. Thöny-Meyer, unpublished). To test whether this \(\Delta\textit{ccmB}\) strain is defective in TMAO respiration, the \textit{dms::kan} locus was transduced via the P1 phage into the \(\Delta\textit{ccmB}\) strain. The strain with the genotype \(\Delta\textit{dms}\) showed red colonies (Fig. 19, panel A), when grown under anaerobic conditions on minimal salt agar plates supplemented with TMAO as the terminal electron acceptor, while the strain with the genotype \(\Delta\textit{ccmB} \Delta\textit{dms}\) showed small white colonies (Fig. 19, panel B + C), indicating a cytochrome \textit{c} deficiency. To examine complementation of a \(\Delta\textit{ccmB} \Delta\textit{dms}\) strain for growth, the strain was transformed with the pACYC184 derived plasmid expressing \textit{ccmB} (pEC517). This strain was streaked out on minimal salt agar plates supplemented with TMAO as the terminal electron acceptor. No colonies were obtained, indicating that the strain was cytochrome \textit{c} deficient (Fig. 19, panel C). The plasmid
pEC517 was also transformed into the ΔccmB mutant EC75 carrying the plasmid pRJ3290, which encodes the *B. japonicum* cytochrome *c*$_{550}$.

**Figure 19. Overproduced CcmB cannot complement a ΔccmB Δdns deletion strain for growth.** The wild-type strain MC1061 Δdns was transformed with pACYC184 (empty vector, panel A). The ΔccmB Δdns strain was transformed with pACYC184 (empty vector, panel B) or pEC517 (CcmB, panel C). The strains were streaked out on minimal salt agar plates supplemented with 10 mM TMAO and incubated under anaerobic conditions for 3 days at 37°C.

Periplasmic fractions were analyzed by absorption difference spectroscopy for the presence of *c*-type cytochromes, but these were not detectable (Fig. 20). We concluded that either (i) the deletion of the *ccmB* gene in the *E. coli* chromosome has a polar effect on the downstream genes, (ii) the gene *ccmB* is not expressed from the plasmid or (iii) the overexpressed CcmB is not active in the cell. It was unlikely that the in frame deletion of 146 amino acid residues would have a polar effect on the downstream genes as the reading frame was confirmed to be intact by sequencing, and the region encompassing the 3’ end of *ccmB* and the 5’ end of *ccmC* was not changed. The gene *ccmC* is located downstream of *ccmB*, separated by 40 nucleotides and codes for another integral membrane protein with six transmembrane helices. As CcmC had been suggested to be another permease of the ABC transporter (Goldman *et al.*, 1997), but this had been disputed (Schulz *et al.*, 1999), the *ccmC* gene was included into our subsequent analysis. Since *ccmB* alone was not complementing the ΔccmB mutant, we tried to introduce it in combination with *ccmA* (pEC100 expressing *ccmAB*) or *ccmA* and *ccmC* (pEC520 expressing *ccmABC*). The ΔccmB strain EC75 carrying cytochrome *c*$_{550}$ (pRJ3290) was transformed with
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these plasmids and tested for holo-cytochrome c formation (Fig. 20). Optical difference spectra of periplasmic proteins showed that only when ccmC was co-expressed with ccmA and ccmB, cytochrome c formation was observed. Conversely, ccmAB was not able to complement a ΔccmB strain for holo-cytochrome c formation (Fig. 20).

These data supported the idea that CcmC is functionally connected to the ABC transporter. The molar ratio of CcmB and CcmC seemed to be critical for optimal cytochrome c maturation.

Figure 20. Cytochrome c is only formed in a ΔccmB strain in presence of overexpressed CcmABC. The ΔccmB strain EC75 was co-transformed with pRJ3290 (cytochrome c550) and either pACYC184 (empty vector) pEC517 (ccmB), pEC100 (ccmAB), pEC520 (ccmABC) or pEC101 (ccmABCD). Periplasmic proteins were isolated from anaerobically grown cells and protein levels were equalized to 500 μg/ml. Dithionite-reduced minus ammonium persulfate oxidized difference spectrum were measured.
Components of the ABC transporter

Overexpressed CcmB suppresses the function of CcmC in heme delivery

CcmC is necessary and sufficient for heme transfer to CcmE (Schulz et al., 1999). In a wild-type situation, it can be assumed that CcmB and CcmC are present in approximately equimolar amounts. However, if CcmB is selectively overproduced, and if it forms a complex with CcmC, it might sequester CcmC such that heme transfer to CcmE cannot occur. To support this assumption, heme incorporation into CcmE was tested in the ΔccmB strain EC75 expressing ccmE from an arabinose inducible promoter and either ccmB, ccmAB or ccmABC from a pACYC184 vector. The ΔccmB strain EC75 was co-transformed with pEC412 (expressing ccmE on a pISC2 vector) and pACYC184 (empty vector), pEC517 (ccmB), pEC100 (ccmAB), pEC520 (ccmABC) or pEC101 (ccmABCD) and grown under anaerobic conditions with nitrite as the terminal electron acceptor. Membrane proteins were isolated and 100 µg were subjected to SDS-15% PAGE, followed by heme stain.

Figure 21. Functional characterization of CcmB. The ΔccmB strain EC75 was co-transformed with pEC412 (ccmE-His6) and pACYC184 (empty vector, lane 1), pEC517 (ccmB, lane 2), pEC100 (ccmAB, lane 3), pEC520 (ccmABC, lane 4) or pEC101 (ccmABCD, lane 5). Membrane proteins were isolated from anaerobically grown cells and subjected to SDS-15% PAGE. First panel, heme stain of membrane proteins (100 µg/ lane). The position of holo-CcmE is indicated on the right. Second panel, immunoblot of membrane proteins (50 µg/lane) probed with antiserum against CcmE. The position of CcmE is indicated on the right. The numbers on the left are molecular masses of marker proteins given in kilodaltons.
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The ΔccmB strain complemented with ccmB or ccmAB did not show the heme-binding form of CcmE (Fig. 21, upper panel, lane 2 and 3, respectively), even though the CcmE polypeptide was present at normal levels (Fig. 21, lower panel, lane 2 and 3), shown by immunoblot analysis. Only when CcmC or CcmCD were present in addition to CcmAB in the membrane, a heme stainable band specific for holo-CcmE could be detected (Fig. 21, upper panel, lane 4 and 5, respectively). These results were in agreement with the previous finding that holo-CcmE formation is a prerequisite of cytochrome c formation (Schulz et al., 1998). They were also consistent with the hypothesis that overexpression of ccmB without concomitant overexpression of ccmC has a negative effect on cytochrome c formation.

Overexpressed CcmB represses cytochrome c formation

Since the plasmid pEC100 (ccmAB) was unable to complement the ΔccmB mutant, we next tested its ability to complement the ΔccmA mutant that had been shown to be non-polar (Fig. 16). The ΔccmA Δdms strain was transformed with pEC100 and was tested for anaerobic growth on minimal salt agar plates supplemented with TMAO. Surprisingly, even though complementation of the ΔccmA Δdms strain with overexpressed CcmA alone was possible, it failed when CcmB was co-expressed (Fig. 22, A + C). This suggested that overexpressed CcmB had a dominant negative effect on cytochrome c maturation. Such an effect was likely the reason why no complementation of the ΔccmB mutant with plasmid-borne ccmB was possible and thus should also be observable in a wild-type background.
Components of the ABC transporter

\[ \Delta \text{ccmA} \Delta \text{cms} \]

\begin{align*}
\text{CcmA} & \quad \text{empty vector} & \quad \text{CcmAB} \\
A & \quad B & \quad C
\end{align*}

Figure 22. Overexpressed CcmAB is unable to complement a \( \Delta \text{ccmA} \Delta \text{cms} \) deletion strain for growth. The \( \Delta \text{ccmA} \Delta \text{cms} \) strain was transformed with pEC71 (ccmA, panel A), pACYC184 (empty vector, panel B) or pEC100 (ccmAB, panel C). The strains were streaked out on minimal salt agar plates supplemented with 10 mM TMAO and incubated under anaerobic conditions for 3 days at 37°C.

The wild-type strain MC1061 expressing cytochrome \( c_{550} \) (pRJ3290) was transformed either with pACYC184 (empty vector), pEC517 (ccmB), pEC100 (ccmAB), pEC520 (ccmABC) or pEC101 (ccmABCD) and was grown under anaerobic conditions in minimal salt media supplemented with nitrite as the terminal electron acceptor. Periplasm was isolated, the protein levels were equalized to 500 \( \mu \)g per ml, and optical difference spectra were recorded. Absorption peaks specific for cytochrome \( c_{550} \) could easily be detected in the periplasm isolated from the wild-type strain carrying the empty vector and pRJ3290 (Fig. 23). When \( ccmB \) or \( ccmAB \) were introduced on a plasmid into the wild-type strain, cytochrome \( c \) formation was repressed (Fig. 23). Only when \( ccmB \) was co-expressed with either \( ccmC \) or \( ccmCD \) on a plasmid, holo-cytochrome \( c \) was formed (Fig. 23). This indicates that the dominant negative effect is the result of a relative overexpression of \( ccmB \) compared to \( ccmC \).
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Figure 23. Dominant-negative effect of CcmB overexpression. The wild-type strain MC1061 was co-transformed with pRJ3290 (cytochrome c_{552}) and either the plasmid pACYC184 (empty vector) pEC517 (ccmB), pEC100 (ccmAB), pEC520 (ccmABC) or pEC101 (ccmABCD). Periplasmic proteins were isolated as described from anaerobically grown cells and protein levels were equalized to 500 µg/ml. Dithionite-reduced minus ammonium persulfate oxidized difference spectra were measured from periplasmic extracts.

Conserved amino acid residues in CcmB

Due to its dominant negative phenotype, ccmB has to be co-expressed with ccmA and ccmC to investigate which amino acids are functionally important. The approach of random mutagenesis using the colony color under conditions of anaerobic growth on TMAO plates as an assay had been successful for the characterization of CcmC. Here, the necessity to coexpress ccmA and ccmB on the same plasmid would have the disadvantage that many random mutation sites would occur in ccmA or ccmC. Thus, the site directed mutagenesis approach was chosen.

To identify conserved amino acid residues, the sequence of the E. coli CcmB protein was aligned with its homologues in 30 other organisms. A representative alignment of six CcmB homologues from close as well distant relatives is presented in Fig. 24. Due to the wide variety of transported substrates, the transmembrane subunits of ABC transporter do not have strictly conserved motifs (Locher et al., 2002).
Many conserved leucine, isoleucine, glycine and alanine residues can be found in the sequence alignment of the CcmB protein. Such residues are thought to be important for structure integrity and appropriate assembly in the membrane. Thus, these residues are not expected to carry out an important function in cytochrome c maturation. Other conserved residues are tryptophane, glutamine, aspartic acid or arginine. These bulky or charged amino acid residues may have a role in transport of a substrate or be part of an interaction site with other proteins. In addition, a histidine at position 100 in CcmB in α- and γ-proteobacteria was strongly conserved and therefore of special interest, as histidine residues are known to be important in proteins involved in heme binding, such as cytochrome c, CemC or CemE.

To assess the question of where the conserved amino acids are located, a transmembrane prediction program was used (see above). Several of the conserved amino acids are located in the

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Figure 24. Amino acid sequence alignment of CcmB homologues from representative organisms. Strictly conserved residues are shaded in black and strongly conserved residues are shaded in grey. Asterisks indicate the amino acid residues which were mutated to alanine in the E. coli CcmB protein.
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second cytoplasmic loop of the membrane protein (Fig. 25). From structural analysis, it is known that this domain interacts with the nucleotide binding domain or subunit (Locher et al., 2002). In CcmB it might be the site of interaction with CcmA.

Figure 25. Proposed topology model of E. coli CcmB. The model was constructed by using the program TMpredict, which predicts six transmembrane helices, four cytoplasmic domains and three periplasmic domains. This is in agreement with the experimental determined topology of the R. capsulatus homologue HelB (Goldman et al., 1997). Amino acid residues characterized in this study are boxed. Black boxes represent the strictly conserved amino acid residues and grey boxes the strongly conserved residues. The conserved residues are from CcmB homologues of the following species, derived from whole genome sequences at NCBI: E. coli, Haemophilus influenzae, Yersinia pestis, Agrobacterium tumefaciens, Rickettsia prowazekii, Pseudomonas aeruginosa, Nitrosomonas europaea, Salmonella typhimurium, Pantoaea citrea, Paracoccus denitrificans, Bradyrhizobium japonicum, Pseudomonas putida, Thermus thermophilus, Shewanella putrefaciens, Rhodobacter sphaeroides, Vibrio cholerae, Caulobacter crescentus, Methanosarcina mazei, Methanosarcina acetivorans and Arabidopsis thaliana.
Analysis of CcmB point mutants co-expressed with CcmA and CcmC

The conserved amino acid residues shown as boxes in Fig. 25 were mutated to alanine. As a template, the plasmid pEC519 was used, expressing CcmAB-His₆CcmC on a pACYC184 vector. The C-terminal His-tag of CcmB was introduced to allow detection of the protein by immunoblot, as point mutants can lead to destabilization of the polypeptide. The mutant constructs were transformed into the ΔccmB strain EC75 expressing cytochrome c₅₅₀ (pRJ3290) and grown anaerobically with nitrite as the terminal electron acceptor. Periplasm was isolated, protein levels were equalized and optical difference spectroscopy was used to analyze the presence of holo-cytochrome c. As shown in Fig. 26, despite some variations in the signal intensities, none of these mutations abolished cytochrome c formation completely.

Figure 26. Spectroscopic characterization of CcmB point mutants overexpressed with CcmA and CcmC. The ΔccmB strain EC75 was co-transformed with pRJ3290 and with variants of pEC519 (ccmAB-His₆ccmC) with the following mutations of CcmB: F28A, W55A, R68A, F70A, D73A, D76A, E80A, K96A, H100A, W101A, F175A. As negative control, pACYC184 (empty vector) was used. Periplasmic proteins were isolated as described from anaerobically grown cells and protein levels were equalized to 500 μg per ml. Dithionite-reduced minus ammonium persulfate oxidized difference spectra were measured from periplasmic extracts. The average percentage of cytochrome c formation of two independent experiments is shown on the right.
Results

This indicates that these amino acid residues in CcmB do not have an essential role in cytochrome c maturation.

Also, holo-CcmE formation was investigated. The ΔccmB strain EC75 with a plasmid expressing ccmE on a pISC2 vector (pEC412) was transformed with the constructs producing the mutated CcmB variants from a pACYC184 vector. The resulting strains were grown anaerobically in the presence of nitrite. Membrane proteins were isolated, subjected to SDS-15% PAGE and analyzed by heme stain. The levels of holo-CcmE were not drastically reduced (Fig. 27).

![Image](image_url)

**Figure 27. Holo-CcmE formation in CcmB point mutants.** The ΔccmB strain EC75 was co-transformed with pEC412 (ccmE-His6) and with variants of pEC519 (ccmAB-His6ccmC, lane 1) with the following mutations in CcmB: F28A (lane 2) W55A (lane 3), R68A (lane 4), F70A (lane 5), D73A (lane 6), D76A (lane 7), E80A (lane 8), K96A (lane 9), H100A (lane 10), W101A (lane 11), F175A (lane 12). As control, membranes of the strain EC75 expressing either ccmABCD (lane 13) or pACYC184 (lane 14) was used. Heme stain of membrane proteins (100 µg/lane) isolated from aerobically grown cells after separation by SDS-15% PAGE. The position of holo-CcmE is indicated on the right. The number on the left is the molecular mass of a marker protein given in kilodalton.

Immunoblot analysis using antibodies directed against either the His6-tag of CcmB or against CcmA showed that these proteins were not affected in a remarkable way (Fig. 28). Note that the running behaviour of CcmB in the gel leads to a band of approximately 21 kDa, while the expected molecular mass is 24.8 kDa. This phenomenon is well known for certain hydrophobic membrane proteins (Thöny-Meyer *et al.*, 1991). In conclusion, these experiments show that none of these mutated amino acids is essential for the function of CcmB in cytochrome c maturation.
Components of the ABC transporter

**Figure 28. Functional analysis of CcmB point mutants.** The ΔccmB strain EC75 was transformed with variants of pEC519 (ccmAB-His₆, ccmC, lane 1) with the following mutations in CcmB: F28A (lane 2), W55A (lane 3), R68A (lane 4), F70A (lane 5), D73A (lane 6), D76A (lane 7), E80A (lane 8), K96A (lane 9), H100A (lane 10), W101A (lane 11), F175A (lane 12). As control, membranes of the strain EC06 expressing either pACYC184 (lane 13) were used. Membrane proteins were isolated from anaerobically grown cells and separated by SDS-15% PAGE. First panel, immunoblot of membrane proteins (50 μg/lane) probed with antiserum against CcmA. Second panel, immunoblot of membrane proteins (50 μg/lane) probed with antiserum against the His₆ tag. The position of CcmA and CcmB are indicated on the right. The numbers on the left are molecular masses of marker proteins given in kilodaltons.

**CcmB point mutants can reverse the dominant negative phenotype**

As overexpressed CcmB shows a dominant negative effect on cytochrome c maturation, the question arose whether a mutation in ccmB would relieve the block in cytochrome c formation caused by overexpression. Mutations were also introduced into the construct pEC517 expressing ccmB. The mutated constructs were transformed into the ΔccmB strain EC75, expressing cytochrome c₅₅₀ (pJ3290) and analyzed for cytochrome c formation as before. Three mutations within CcmB, R68A, D73A and D76A, restored cytochrome c maturation (Fig. 29).
Results

Figure 29. Spectroscopic characterization of CcmB point mutants overexpressed in the absence of additional Ccm proteins. The ΔccmB strain EC75 was co-transformed with pRJ3290 (cytochrome c550) and either the plasmid pEC517 (ccmB) and its variants F28A, R68A, F70A, D73A, D76A, H100A, W101A, F175A. As control, pEC101 (ccmABCD) was used. Periplasmic proteins were isolated as described from anaerobically grown cells and protein levels were equalized to 500 µg per ml. Dithionite-reduced minus ammonium persulfate oxidized difference spectra were measured from periplasmic extracts. The percentage of formed cytochrome c550 is shown on the right.

Interestingly, all mutations were located in the cytoplasmic domain of CcmB, which might be important for interaction with CcmA (Fig. 25). To answer the question of whether these mutations could also restore heme incorporation into CcmE, periplasmic extracts were isolated from the anaerobically grown ΔccmB strain EC75 harboring pEC415 (ccmEsoi-His6) and the constructs producing the mutant CcmB version. After SDS-15% PAGE, the gel was stained for covalently bound heme. Holo-CcmE formation could only be obtained in the membranes of ΔccmB strains expressing the proteins CcmB R68A, D73A or D76A (Fig. 30).

In conclusion, CcmB R68A, D73A and D76A maintain the activity necessary for cytochrome c maturation, while loosing their dominant negative phenotype on cytochrome c formation when overexpressed.
Components of the ABC transporter

\[ \Delta ccmB / ccmE \]

Table 1. CcmE formation in a \( \Delta ccmB \) strain overexpressing CcmB point mutants. The \( \Delta ccmB \) strain EC75 was co-transformed with pEC415 (soluble CcmE-His6) and either the plasmid pEC517 (ccmB, lane 1) and the CcmB variants F28A (lane 2), R68A (lane 3), F70A (lane 4), D73A (lane 5), D76A (lane 6), H100A (lane 7), W101A (lane 8), F175A (lane 9). As control, pACYC184 (empty vector, lane 10) or pEC101 (CcmABCD, lane 11) were used. 100 \( \mu \)g/lane periplasmic proteins isolated from aerobically grown cells were separated by SDS-15\% PAGE, followed by heme stain. The position of holo-CcmE is indicated on the right. The number on the left is the molecular mass of the marker protein given in kilodaltons.

Figure 30. CcmE formation in a \( \Delta ccmB \) strain overexpressing CcmB point mutants. The \( \Delta ccmB \) strain EC75 was co-transformed with pEC415 (soluble CcmE-His6) and either the plasmid pEC517 (ccmB, lane 1) and the CcmB variants F28A (lane 2), R68A (lane 3), F70A (lane 4), D73A (lane 5), D76A (lane 6), H100A (lane 7), W101A (lane 8), F175A (lane 9). As control, pACYC184 (empty vector, lane 10) or pEC101 (CcmABCD, lane 11) were used. 100 \( \mu \)g/lane periplasmic proteins isolated from aerobically grown cells were separated by SDS-15\% PAGE, followed by heme stain. The position of holo-CcmE is indicated on the right. The number on the left is the molecular mass of the marker protein given in kilodaltons.
Results

Components of the ABC transporter

As predicted from sequence alignments (Fig. 14), CcmA is thought to be the nucleotide binding subunit of an ABC transporter. The predicted six transmembrane α helices of CcmB make it a likely candidate to be the corresponding transmembrane permease subunit. In addition to CcmB, CcmC was also predicted to contain six transmembrane α helices (Ahuja and Thöny-Meyer, 2003). Furthermore, Goldman et al. demonstrated with the CcmABC homologues HelABC of *Rhodobacter capsulatus* that the ABC transporter involved in cytochrome c maturation consists of at least the three components HelA, HelB and HelC. This conclusion was based on co-immunoprecipitation experiments with LacZ fusions to HelB and HelC, using the monoclonal antibody against β-galactosidase for the pull down and the monoclonal antibody against HelA (CcmA homologue) for detection (Goldman *et al.*, 1997). However, Schulz *et al.* showed that the *E. coli* CcmC incorporates heme into CcmE in the absence of CcmAB (Schulz *et al.*, 1999), indicating that CcmC has a function independent of CcmAB. It was therefore more likely that the ABC transporter consists of CcmA and CcmB only.

CcmA and CcmB are the subunits of the ABC transporter

To further test this hypothesis, experiments were performed to see whether CcmA interacts directly with CcmB and/or with CcmC. CcmA does not contain an obvious transmembrane or hydrophobic domain for association with the membrane, and thus CcmA must interact with a membrane partner to be associated with the membrane, as already shown in the first chapter. CcmA should not be detected in the membrane in the absence of its interacting partners, CcmB and/or CcmC. If CcmA interacts with both proteins CcmB and CcmC, then the presence of either CcmB or CcmC should be sufficient for the detection of CcmA in the membrane. The ΔccmA-H strain EC06 harboring the vector pACYC184 or plasmids expressing either *ccmABCD* (pEC101), *ccmA* (pEC71), *ccmACDE* (pEC102) or *ccmABDE* (pEC105) were used to isolate membranes and soluble proteins. These fractions were tested for the presence or absence of CcmA by immunoblot probed with antibodies specific for CcmA. Fig. 31 shows that CcmA is detected in...
Components of the ABC transporter

the membrane when CcmB, CcmC and CcmD are co-produced (lane 1). In the absence of any other Ccm proteins, CcmA was detected neither in the membrane fraction nor in the soluble fraction (Fig. 31, lane 2 and 3). Next, the presence of CcmA was tested using plasmids from which either ccmCDE (Fig. 31, lane 6 and 7) or ccmBDE (Fig. 31, lane 8 and 9) were co-expressed with ccmA in order to determine whether CcmB or CcmC were required for assembly of CcmA in the membrane. The signal appeared only in membranes of a strains producing CcmB and was not dependent on CcmC. The absence of CcmA in the soluble fractions shows that CcmA is not stably maintained in the cytoplasm in the absence of its interacting membrane integral partner. This experiment clearly shows that CcmA interacts with CcmB, but not necessarily with CcmC. CcmA and CcmB most likely assemble to an ABC transporter consisting of CcmA<sub>2</sub>B<sub>2</sub>.

Figure 31. CcmA requires the presence of CcmA for assembly in the membrane. The ΔccmA-H strain EC06 was transformed with plasmid pEC101 (CcmABCD, lane 1), pEC71 (CcmA, lane 2+3), pEC94 (CcmB-H, lanes 4 and 5), pEC102 (CcmACDE, lanes 6 and 7) or pEC105 (CcmABDE, lanes 8 and 9). 50 μg/lane of membrane proteins (Me, lanes 2, 4, 6 and 8) and soluble proteins (Sol, lanes 3, 5, 7 and 9) from aerobically grown cells were separated by SDS-15% PAGE, followed by immunoblot probed with antiserum against CcmA. The position of CcmA is indicated on the right. The numbers on the left are molecular masses of marker proteins given in kilodaltons.
Results

**CcmC acts as an accessory protein of the ABC transporter**

Previously, the dominant negative effect of overexpressed CcmB was shown to be relieved only by also overexpressing CcmC. Thus, CcmC was expected to exhibit some interaction with CcmB, and such a possible interaction was tested in a pull down experiment. Since only a peptide antibody against CcmA was sufficiently specific, the plasmid pEC515 was constructed, which expressed the proteins CcmA, CcmB-strep and CcmC-His6. As a positive control, the plasmid pEC514 was used, expressing CcmA and CcmB-strep. As a negative control, the empty vector pBR322 was used. The strep-tag has an affinity for StrepTactin beads (Schmidt et al., 1996; Voss and Skerra, 1997), while the His6-tag has an affinity for Ni-NTA agarose (Janknecht et al., 1991). The ΔccmA-H strain EC06 harbouring either pBR322, pEC514 or pEC515 was grown aerobically in LB medium, and membrane proteins were isolated and solubilized with 1% n-dodecyl-β-D-maltoside. Membrane proteins were separated by SDS-15% PAGE, followed by immunoblot probed with antibodies against either CcmB-strep or CcmC-His6. Signals specific for a protein corresponding to the size of CcmB could only be detected in membranes containing overexpressed CcmB-strep (Fig. 32, upper panel, lane 2 and 3). In addition, signals corresponding to the size of CcmC were detected in membranes containing overexpressed CcmC-His6, but not in membranes of strains expressing only CcmA and CcmB-strep (Fig. 32, lower panel, lane 2 and 3). In a first pull down experiment, solubilized membranes were treated with Ni-NTA agarose and separated by SDS-15% PAGE. CcmB-strep was only detected in the membranes containing both CcmB-strep and CcmC-His6 (Fig. 32, upper panel, lane 6). Apparently, the CcmC-His6 interacted with CcmB-strep. As expected, CcmC-His6 could only be detected in membranes containing CcmC-His6 (Fig. 32, lower panel, lane 6). Next, the StrepTactin beads were used to pull down CcmB-strep. When the immunoblot was probed with antibodies against the strep-tag of CcmB, specific signals could be obtained in the pull down fractions of solubilized membranes containing CcmB-strep (Fig. 32, upper panel, lane 8 and 9). The protein CcmC-His6 was only detected in the pull down experiment with solubilized membranes containing CcmA, CcmB-strep and CcmC-His6 (Fig. 32, lower panel, lane 9). These results confirm that CcmC interacts with CcmB and thus might be an accessory protein of the ABC transporter.
Components of the ABC transporter

**Figure 3.2.** CcmB interacts with CcmC. The ΔccmA-H strain EC06 was transformed with plasmid pBR322 (empty vector, lane 1, 4 and 7), pEC514 (CcmA, CcmB-strep, lanes 2, 5 and 8) or pEC515 (CcmA, CcmB-strep, CcmC-His6, lanes 3, 6 and 9). Membrane proteins were isolated from aerobically grown cells. Lanes 1-3, 50 μg of membrane proteins were separated by SDS-15% PAGE. Lanes 4 to 6, 1 mg of solubilized membrane proteins were pulled down with 5 mg nickel-nitritriacetic acid agarose beads and separated by SDS-15% PAGE. Lanes 7 to 9, 1 mg of solubilized membrane proteins were pulled down with 5 mg StrepTactin beads and separated by SDS-15% PAGE. *First panel*, immunoblot probed with antiserum against the strep-tag of CcmB. The position of CcmB is indicated on the right. *Second panel*, immunoblot probed with antiserum against the His6-tag of CcmC. The position of CcmC is indicated on the right. The numbers on the left are molecular masses of marker proteins given in kilodaltons.
Function of CcmAB in vivo

In the previous chapter, CcmA and CcmB were predicted to be the subunits of the ABC transporter involved in cytochrome c maturation, while CcmC may act as an accessory protein. One of the goals of this study was to identify the transported substrate. For cytochrome c maturation, two components must be translocated across the membrane: apo-cytochrome c and heme. While apo-cytochrome c was shown to be transported across the membrane via the general secretion (sec) pathway (Thöny-Meyer and Künzler, 1997), the translocation pathway for heme is still unknown. Therefore, the most likely substrate to be transported by CcmAB would be heme. If heme is in fact the substrate exported to the periplasm, it might be possible to complement the defect of an ABC transporter mutant by the addition of extracellular heme. However, the outer membrane of E. coli is impermeable to heme.

Complementation for growth of a ΔhemA strain with extracellular heme

An E. coli ΔhemA strain deficient in heme biosynthesis grows poorly unless 5-aminolevulinic acid is supplied exogenously. Also, the ΔhemA strain does not grow on a hemin-containing medium (McConville and Charles, 1979). Reidl and Mekalanos described in 1996 that the plasmid pJRH1, encoding a fusion lipoprotein, e(P4)-TGT derived from Haemophilus influenzae, enabled the E. coli strain ΔhemA to grow on hemin plates (Reidl and Mekalanos, 1996). It seems likely that the outer membrane permeability for heme increases when e(P4)-TGT is present in the outer membrane.

First, complementation of the deletion strain ΔhemA with hemin was tested in our plate assay. The strain ΔhemA was transformed with either the plasmid pJRH1 or the empty vector pACYC184 and grown anaerobically on minimal salt agar plates supplemented with either hemin, 5-aminolevulinic acid (δ-ALA), or not supplemented as a negative control. As shown in Fig. 33, panel B, the ΔhemA strain containing the vector was growing only when the plates were supplemented with δ-ALA, but not when they were supplemented with hemin. By contrast, the ΔhemA strain transformed with the plasmid pJRH1 formed colonies when either δ-ALA or hemin...
were added (Fig 33, panel A), indicating that hemin was taken up with the help of the fusion lipoprotein.

**Figure 33.** Hemin is able to complement a ΔhemA strain for heme biosynthesis. The ΔhemA strain was transformed either with the plasmid pJRHI (expressing e(P4)-TGT, *panel A*) or pACYC184 (empty vector, *panel B*). The strain was streaked out on minimal salt agar plates supplemented with 10 mM TMAO and incubated under anaerobic conditions for 3 days. *Upper panel* not supplemented. *Middle panel*, supplemented with 50 μg/ml 5-aminolevulinic acid (δ-ALA). *Lower panel*, supplemented with 20 μg/ml hemin.
Results

A ΔccmAΔdms strain cannot be complemented for growth with extracellular heme

If heme can diffuse into the periplasm, a transporter would not be needed to direct heme to the periplasm during cytochrome c maturation. The defect of a ΔccmA strain in cytochrome c maturation might then be bypassed in the presence of hemin in the medium. The ΔccmAΔdms strain was transformed with either pJRH1 or with the empty vector pACYC184. As a positive control, the cytochrome c proficient strain MC1061 Δdms was used. The strains were grown anaerobically on minimal salt agar plates containing TMAO in the presence or absence of heme. As expected, the MC1061 Δdms strain expressing the genes ccmA-H was able to grow independent of the presence of hemin due to its ability to produce heme and to form cytochrome c (Fig. 34, panel A). In contrast, the ΔccmA Δdms strain transformed with the empty vector pACYC184 was not able to grow, even when hemin was added (Fig. 34, panel B). The same strain additionally expressing e(P4)-TGT from pJRH1 was also unable to grow in the presence of hemin (Fig. 34, panel C).

![Figure 34. Hemin is not able to complement a ΔccmA Δdms strain for cytochrome c formation. The MC1061 Δdms strain was transformed with pACYC184 (empty vector, panel A). The ΔccmA Δdms strain was transformed either with pACYC184 (empty vector, panel B) or pJRH1 (expressing e(P4)-TGT, panel C). The strains were streaked out on minimal salt agar plates supplemented with 10 mM TMAO and incubated under anaerobic conditions. Upper panel not supplemented. Lower panel, supplemented with 20 μg/ml hemin.](image-url)
We conclude that heme is able to penetrate the outer membrane and then either is taken up into the cytoplasm without being released into the periplasm, or is released into the periplasm but not accessible to the cytochrome c maturation apparatus. To exclude the possibility that the plate assay with supplemented hemin was not sensitive enough, formation of cytochrome c in the periplasm was investigated. The \( \Delta ccmA \) strain EC21 expressing cytochrome \( c_{550} \) (pRJ3290) was transformed with pJRHI. The wild-type strain MC1061 carrying pRJ3290 was used as positive control. The strains were grown anaerobically with nitrite as the terminal electron acceptor.

Cytochrome c production was induced, and hemin was added to one part of the culture, whereas to the other part of the culture and to the positive control no hemin was added. After another 12 hours of anaerobic growth, the periplasmic fraction was isolated, and optical difference spectra were recorded. While the periplasm of the positive control showed absorption peaks at 550 nm specific for cytochrome \( c_{550} \), the periplasm of the \( \Delta ccmA \) strain did not reveal the typical peaks for cytochrome \( c_{550} \), independent of whether or not hemin had been added (Fig. 35).

This also confirmed that heme cannot complement a \( \Delta ccmA \) deletion strain for cytochrome c formation.

![Figure 35. Hemin does not complement a ccmA mutant for cytochrome c formation.](image-url)
Results

**Function of CcmAB in vitro**

To assess the question of whether CcmA has ATPase activity and whether the point mutation K40D in CcmA affects ATP hydrolysis, the wild-type protein CcmA and the mutant protein CcmA(K40D) were purified. The plasmids pEC23 (pET28c, ccmA) and pEC98 (pET28c, ccmA(K40D)) were constructed and transformed into the ΔccmA-H strain EC06. Expression of the proteins was optimized by growth temperature, IPTG concentrations and induction times. The soluble and insoluble fractions were isolated after cell lysis by French press treatment and the proteins were separated and analyzed by SDS-15% PAGE, followed by immunoblot probed with antiserum against CcmA. Neither the wild-type nor the mutant CcmA were present in the soluble fractions, but were detected in the insoluble fractions containing cell debris and inclusion bodies (data not shown). This is in agreement with the previous observation that CcmA was not found in the soluble fraction in the absence of its interaction partner CcmB. Therefore, CcmA was purified in the presence of CcmB. Two questions were addressed: does the purified CcmAB protein complex reveal specific ATPase activity characteristic for the ABC transporter family? Can ATP hydrolysis be stimulated by a substance, i.e. the substrate to be transported? This latter possibility was considered because of previous studies on ABC transporters, which had shown that the intrinsic ATPase activity can be stimulated by adding the substrate (Liu and Ames, 1997; Landmesser et al., 2002). Therefore, if the CcmAB complex can be purified in its active form and exhibits an intrinsic ATPase activity, then it should be possible to stimulate this activity by adding the substrate. Although in vivo complementation of the ΔccmA strain with extracellular heme had not been successful, this still did not exclude heme as the transported substrate. Therefore, it was of particular interest to test heme as a potential substrate for the ABC transporter CcmAB.
Purification of the ABC transporter CcmAB and the mutant CcmA(K40D)B

Plasmid pEC512 was constructed, that expresses an N-terminally His6-tagged CcmA and a C-terminally strep-tagged CcmB on the vector pET28c. To differentiate the ATP hydrolysis of CcmAB from contaminating ATPases, the plasmid pEC516 was constructed, which encodes the point-mutated His6-tagged CcmA(K40D) and the strep-tagged CcmB on the vector pET28c. Mutation of the amino acid residue K at position 40 to D in the ATP binding motif had been previously shown to affect the function of CcmA in cytochrome c maturation (see Fig. 17), most probably due to its inability to bind ATP. Thus, the mutated CcmA protein was the ideal control to distinguish between specific ATPase activity of CcmA and activity of other ATPases.

The plasmids pEC512 or pEC516 were transformed into the E. coli strain C43, which is particularly suitable for purification of membrane proteins (Miroux and Walker, 1996). Cells were grown at 30°C. When OD{sub}600 = 0.8 was reached, expression of His6-CcmACcmB-strep or His6-CcmA(K40D) CcmB-strep was induced with 1 mM IPTG, followed by further incubation over night at 30°C. Membrane proteins were isolated and solubilized with 1% n-Dodecyl-ß-D-maltoside. The protein His6-CcmA was co-purified with its binding partner CcmB-strep by nickel affinity chromatography. Proteins of the load, flow through, wash and elution fractions were separated by SDS-15% PAGE, followed by Coomassie stain (Fig. 36 and 37). His6-CcmA and CcmB-strep could easily be detected in the elution fractions, which however contained minor contaminants. His6-CcmA(K40D) and CcmB-strep could also be obtained in the same elution fractions at similar concentrations. An immunoblot probed with antibodies against the His6-tag of CcmA and against the strep-tag of CcmB confirmed the Coomassie stained bands to be CcmA and CcmB (data not shown). This was important, because CcmA and CcmB have the theoretical molecular weight of 25.3 kDa and 24.8 kDa, respectively, but CcmB runs lower than expected. In addition, mass spectroscopy of the elution fractions resulted in a mass of 24810 m/z for CcmB, showing that CcmB is not degraded.

Fractions (elution fractions 4-10) containing highly enriched His6-CcmA and CcmB-strep respectively His6-CcmA(K40D) and CcmB-strep proteins, respectively, were pooled. From a total of 76.5 mg protein present in the solubilized membranes (load), 12 mg His6-CcmACcmB-strep were obtained with the wild-type CcmA, whereas with the mutant CcmAB from a total of
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72.5 mg protein present in the solubilized membranes (Load), 9.6 mg His<sub>6</sub>-CcmA(K40D)CcmB-strep were obtained.

Figure 36. Affinity purification of His<sub>6</sub>-CcmA and CcmB-strep. The solubilized membranes from the aerobically grown strain C43 expressing His<sub>6</sub>-CcmACcmB-strep (pEC512) were subjected to nickel-nitrilotriacetic acid affinity chromatography. 10 µl of each fraction (except in lane 2, where 600 µl of cells were TCA precipitated) was subjected to SDS-15% PAGE, followed by Coomassie stain. Lane 1, broad range marker; lane 2, cells non-induced; lane 3, solubilized membranes, 15 µg; lane 4, flow through, 15 µg; lane 5, wash, 5 µg; lane 6, elution fraction 2, 2.5 µg; lane 7, elution fraction 4, 10 µg; lane 8, elution fraction 6, 15 µg; lane 9, elution fraction 8, 14 µg; lane 10, elution fraction 10, 6.5 µg. The position of His<sub>6</sub>-CcmA and CcmB-strep are indicated on the right. The numbers on the left are molecular masses of marker proteins given in kilodaltons (kDa).
Coupled ATP hydrolysis assay

The activity of the ATPase was determined by a coupled spectrophotometric assay containing pyruvate kinase and lactate dehydrogenase (Laubinger and Dimroth, 1987). When ATP is hydrolyzed, ADP and free phosphate are liberated. In the presence of ADP, the pyruvate kinase removes the phosphate from its substrate phosphoenolpyruvate (PEP), thus generating pyruvate and ATP. Pyruvate is a substrate for lactate dehydrogenase, which converts pyruvate to lactate by the consumption of NADH (Fig. 38). The decrease of NADH to NAD⁺ can be measured at 340 nm, as NADH has a specific absorption at this wavelength. Thus, ATP hydrolysis can be measured indirectly by the decrease of absorption at 340 nm over time.
Results

Figure 38. Scheme of the coupled ATP hydrolysis assay. The activity of the ATPase was determined by a coupled spectrophotometric assay containing pyruvate kinase and lactate dehydrogenase. When ATP is hydrolyzed, ADP and free phosphate are liberated. The pyruvate kinase removes the phosphate from its substrate phosphoenolpyruvate (PEP) and transfers it to the released ADP, thus generating pyruvate and ATP. The pyruvate is a substrate for lactate dehydrogenase, which converts pyruvate to lactate by the consumption of NADH, which can be measured at 340 nm, as NADH has a specific absorption at this wavelength.

ATP hydrolysis activity of wild-type CcmAB and mutant CcmA(K40D)B

Initially, 10 μl of the load, flow through, wash and pooled elution fraction were analyzed for ATPase activity. As shown in Fig. 39, the fractions of the load and the flow through exhibited strong ATPase activity, while the wash fraction did not reveal any activity. Moreover, the pooled fractions of His6-CcmA and CcmB-strep showed an ATPase activity that could be stimulated by increasing the amount of protein.

The activity of wild-type CcmAB was measured with the pooled fraction of His6-CcmA(K40D)CcmB-strep. This was of interest, as it was not clear whether the activity measured for the pooled His6-CcmACcmB-strep fractions was specific and was not the result of ATP hydrolysis by contaminating ATPases. In addition, it was of interest to determine whether the cytochrome c deficiency of CcmA(K40D) was due to the inability of the mutant protein to bind
and hydrolyze ATP. The ATPase activity of the load, flow through, wash and pooled fractions of purified His<sub>6</sub>-CcmA(K40D)CcmB-strep was measured as shown in Fig. 39.

**Figure 39.** ATPase activity of different fractions obtained from the affinity purification of His<sub>6</sub>-CcmACcmB-strep and His<sub>6</sub>-CcmA(K40D)CcmB. 10 μl of each fraction from the affinity purification was used for the measurements except for the pooled elution fraction for which the measurements were made according to the protein amount. **Upper panel,** activity of wild-type CcmAB. The measurements were done with load, 15 μg; FT (Flow through), 15 μg; Wash, 5 μg; for the pooled elution fraction, 20 μg or 10 μl and 40 μg or 20 μl were measured. **Lower panel,** Activity of mutant CcmA(K40D)CcmB. The measurements were done with Load, 14.5 μg; FT (Flow through), 14.6 μg; Wash, 4 μg; for the pooled elution fraction, 16 μg or 10 μl and 32 μg or 20 μl were measured. The x axis indicates the measured time in seconds. The y axis shows the absorption at 340 nm corresponding to the NADH concentration present at a certain time point.

Load, flow through and wash exhibited similar activities as obtained for fractions obtained during purification of the wild-type CcmAB. Despite these activities, the pooled purified fractions containing mutant CcmAB did not show a significant ATPase activity and could not be
Results

stimulated by increasing the amounts of His6-CcmA(K40D)CcmB-strep. This shows clearly, that the ATP hydrolysis activity obtained for the wild-type CcmAB complex is specific, while the mutant complex is not able to hydrolyze ATP due to the point mutation K40D.

To verify that the mutant was unable to hydrolyze ATP and to better characterize the activity of the wild type CcmAB, the ATPase activity of different amounts of the purified His6-CcmA(K40D)CcmB-strep proteins was compared with the same levels of the purified wild-type His6-CcmACcmB-strep proteins (Fig. 40). As a negative control, the activity of the reaction buffer in the absence of any protein was measured (data not shown). While the activity of His6-CcmACcmB-strep increased proportionally with the concentrations in the assay, no significant increase in ATP hydrolysis activity could be detected for His6-CcmA(K40D)CcmB-strep.

To determine the specific ATP hydrolysis activity of the ABC transporter CcmAB, ATP hydrolysis was measured over time, and the μmoles NADH converted per minute (units) were calculated per mg ATPase protein.

Figure 40. ATP hydrolysis activity of different amounts of wild-type or mutant proteins after 10 minutes. 2 μg, 20 μg, 40 μg and 80 μg protein of pooled fractions from either wild-type His6-CcmACcmB-strep or mutant His6-CcmA(K40D) CcmB-strep were added to the reaction buffer and the reaction was started with 2.5 mM ATP. Diamonds, activity of His6-CcmACcmB-strep; Squares, activity of His6-CcmA(K40D)CcmB-strep. The x axis indicates the amount of protein CcmA and CcmB in μg added to the reaction mixture. The y axis shows the absorption at 340 nm corresponding to the NADH concentration present at a certain time point.

20 μg of purified CcmA and CcmB were added to 1 ml reaction mixture and the reaction was started with 2.5 mM ATP. The NADH decrease was recorded for 10 minutes and measurements were done three times.
The specific activity obtained was 0.025 U/mg, which correspond well to several other ATPase activities found for other ABC transporters (0.1 - 0.001 U/mg) (Doerrler and Raetz, 2002; Landmesser et al., 2002; Liu et al., 1997).

**Heme does not stimulate ATP hydrolysis activity of CcmAB**

CcmA and CcmB have been predicted to transport heme (Goldman et al., 1997). So far, no experimental evidence supported this hypothesis. As it was shown that CcmA and CcmB exhibit an intrinsic ATPase activity, it was of interest to see whether this activity could be stimulated by heme. As it was shown that oxidized as well as reduced heme can bind to CcmE, but in vitro heme binding occurs more efficiently with reduced heme, the redox state of heme may be critical also for the transport of the molecule across the membrane (Daltrop et al., 2002; Nicholson and Neupert, 1989; Tong and Margoliash, 1998). Therefore, reduced and oxidized hemin was tested.

![Figure 41](image_url)

**Figure 41. ATPase activity of CcmAB in the presence of oxidized or reduced hemin.** The reaction mixture was supplemented with either 0, 1, 4 and 8 μM reduced or oxidized hemin as indicated on the right and the reaction was started with 2.5 mM ATP. Buffer contains 8 μM hemin, but no His6-CcmACcmB-Strep. CcmA*B contains 8 μM hemin and 20 μg His6-CcmA(K40D)CcmB-Strep. The x axis indicates the measured time in seconds. The y axis shows the absorption at 340 nm corresponding to the NADH concentration present at a certain time point.
Results

Different amounts of reduced or oxidized hemin ranging from 1 μM to 8 μM were added to the reaction mixture containing 20 μg of purified His6-CcmACcmB-strep. As a negative control, buffer without heme or 20 μg CcmA-His6(K40D)CcmB-strep with 8 μM hemin were used (Fig. 41). Neither reduced nor oxidized hemin could stimulate ATPase activity, suggesting that heme is very unlikely to be translocated across the membrane by CcmAB.

No stimulation of the ATPase activity by redox-active substances

As CcmA and CcmB are also involved in the cytochrome c maturation pathway after heme is attached to CcmE, it is possible that CcmA and CcmB are required to provide a redox-active substance controlling the redox state of heme. Thus, it is possible that CcmA and CcmB transport reducing or oxidizing substances. Therefore, several substances of this type produced in the cell were tested for stimulation of the ATPase activity of CcmA. Concentrations of 0.01 mM to 1 mM of the flavins FAD and FMN as well as of 0.01 mM to 5 mM cysteine, cystine, malate, fumarate, succinate, reduced glutathione or lactate were tested. As before, 20 μg of purified CcmA-His6CcmB-strep was incubated with the reaction mixture containing different amounts of substrate and the reaction was started with 2.5 mM ATP. In neither case stimulation of ATP hydrolysis could be observed (Fig. 42, for simplicity, activity measurements of 0.5 mM are shown). This indicates that either the tested substrates are not the molecules transported by the ABC transporter or the conditions have not been appropriately chosen.
Function of CcmAB in vitro

CcmAB stimulates the ATP hydrolysis activity of CcmAB

Holo-CcmE formation takes place in the presence of CcmC, even in the absence of CcmAB (Schulz et al., 1999). However, in the absence of CcmA or CcmB, cytochrome c formation was not observed. This indicated a role of the ABC transporter in the later steps of cytochrome c maturation, i.e. after heme is transferred to CcmE. In a previous chapter, it was shown, that CcmC interacts with CcmB. As CcmC also interacts with CcmE for heme attachment, it was of interest, whether CcmA and CcmB in a complex with CcmC and/or CcmE participate in the release of heme from CcmE by providing a substrate. In the literature, the activity of the maltose importer was shown to be stimulated by its periplasmic binding protein MalE (Landmesser et al.,...
Results

2002) also in the absence of maltose. Thus, it seemed possible that CcmE stimulates the ABC transporter so that CcmAB hydrolyzes ATP to use the energy for transport of a substrate responsible for the later steps in cytochrome c formation.

ATP hydrolysis measurements were done as described previously. As a substrate, purified soluble CcmE-His$_6$ (a mixture of apo- and holo-forms), was used at end-concentrations of 5, 10, 20, or 40 μM. As negative control, 40 μM of soluble CcmE-His$_6$ was used in the absence of His$_6$-CcmACcmB-strep or in the presence of mutant His$_6$-CcmA (K40D)CcmB-strep. As shown, in Fig. 43, ATPase activity could be slightly stimulated by CcmE (~1.2 fold), whereas no stimulation was obtained in the control reaction.

![Figure 43. CcmE stimulates the ATPase activity of CcmA.](image)

To test whether the stimulation was specific for CcmE, several other proteins were tested for stimulation of ATP hydrolysis. These control experiments included heme binding proteins as well as other Ccm proteins. The protein BSA was used to investigate, whether CcmA activity can be stimulated by any unrelated protein. No stimulation was found, when BSA was added at
Function of CcmAB in vitro

concentrations of 5, 10, 20 or 40 μM (Fig. 44). To answer the question of whether heme-containing proteins can stimulate the activity, the *B. japonicum* cytochrome c₅₅₀ was used at concentrations of 5, 10, 20 and 40 μM (Fig. 44). Also, this protein was not able to stimulate ATPase activity. Next, soluble CcmG-His₆ and soluble CcmH-His₆ were tested (gift from Umesh Ahuja). These two polypeptides are the periplasmic domains of two Ccm proteins believed to be involved in redox reactions. Again, no stimulation was observed, when these proteins were added at concentrations of 5, 10, 20, 40 μM (Fig. 44).

![Figure 44. No stimulation ATP hydrolysis activity by other proteins.](image)

Table 44. No stimulation ATP hydrolysis activity by other proteins. 0, 5, 10, 20 and 40 μM of protein was added to the reaction mixture containing 20 μg purified CcmA-His₆-CcmB-strep and the reaction was started with 2.5 mM ATP. The used proteins are cytochrome c₅₅₀, BSA, CcmG-His₆, CcmH-His₆ or CcmE-His₆ and indicated on the right. For simplicity, the measurement with 40 μM substrate is shown. Buffer, reaction buffer in the absence of His₆-CcmACcmB, but in the presence of substrate. Control, reaction mixture in the absence of substrate. The x axis indicates the measured time in seconds. The y axis shows the absorption at 340 nm corresponding to the relative NADH concentration present at a certain time point. The absorption per minute is shown on the right.

![Sample AAAn in -](image)

Figure 44. No stimulation ATP hydrolysis activity by other proteins. 0, 5, 10, 20 and 40 μM of protein was added to the reaction mixture containing 20 μg purified CcmA-His₆-CcmB-strep and the reaction was started with 2.5 mM ATP. The used proteins are cytochrome c₅₅₀, BSA, CcmG-His₆, CcmH-His₆ or CcmE-His₆ and indicated on the right. For simplicity, the measurement with 40 μM substrate is shown. Buffer, reaction buffer in the absence of His₆-CcmACcmB, but in the presence of each substrate. Control, reaction mixture in the absence of substrate. The x axis indicates the measured time in seconds. The y axis shows the absorption at 340 nm corresponding to the relative NADH concentration present at a certain time point. The absorption per minute is shown on the right.

Although the stimulation by CcmE was not strong, it was significant (Fig. 45). Stimulation of the ATPase CcmA was already observed with 5 μM CcmE, but reached a maximum with approximately 20 μM. The concentration in the reaction mixture was 1 μM CcmA and 1 μM CcmB. Saturation of stimulation occurred with a 20-fold molar excess of CcmE. Thus it seems reasonable that no further increase was obtained with higher amounts of added CcmE.

Stimulation of ATP hydrolysis is in agreement with a role of the ABC transporter in the later steps of cytochrome c maturation and suggests a direct interaction with CcmE.
Figure 45. Stimulation of ATPase activity of CcmA-His6-CcmB-strep with CcmE. 0, 5, 10, 20 and 40 μM of either protein was added to the reaction mixture containing 20 μg purified His6-CcmACcmB-strep and the reaction was started with 2.5 mM ATP. The measured proteins are BSA, soluble CcmE-His6 and soluble CcmG-His6 and are indicated right. Measurements were done at least three times; average and standard deviation was calculated. The y axis shows the decrease of absorption at 340 nm corresponding to NADH consumption divided by minutes. The x axis indicates the used μM of purified protein.
Characterization of CcmE dimer

CcmE is a heme chaperone that transiently binds heme via a covalent bond and delivers it further to the apo-cytochrome c. The binding of heme occurs at position H130 of CcmE (Schulz et al., 1998) and at the β-C-atom of one of the vinyl groups of heme (Lee et al., 2005). None of the chemical reactions involved in binding to and release from CcmE is known. As shown, the ABC transporter CcmAB is important for heme transfer to and from CcmE. CcmE was shown previously to have a particular running behavior after separation by SDS-PAGE in that minor bands corresponding to a dimer were present in addition to the band running like a monomer (Schulz et al., 1998). It seemed that the dimers were always detected when the other Ccm proteins were also overproduced. Preliminary data suggested that in a ΔccmA or ΔccmB background, no CcmE dimers were found.

Dimer formation is dependent on the ABC transporter CcmA and CcmB

The first experiment was to answer the question of whether the absence of CcmA abolishes the formation of CcmE dimer even in the presence of all other Ccm proteins. The ΔccmA-H strain EC06 was transformed with either pEC86 (encoding CcmA-H), pEC94 (encoding CcmB-H) or pEC97 (encoding CcmA(K40D)-H). Membrane proteins isolated from aerobically grown cells were subjected to SDS-15% PAGE, followed by heme staining. As shown in Fig. 46 (upper panel), all three lanes showed a heme staining band specific for holo-CcmE (18 kDa). However, the amount of holo-CcmE monomer was decreased in membranes of cells expressing CcmB-H (lane 2) and cells expressing CcmA(K40D)-H (lane 3), indicating that a functional ABC transporter was somehow important for holo-CcmE formation. To analyze whether this decrease in holo-CcmE formation was due to a deficiency of heme incorporation or to a decreased level of CcmE polypeptide, an immunoblot was probed with antibodies against CcmE. As shown in Fig. 46 (lower panel), the levels of CcmE in strains expressing either CcmB-H (lane 2) or CcmA(K40D)-H (lane 3) were also decreased when compared with those from strains expressing
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CcmA-H (lane 1). The 36 kDa band reacted with the CcmE-specific antibodies, revealing the identity of this band as a CcmE dimer. The formation of the dimer was only observed in membranes isolated from strains expressing CcmA-H (lane 1), but not in membranes isolated from strains expressing the defective ABC transporter (lanes 2 and 3).

\[ \Delta ccmA-H \]

![Image of gel electrophoresis and immunoblot analysis](image_url)

**Figure 46.** CcmE dimer formation is dependent on CcmA. The \( \Delta ccmA-H \) strain EC06 was transformed with plasmid pEC86 (CcmA-H, lane 1), pEC94 (CcmB-H, lane 2) or pEC97 (CcmA(K40D)-H, lane 3). Membrane proteins were isolated from aerobically grown cells and subjected to SDS-15% PAGE. Upper panel, heme stain of membrane proteins (100 µg/lane). Lower panel, immunoblot (50 µg/lane) probed with antiserum against CcmE. The position of CcmE dimer and monomer are indicated on the right. The numbers on the left are molecular masses of marker proteins given in kilodaltons.

As the dimer was not observed in strains expressing mutant CcmA, it was important to analyze whether this was only due to decreased levels of CcmE. Different amounts of membrane proteins isolated from aerobically grown \( \Delta ccmA-H \) cells expressing either CcmA-H or CcmA(K40D)-H were subjected to SDS-15% PAGE, followed by immunoblot analysis probing with antibodies against CcmE. As shown in Fig. 47, CcmE dimer can already be seen when 5 µg membrane protein...
proteins from cells expressing CcmA-H were loaded (lane 2). No CcmE dimer could be detected in membrane proteins isolated from cells expressing CcmA(K40D)-H, even when up to 250 μg protein was loaded (lanes 5 to 9). This demonstrates that the observed dimer formation is not dependent on the level of CcmE. The point mutation K40D in CcmA abolishes not only the formation of holo-cytochrome c, but also that of CcmE dimer.

Figure 47. CcmE dimer formation is not dependent on the amount of protein. The ΔccmA-H strain EC06 was transformed with plasmid pEC86 (CcmA-H) or pEC97 (CcmA (K40D)-H). Membrane proteins were isolated from aerobically grown cells and subjected to SDS-15% PAGE and immunoblot analysis as follows. Membranes from the strain producing CcmA-H: 1 μg (lane 1); 5 μg (lane 2); 10 μg (lane 3); 20 μg (lane 4). Membranes from the strain producing CcmA (K40D)-H: 50 μg (lane 5); 100 μg (lane 6); 150 μg (lane 7); 200 μg (lane 8); 250 μg (lane 9). For detection, antiserum against CcmE was used. The position of CcmE dimer and monomer are indicated on the right. The numbers on the left are molecular masses of marker proteins given in kilodaltons.

Dimer formation is also dependent on CcmC

The next experiment was done to investigate whether dimer formation of CcmE is dependent on other Ccm proteins. Several plasmids were constructed from which combinations of CcmA-E proteins were expressed from the same vector (pACYC184). The ΔccmA-H strain EC06 was transformed with either pEC78 (ccmABCDE), pEC101 (ccmABCD), pEC102 (ccmACDE), pEC105 (ccmABDE), pEC406 (ccmCDE), pEC408 (ccmDE) or pEC410 (ccmE). Membrane proteins were isolated from aerobically grown cells and subjected to SDS-15% PAGE, followed by heme staining. In addition, an immunoblot was probed with antibodies against CcmE. Holo-
Results

CcmE formation was only observed in the presence of CcmC (Fig. 48, upper panel, lanes 1, 3 and 5), which is in agreement with previous reports (Schulz et al., 1999).

Figure 48. CcmE dimer is only observed when CcmA, CcmB and CcmC are coexpressed. The ΔccmA-H strain EC06 was transformed with plasmid pEC78 (CcmABCD, lane 1), pEC101 (CcmABCD, lane 2), pEC102 (CcmACDE, lane 3), pEC105 (CcmABDE, lane 4), pEC406 (CcmCDE, lane 5), pEC408 (CmDE, lane 6) or pEC410 (CcmE, lane 7). The membrane proteins were isolated from aerobically grown cells and separated by SDS-15% PAGE. Upper panel, heme stain of membrane proteins (100 µg/lane). Lower panel, immunoblot of membrane proteins (50 µg/lane) probed with antiserum against CcmE. The position of CcmE dimer and monomer are indicated on the right. The numbers on the left are molecular masses of marker proteins given in kilodaltons.

CcmE dimer was only observed in the presence of CcmA, CcmB and CcmC (Fig. 48, upper and lower panel, lane 1), indicating that besides the role of the ABC transporter CcmAB in formation of CcmE dimer a role of CcmC in this process must be postulated.
Growth conditions influence CcmE dimer formation

From early experiments, where dimers had been observed under some conditions but not under others, it appeared that growth conditions, and in particular the presence or absence of oxygen, might influence the relative amounts of dimers. Hence, a systematic analysis of CcmE dimer formation under different growth conditions was conducted. The ΔccmA-H strain EC06 expressing different constructs was grown either under anaerobic conditions, supplemented with 5 mM nitrite, or under aerobic conditions. Membrane proteins were subjected to SDS-15% PAGE, followed by heme stain and immunoblot probed with antibodies against CcmE. The results of this comparative study are presented in Table III. The strongest signals for holo-CcmE monomer were observed in membranes containing overexpressed CcmABCDE or CcmA-H, while weaker signals were obtained in membranes containing either CcmCDE or CcmACDE. Also, the absence of functional CcmA in the membranes led to a significant decrease in holo-CcmE monomer formation. No apparent difference was observed for monomers when membranes isolated from aerobically grown strains were compared to those from anaerobically grown strains. Holo-CcmE dimers were observed only in membranes containing CcmABCDE or CcmA-H. Membranes isolated from aerobically grown cells showed stronger dimer signals. Interestingly, the formation of holo-CcmE dimer was below the limit of detection in membranes from cells grown anaerobically in LB.

In the immunoblot, in all membranes, except for membranes of strains expressing ccmA alone or ccmABCD (without ccmE), CcmE monomer was found (Table III). Again, the strongest signals were found in the membranes of strains expressing either ccmABCDE or ccmA-H. Also, CcmE dimer was only observed in membranes of strains expressing either ccmABCDE or ccmA-H. In membranes of strains grown anaerobically in LB and expressing ccmABCDE or ccmA-H, only weak signals were obtained for CcmE dimers. Thus, the absence of a signal for holo-CcmE dimer in these membranes was most probably due to low protein levels according to the immunoblot. In summary, the ratio of dimer to monomer was highest when cells were grown aerobically in LB medium. Under anaerobic conditions, more dimer was formed when cells were grown in MS as compared to LB medium. Hence, there is an effect of both oxidative conditions and media composition that influences dimer formation.
### Results

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<thead>
<tr>
<th>Strains/Genotype</th>
<th>Monomer</th>
<th>Dimer</th>
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<tr>
<td>Strains/Genotype</td>
<td>MS anaerobic</td>
<td>LB anaerobic</td>
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<tr>
<td>Strains/Genotype</td>
<td>Holo-E</td>
<td>CcmE</td>
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<td>EC06/pEC410 ccmE</td>
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Table III. CcmE monomer and dimer formation under different growth conditions. Membrane proteins were isolated from aerobically and anaerobically grown ΔccmA-H strain EC06 expressing different constructs as shown in the left column. To investigate holo-CcmE formation, 100 μg of membrane proteins were separated by SDS-15% PAGE, followed by heme stain. To investigate CcmE monomer and dimer formation, 50 μg of membrane proteins were separated by SDS-15% PAGE, followed by immunoblot probed with antiserum against CcmE. + is a qualitative measure for signal intensities. MS: minimal salt media supplemented with 5 mM nitrite. LB: Luria-Bertani media.

**CcmE dimer formation is dependent on heme incorporation**

As shown before, CcmE dimer formation is not only dependent on the ABC transporter CcmA and CcmB but also on CcmC. Schulz et al. have shown that CcmC delivers heme to CcmE (Schulz et al., 1999). Therefore, it was possible that CcmE dimer formation might also be dependent on heme incorporation into CcmE. To assess the question of whether heme is required for dimer formation, a ΔhemA mutant strain was used, which is unable to synthesize the heme precursor 5-aminolevulinic acid (δ-ALA) and thus cannot incorporate heme into CcmE.
Characterization of CcmE dimer

The ΔhemA ΔccmE strain, carrying pEC78 (ccmABCDE), was grown anaerobically in LB supplemented with 20% glucose in the presence or absence of 50 μg/ml δ-ALA. Membrane proteins were isolated, and different amounts were subjected to SDS-15% PAGE, followed by immunoblot analysis probing with antibodies against CcmE. As shown in Fig. 48, dimer formation was already observed with 10 μg membrane proteins from strains supplemented with δ-ALA (Fig. 48, lane 1). No dimer formation was observed in membranes of strains not supplemented with δ-ALA, even with increasing amounts of membranes (Fig. 48, lanes 5 to 8), indicating the dependence of CcmE dimer formation on heme incorporation.

If the dimer is formed only in the presence of heme, then no dimer should be observed when CcmE is produced in its apo-form. To purify the CcmE protein in a soluble form, the DNA encoding the first 29 amino acids, representing the membrane anchor, was replaced by the DNA encoding the 21 N-terminal amino acids of the outer membrane protein (Schulz et al., 1998). The polypeptide with the OmpA signal sequence is targeted to the membrane via the general secretion pathway.
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machinery (Sec) which translocates the protein across the cytoplasmic membrane. The leader peptidase (Lep) cleaves the signal sequence, thereby releasing the CcmE-His\textsubscript{6} into the periplasm. The \( \Delta ccmA-H \) strain EC06 carrying pEC415 (producing CcmE\textsubscript{sol} -His\textsubscript{6}) was transformed with either empty plasmid or pEC101 expressing \( ccmABCD \). The cells were grown aerobically, the periplasm was isolated and CcmE\textsubscript{sol} -His\textsubscript{6} was purified by Ni\textsuperscript{2+}-affinity chromatography.

The fractions containing CcmE\textsubscript{sol} -His\textsubscript{6} were pooled and different amounts of protein were separated by SDS-15% PAGE. The Coomassie stain showed that only in the presence of CcmABCD CcmE dimer is visible in a SDS polyacrylamide gel (Fig. 49, lanes 6 to 9). Clearly, CcmE dimer formation is only possible when CcmE is produced as a holo protein. This depends on heme incorporation, which is catalyzed at low levels by CcmC and stimulated by CcmAB and CcmD. If in fact CcmE dimer formation is dependent on heme, we can conclude that all dimers observed contain holo-CcmE only. Moreover, soluble CcmE does not contain any cysteines, whereas the membrane-bound CcmE has a cysteine at position 13, i.e. in the non-conserved membrane anchoring domain. The fact that the CcmE dimers are also observed with the soluble...
Characterization of CcmE dimer

protein excludes the possibility that the dimers are the result of intermolecular disulfide bond formation.

**CcmE dimer might be a prerequisite of cytochrome c formation**

The next question to be answered was whether dimer formation of CcmE is a prerequisite for cytochrome c maturation. It was shown previously that several point mutants of CcmE were able to form cytochrome c with one exception: the point mutation at residue H130 (Schulz et al., 1998). However, at that time no attention had been drawn to the dimers. In this work, these mutant constructs were used to investigate dimer formation. The ΔccmA-H strain EC06 carrying the plasmid pEC101 (ccmABCD) was transformed with the plasmids expressing constitutively different variants of CcmE. Cells were grown under aerobic conditions and membrane proteins were isolated and separated by SDS-15% PAGE, followed by heme staining. An immunoblot was probed with antibodies against CcmE. As expected, only membranes containing the CcmE H130A variant were unable to form holo-CcmE (Fig. 50, upper panel, lane 2). In addition, the immunoblot revealed that the H130A mutant was not able to form CcmE dimer (Fig. 50, middle panel, lane 2). All the other variants were not only able to form cytochrome c (Enggist et al., 2003) but also were capable of forming CcmE dimer despite the presence of apo-CcmE monomer (Fig. 50, middle and lower panel, lanes 4 to 8). In conclusion, it has been shown that CcmE dimer formation is dependent on the ABC transporter, on heme incorporation into CcmE with the help of CcmC and on heme binding to the H130 of CcmE. These results do not exclude the possibility that the formation of CcmE dimer is essential for cytochrome c maturation.
Results

Figure 50. Dimer formation in CcmE point mutants. The ccmA-H deletion strain EC06 was co-transformed with plasmid pEC101 (CcmABCD) and pEC458 (wild-type CcmE; lane 1), pEC467 (CcmE(H130A); lane 2), pBR322 (empty plasmid; lane 3), pEC472 (CcmE(K129A); lane 4), pEC493 (CcmE(Y95A); lane 5), pEC494 (CcmE(F103A); lane 6), pEC495 (CcmE(L127A); lane 7) or pEC499 (CcmE(D131A E132A); lane 8). Cells were grown aerobically and membrane proteins were isolated. Upper panel, heme stain of membrane proteins (100 µg/lane) after separation by SDS-15% PAGE. The position of holo-CcmE is indicated on the right. Middle panel, immunoblot of membrane proteins (50 µg/lane) probed with antiserum against CcmE. The position of CcmE dimer and monomer are indicated on the right. The numbers on the left are molecular masses of marker proteins given in kilodaltons. Lower panel, cytochrome c deficiency (-) and proficiency (+).

Stability of CcmE dimer

As CcmE runs as a dimer in a denaturing SDS-polyacrylamide gel, it was of interest to know the nature and stability of this form. A first attempt was made to investigate whether specific treatments could destroy the dimer. The ΔccmA-H strain EC06 was transformed with pEC86 (ccmA-H) and was grown aerobically. Membrane proteins were isolated and either boiled or subjected for at least 10 minutes to the following treatments: i) 100% TCA, ii) methanol, iii) 6 M urea, iv) 1 M DTT, v) 1 M APS, vi) 1% SDS, vii) 1 M EDTA, viii) 1 M NaOH. The samples were precipitated with TCA, resuspended in 2x SDS loading dye with 20 mM DTT and were separated by SDS-15% PAGE, followed by immunoblot analysis using antibodies against CcmE. Figure 51 shows the results of some of the treatments. None of the treatments destroyed the
Characterization of CcmE dimer

dimers, suggesting a strong interaction between two CcmE molecules, probably via a covalent bond.

Figure 51. Attempts to dissociate the CcmE dimer. The ΔccmA-H strain EC06 was transformed with plasmid pEC86 (ccmA-H). Membrane proteins were isolated from aerobically grown cells. 100 μg (3 μl) membrane proteins were treated as follows for 10 minutes and separated by SDS-15% PAGE, followed by immunoblot probed with antiserum against CcmE. Lane 1, untreated membrane proteins; lane 2, 100 μl of 100% TCA; lane 3, 100 μl of 100% methanol; lane 4, 100 μl of 6 M urea; lane 5, heat at 100°C. The position of CcmE dimer and monomer are indicated on the right. The numbers on the left are molecular masses of marker proteins given in kilodaltons.
Purification and characterization of CcmE dimer

It was previously shown that heme needs to be attached for CcmE to form dimer. Two main models are considered feasible for the formation of CcmE dimers and are shown in Fig 52. One model involves the covalent attachment of two CcmE polypeptides to a single heme by the heme vinyl groups. The second model proposes that two holo-CcmE molecules are attached to each other via the two hemes involving heme:heme stacking or another strong interaction.

Fig. 53 shows the amino acid sequence and its main features. As the two models predict different sizes of the products, it was of interest to calculate the molecular weights.

To biochemically characterize the CcmE dimer, it was produced from the ccm deletion strain EC06 carrying pEC415 (ccmE-His6) and pEC101 (ccmABCD). Cells of 6 liters of culture were grown aerobically and induced with 0.1% arabinose to express soluble CcmE-His6. The CcmE-His6 protein was purified from the periplasm by Ni²⁺ affinity chromatography and the eluted fractions containing CcmE-His6 were pooled and concentrated, yielding 50 mg of pure protein. This preparation was used to purify the dimer.

The theoretical molecular weight of apo-CcmE-His6 monomer is 15325 Da, and that of holo-CcmE-His6 monomer is 15942 Da. These forms are not separable by SDS-PAGE. The dimer is
Characterization of CcmE dimer

predicted to have a molecular weight of 31267 Da or 31884 Da for one or two hemes per dimer, respectively.

MKKTAIAIAVALAGFATVAQALRSNIDLFYTPGEILYGKRETQQMPE
VGQRLRVGGMVPGSVQRDPNSLKVTFTIYDAEGSVDVSYEGILP
DLFREGQQGVVVQGELEKGNHILAKEVLAKDENYTPPEVEKAME
ANHRRPASYVDPA SHHHHHHH

Figure 53. Amino acid sequence of soluble CcmE-His. The underlined amino acid residues indicate the cleavable OmpA signal sequence. The highlighted H is the conserved Histidine at position 130 of CcmE responsible for heme binding.

Therefore, it was expected that monomers and dimers could be separated by gel filtration. A Superdex 75 Prepgrade column was selected because of its high resolution for the separation of globular proteins in the fractionation range of 3 to 70 kDa and for its good recovery. The absorption profile during purification at 280 nm and 415 nm for the detection of protein and heme, respectively, is shown in Fig. 54A. Approximately 10 µg of the peak fractions were analyzed by SDS-15% PAGE (Fig. 54 B). The elution fraction A12 (lane 2) contained increased amounts of dimer compared to the purified protein before gel filtration (lane 4), but was still contaminated with monomer. Therefore, the elution fraction A12 was concentrated to 100 µl (2.5 mg) and subjected a second time to gel filtration at a lower flow rate and smaller fractions were collected to obtain better resolution. Samples of 15 µl of peak fractions, of affinity purified protein and of the concentrated A12 fraction of the first gel filtration were subjected to SDS-15% PAGE. The Coomassie stain presented in Fig 55 B shows that after the second gel size exclusion column no monomer band was visible in the fraction D4 (lane 1), D8 (lane 2) or E6 (lane 4). The peak fraction E11 (lane 5) contained only monomer whereas the D14 fraction consisted of almost
Results

pure CcmE dimer (lane 3). This was confirmed in an immunoblot, where traces of monomer were detected in fraction D14 and traces of dimer in fraction E11 (data not shown).

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Figure S4. Separation of soluble CcmE-His$_6$ dimer from monomer by size exclusion chromatography. 50 mg of Ni$^{2+}$-affinity purified soluble CcmE-His$_6$ was loaded onto a 16/60 Superdex 75 prepgrade column. After loading, the column was washed with 1.5 column volumes (200 ml) and 5 ml fractions were collected at 1 ml/min. 

A, Elution profile. The absorption at 280 nm and 415 nm are indicated. A7-B6 corresponds to the elution fractions. The elution volume is indicated in ml. Peak 1, elution fraction A11; peak 2, elution fraction A12; peak 3, elution fraction B2 + B3; B, Coomassie stain. 10 µg protein of the peak fractions were loaded onto a SDS-15% PAGE. Lane 1, elution fraction A11; lane 2, elution fraction A12; lane 3, elution fraction B2; lane 4, affinity purified CcmE-His$_6$ before gel filtration (Load). The position of CcmE dimer and monomer are indicated on the right. The numbers on the left are molecular masses of marker proteins given in kilodaltons.
Characterization of CcmE dimer

Figure 55. Separation of soluble CcmE-His$_6$ dimer from monomer by a second size exclusion chromatography step. 2.5 mg of soluble CcmE-His$_6$ obtained from peak fraction 2 (A12) of the first gel size exclusion chromatography was loaded onto a 16/60 Superdex 75 prepgrade column. After loading, the column was washed with 1.5 column volumes (200 ml) and 1 ml fractions were collected. A, Elution profile. The absorption at 280 nm and 415 nm are indicated. C8-F9 corresponds to the elution fractions. Peak 1, elution fraction D4; peak 2, elution fraction D8; peak 3, elution fraction D14; peak 4, elution fraction E6; peak 5, elution fraction E11. B, Coomassie stain. 15 µl of the peak fraction were loaded onto a SDS-15% PAGE. Lane 1, elution fraction D4, 2.4 µg protein; lane 2, elution fraction D8, 2.7 µg protein; lane 3, elution fraction D14, 5.7 µg protein; lane 4, elution fraction E6, 0.0 µg protein; lane 5, elution fraction E11, 1.65 µg protein; lane 6, affinity purified CcmE-His$_6$ before first gel filtration, 750 µg protein; lane 7, empty lane; lane 8, fraction A12 after first gel size exclusion chromatography, 375 µg protein. The position of CcmE dimer and monomer are indicated on the right. The numbers on the left are molecular masses of marker proteins given in kilodaltons.
Results

Mass spectroscopy of CcmE dimer

In Fig. 56, a mass spectrum of the undigested fraction D14 is presented showing a peak of 31658.5 m/z. This mass corresponds neither to the calculated mass for a dimer consisting of two holo-CcmE (31884 Da) nor to a dimer consisting of two CcmE polypeptides with one heme (31267 Da). Calculations for possible degradation either at the N-terminus or at the C-terminus were made for soluble CcmE-His6. Indeed, when removal of the first leucine (L22) at the N-terminus is assumed, then the calculated mass of two holo-CcmE(R23-S151)-His6 is 31658.08 Da and corresponds very accurately to the observed mass of 31658.5 m/z. The conclusion was therefore that the dimer consists of two holo-CcmE polypeptides, missing the two N-terminal leucine residues.

![Mass spectrum of the CcmE dimer.](image)

**Figure 56. Mass spectrum of the CcmE dimer.** The observed mass is 31658.529 m/z. The calculated mass is for CcmE: 15325 Da, for CcmE-heme: 15942 Da, for CcmE-heme-CcmE: 31267 Da and for CcmE-heme-heme-CcmE: 31884 Da. When a dimer is expected to consist of two CcmE polypeptides and two heme, but the first leucine at the N-terminus is degraded, and then a mass of 31658.08 Da is calculated.
Chapter IV

Discussion
Discussion

In *E. coli*, all eight genes of the *ccm* operon are essential for cytochrome *c* formation. This study concerns the characterization of the role of the two genes *ccmA* and *ccmB*, which encode a putative ABC transporter whose function is not yet understood. From initial studies on the *ccmABC* homologues *helABC* in *R. capsulatus* and *B. japonicum* these genes were found to be essential for the biogenesis of cytochrome *c* and sequence analysis suggested that they encode the subunits of an ABC transporter (Ramseier et al., 1991; Beckman et al., 1992). Because heme was the most obvious substrate for an ABC transporter involved in cytochrome *c* biogenesis, the *R. capsulatus* transporter was named “Hel”, initially standing for “heme ligation”, later for “heme export for ligation” (Goldman et al., 1997). Unfortunately, despite the lack of experimental proof, the function of the transporter is firmly established in the literature and in databases as “heme transporter”. Thus, an important challenge for the field of cytochrome *c* biogenesis started already more than a decade ago, namely to define the structure of the transporter and the substrate that is transported during cytochrome *c* biogenesis.

A genetic system to investigate the function of CcmAB

A genetic system based on complementation of in frame gene deletion mutants needed to be established for the functional analysis of *ccmA* and *ccmB*. While it was possible to complement a Δ*ccmA* mutant with a plasmid-borne *ccmA* gene, the analogous experiment of complementing a Δ*ccmB* mutant with *ccmB* failed. The combination of the Δ*ccmA* and Δ*ccmB* mutants with a Δ*dms* allele allowed the use of a direct colony assay to detect cytochrome *c* formation. The Δ*ccmA*Δ*dms* strain was not able to grow anaerobically on MS plates containing TMAO as terminal electron acceptor as expected from previous work (Ahuja and Thöny-Meyer, 2003; Thöny-Meyer et al., 1995). When the strain Δ*ccmA*Δ*dms* was complemented with a plasmid-borne copy of *ccmA*, cytochrome *c* formation was restored as indicated by colony growth. Again, the Δ*ccmB*Δ*dms* strain was not able to grow on TMAO plates when transformed with a plasmid-
Attempts to identify the substrate of CcmAB

borne copy of ccmB or with a plasmid-borne copy of ccmAB. It was known from previous work that the in-frame deletion of ccmB was not polar on the expression of the genes located downstream of ccmB, because in a strain with a co-integrated ccmA gene expressed from a stronger promoter in the ΔccmB background, a ccmA-H transcript with the partially deleted ccmB gene gave rise to detectable levels of CcmE protein (Henk Schulz and Linda Thöny-Meyer, unpublished work). Moreover, even overexpression of ccmAB or ccmB in the wild-type strain MC1061 led to loss of cytochrome c maturation, indicating a dominant-negative effect of overexpressed ccmB. Cytochrome c maturation could be rescued by co-expression of ccmB with ccmA and ccmC, which indicates that the overexpressed CcmB might sequester CcmC, thereby preventing CcmC from delivering heme to the heme chaperone CcmE.

The finding that complementation of the ΔccmB mutant could be achieved only with coexpression of all these genes, i.e. ccmA, ccmB and ccmC, led to the decision to use site-directed rather than random mutagenesis of ccmB to identify important amino acid residues of CcmB. Conserved and possibly important residues of CcmB were determined by topology prediction of the E. coli CcmB protein and sequence alignment with its homologues in other bacteria. Many hydrophobic residues (I, L, V) were found to be conserved. These residues probably have a structural role for folding of the protein in the membrane. However, the possibility that these residues form a channel for a hydrophobic substrate cannot be ruled out. If these residues would be mutated to alanine, then the hydrophobic channel still might exist. Mutations of these residues to hydrophilic amino acid residues would disable the transport of a hydrophobic substrate, but might also increase the risk of improper folding of CcmB in the membrane. Many conserved amino acid residues are found to be located in the first cytoplasmic loop of CcmB, indicating a role in the interaction with CcmA. From structural analysis, it is generally accepted that the first cytoplasmic loop in the transmembrane subunit is needed to establish specific contacts with the nucleotide binding subunit (Locher and Borths, 2004). The corresponding region of the transmembrane subunit in MsbA, a protein involved in lipid A transport, has indeed been found to be in contact with the ABC cassette in the recently published crystal structure (Chang and Roth, 2001). Mutations introduced by site directed mutagenesis of the maltose transporter transmembrane subunits (MalF and MalG) in the corresponding cytoplasmic loop were shown to result in loss of the nucleotide binding subunit (MalK). Interestingly, MalK mutants could be found which not only restored MalFGK2 assembly but also maltose transport. Furthermore, some
mutations in either MalF or MalG abolished maltose transport, while MalK was still assembled (Mourez et al., 1997). In the cytoplasmic domain of CcmB mainly negatively charged residues (D and E) are conserved, but in addition, an aromatic residue (F) was also found to be conserved. Two conserved tryptophane residues were found in the second and third transmembrane segment of CcmB. Such residues were shown to be important in the proteins CcmC and CcmF (Schulz et al., 2000; Ren et al., 2002) and in the CcsA protein of system II (Hamel et al., 2003) which all contain a tryptophane-rich motif on the periplasmic side of the membrane. The conserved H100 in CcmB is located next to the cytoplasmic loop. Many heme binding proteins are known to require histidines as heme ligands (Schulz et al., 1998; Ren and Thöny-Meyer, 2001). No strictly conserved sequence motif was found in all CcmB homologues, indicating that the substrate binding site might be defined over the three dimensional structure rather than by distinct amino acid residues. This is in agreement with previous reports that ABC transporters in general are defined over their nucleotide binding domain, but show only weakly conserved regions in their transmembrane domains (Linton and Higgins, 1998; Locher et al., 2002). Site directed mutagenesis of the conserved residues in CcmB (F28A, W55A, R86A, F70A, D73A, D76A, E80A, K96A, H100A, W101A, F175A) was done in a plasmid encoding ccmA, ccmB and ccmC. None of these mutations showed an effect, neither in cytochrome c formation, nor in holo-CcmE formation, in stability of CcmB or in interaction with the nucleotide binding subunit CcmA. This indicated that either these mutations do not have an essential role in maintaining the function of CcmB or that the function of these residues is not affected when they are substituted by alanine. Therefore, multiple mutations in CcmB might result in an altered phenotype. Alternatively, if the conserved amino acid residues were mutated to amino acid residues with more significant differences in their physico-chemical properties, an effect might be observed. For example, mutation of the positively charged residues arginine or lysine in the cytoplasmic loop of CcmB to the negatively charged residues aspartate or glutamate could have a more severe effect and might interfere with the interaction of CcmB with CcmA.

As overexpressed ccmB causes a dominant negative phenotype, it was of interest to investigate whether this effect can be relieved by mutations of the conserved amino acids of CcmB. Indeed, three mutations (R68A, D73A and D76A) suppressed the dominant negative phenotype. All of them were located in the cytoplasmic domain, which is believed to bind CcmA. The meaning of
Attempts to identify the substrate of CcmAB

this observation is not clear and may have to do with the assembly of super complexes containing many Ccm proteins (see below).

CcmA functions as an ATPase

Sequence analysis of CcmA revealed the three conserved motifs (Walker A, Walker B and the C motif), which are known to be characteristic for the nucleotide binding domains of ABC transporters. Further analysis of CcmA showed that the mutation of K to D in the Walker A motif abolished cytochrome c formation. This indicated that the mutation affects ATP hydrolysis. Mutation of this residue in other ABC transporters also severely affected ATP hydrolysis (Azzaria et al., 1989; Davidson and Sharma, 1997; Lapinski et al., 2001), indicating the importance of ATP hydrolysis for active transport. A similar mutation in the CcmA homologue HelA of R. capsulatus, where a conserved residue of the C-motif that is also involved in nucleotide binding had been exchanged, abolished cytochrome c formation (Goldman et al., 1997). In this work, CcmA and CcmB were co-purified and the preparation indeed showed ATP hydrolysis activity of the wild-type but not of the mutant enzyme, confirming that CcmA is the nucleotide binding subunit of an ABC transporter.

Subunit composition

One important goal of this work was to identify the subunit composition of the ABC transporter. Initially, Goldman et al. postulated, that the ABC transporter of Rhodobacter capsulatus consists of CcmA, CcmB and CcmC with CcmD as an accessory protein (Goldman et al., 1997). These results were obtained by studies on the membrane association of CcmA in the presence or absence of the putative transmembrane subunits. In their experiment, CcmA only associated with the membrane when both, CcmB and CcmC, were present, indicating that CcmA interacts with
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CcmB and CcmC. Furthermore, co-immunoprecipitation experiments, where CcmC was precipitated and CcmA was detected, further indicated that CcmC interacts with CcmA. This is in contradiction to the results obtained in this study. Experiments presented in this work showed that CcmA was only associated with the membrane when CcmB, but not when CcmC alone was present in the membrane. This indicates that the ABC transporter assembles to a complex consisting of CcmA and CcmB, but not of CcmA, CcmB and CcmC. This model is further supported by the study of Schulz et al., where overexpressed CcmC was shown to incorporate heme into CcmE in the absence of CcmA and CcmB (Schulz et al., 1999). In addition, Page et al. described a phenotype of a P. denitrificans ccmC mutant strain to be different from those of either ccmA and ccmB mutant strains (Page and Ferguson, 1999). This observation supports the model of an ABC transporter consisting of CcmAB only. In this work, CcmC was also shown to interact with CcmB in a pull down experiment, indicating that CcmC is interacting with CcmB to some extent. The finding of Goldman et al. might be explained by assuming that the absence of HelC leads to an unstable HelB protein in the membrane of R. capsulatus, leading to a loss of HelA in the membrane. In this case, CcmC is needed for the stability of the whole complex rather than as a subunit of the ABC transporter.

Further evidence that the ABC transporter consists of the subunits CcmA and CcmB, is that purified CcmA and CcmB have ATPase activity in the absence of CcmC.

Another support for the CcmAB model can be taken from the two topology models of CcmB and CcmC (Ahuja and Thöny-Meyer, 2003 and this work). CcmB possesses a large cytoplasmic loop (~33 amino acid residues) with conserved residues between the second and third transmembrane segment, while the protein CcmC only contains small cytoplasmic loops (~8 amino acid residues) with non-conserved residues, thus making them very unlikely to interact with CcmA. Attempts to show that the cytoplasmic loop of CcmB interacts with CcmA could be to either delete this cytoplasmic region or to replace more than one charged amino acid residue in the cytoplasmic region at a time.

Last, Goldman showed that the CcmD homologue HelD co-precipitated with HelA (Goldman et al., 1997). However, later work of these authors does not refer to this result anymore. The E.coli HelD homologue CcmD interacts with the HelC homologue CcmC (Ahuja and Thöny-Meyer, 2005). This work shows that CcmB interacts with CcmC and CcmA. Therefore, a larger complex
Attempts to identify the substrate of CcmAB consisting of CcmABCD may be formed, either transiently or at steady state levels. Nevertheless, the true ABC transporter is most likely a complex of CcmA₂B₂ with CcmC and perhaps CcmD as accessory subunits.

The mechanism of substrate translocation in ABC transporters is generally assumed to occur via conformational changes of the transmembrane subunit upon ATP hydrolysis of the nucleotide binding domain (Davidson, 2002). Thus, the relieve of the dominant negative phenotype of overexpressed CcmB by mutations in the cytoplasmic domain might be explained as follows: first, the point mutated residues induce an overall conformational change of CcmB, so that possible amino acid residues of CcmB that normally interact with CcmC are no longer accessible for CcmC. The question, why these mutations do not abolish cytochrome c formation, remains to be answered. Even though the cytoplasmic loop of CcmB is envisaged to be the site of interaction with CcmA, the possibility cannot be ruled out, that CcmC also interacts with the cytoplasmic loop of CcmB. Second, the mutations in CcmB might weaken the interaction with CcmC, enabling CcmC to readopt its function in heme delivery that is blocked by overexpressed wild-type CcmB. Third, overproduced CcmB might interact with CcmE, thereby blocking the transfer of heme to CcmE, which is catalyzed by CcmC. It was shown that CcmE dimer formation is dependent on CcmAB, and that ATP hydrolysis activity is stimulated by CcmE. The mutation of CcmB might weaken the interaction of CcmB with CcmE so that CcmC can transfer heme to CcmE. Obviously, it would be interesting to test an interaction of CcmB with CcmE experimentally, for example by co-immunoprecipitation.
Discussion

Attempts to identify the substrate of CcmAB

Is heme the substrate?

Since the discovery of an ABC transporter involved in cytochrome c maturation it was postulated that this transporter uses heme as its substrate. However, all experiments so far failed to support this assumption. Throne-Holst et al. showed that a ccmA mutant still was able to form periplasmic b-type cytochromes (Throne-Holst et al., 1997). Furthermore, Schulz reported that heme can be transferred to CcmE in ccmA mutants when CcmC was overproduced (Schulz et al., 1999). However, as it was still not undisputedly clear whether or not CcmAB transport heme, several approaches were tried to answer this question.

The plasmid pJRH1 (Reidl and Mekalanos, 1996) was previously reported to enable E. coli to take up heme from the extracellular medium. Thus, it was of interest to see whether a ccmA mutant can be complemented by heme. A ΔhemA strain, deficient in heme biosynthesis, carrying pJRH1 was complemented with heme, but the ΔccmAΔdms strain carrying pJRH1, was not able to grow on TMAO plates supplemented with heme. This points to an inability of heme to complement a ccmA mutant for cytochrome c formation, which is in agreement with previous observations in Paracoccus denitrificans. Page et al. used the P. denitrificans ccmA and ccmB deletion strains to investigate cytochrome c formation by complementing these strains with extracellular heme, but could not detect holo-cytochrome c (Page et al., 1997). However, they were not able to show that heme passes the outer membrane. By contrast, in our experiments heme was shown to pass the outer membrane with the help of the plasmid pJRH1. As heme is toxic for the cell and is rarely found free in the cell, it is very unlikely that heme is translocated across the membrane and then is released unbound into the periplasm. It is more reasonable that heme is taken up directly into the cytoplasm and then is transferred following its normal route to CcmC or CcmE, conceivably involving an ABC transporter, and then is further transferred to the cytochrome c. Alternatively, if heme can be released into the periplasm, it still might not be available to the Ccm machinery. Thus, the heme complementation experiment does not exclude the possibility of heme being the transported substrate. An analogous experiment done with the
Attempts to identify the substrate of CcmAB

*ccmC* mutant had also failed to show a dependence of heme transport by CcmC (Ren and Thöny-Meyer, 2001).

As in vivo complementation with heme was not successful, the ABC transporter consisting of CcmA and CcmB was purified. The ATPase activity of several purified ABC transporters has been characterized so far (Davidson and Nikaido, 1990; Liu et al., 1997; Doerrler and Raetz, 2002). Some of these were shown to have intrinsic ATPase activity when reconstituted in detergent solutions (Chen et al., 2001). This intrinsic ATPase activity could further be stimulated by adding the substrate (Liu et al., 1997; Doerrler and Raetz, 2002; Landmesser et al., 2002). The aim of this work was to reconstitute CcmA and CcmB in detergent solution, to measure its ATPase activity and to test whether this activity can be stimulated by heme. At least 57 different ABC transporters are found in the cytoplasmic membrane of *E. coli* (Linton and Higgins, 1998). To distinguish between contaminating ATPases and the purified ABC transporter, a plasmid was constructed which exhibits the same features as the plasmid expressing CcmAB but contains the mutation K40D in the Walker A motif of CcmA. This mutation was shown to abolish cytochrome c formation. Thus, the mutant transporter CcmA(K40D)B was the ideal control to distinguish between contaminant ATPases and the purified transporter. After solubilization and purification of wild-type and mutant proteins, ATPase activity was measured. Similar amounts of purified protein were compared. Only the wild-type CcmAB exhibited activity, whereas no activity was obtained for the mutant derivative, even with increasing amounts of protein. Further analysis of the wild-type protein complex CcmAB showed a specific ATP hydrolysis activity of 0.025 Units/mg protein. This activity is in the range of other activities observed in purified ABC transporters (0.1 to 0.01 Units/mg) (Liu et al., 1997; Landmesser et al., 2002). The activity of CcmAB might be increased by optimizing pH, salt and MgCl₂ concentrations. Although the activity measurements were not optimized, the assay was sufficiently sensitive to measure stimulation by possible substrates such as heme.
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Reduction of heme is reported to be a necessary step prior to the covalent attachment of heme to the apo-cytochrome c (Nicholson and Neupert, 1989). In addition, Daltrop et al. reported that apo-CcmE has a preference for ferric rather than ferrous heme for initial non-covalent binding, but only reduced heme leads to the formation of the covalent bond (Daltrop et al., 2002). Thus, reduced and oxidized heme was tested at different concentrations, but no stimulation was detected. This result is in agreement with the approach used by Cook and Poole (Cook and Poole, 2000). They isolated membrane vesicles of wild-type and the ccmA mutant strain and tried to detect differences in heme uptake, either in the presence or absence of ATP. However, no differences in heme uptake of membrane vesicles were observed between the strains, in the presence or absence of ATP. Again, these negative results support, but do not confirm, that CcmAB is not a heme transporter.

Three main models can be proposed for the heme translocation pathway: in the first model, heme diffuses passively through the membrane. Although the rate of diffusion might be too slow (Cook and Poole, 2000) in the presence of overexpressed cytochromes, this possibility cannot be excluded. Second, heme is taken up spontaneously by the membrane and associates with a specific binding protein, and third, heme is actively transported through the membrane, independent of CcmAB. It is possible that CcmC is such a heme transporter.

Ferrochelatase is the enzyme catalyzing the last step of heme biosynthesis and releases ferrous heme. The determination of interaction partners with the ferrochelatase HemH might give an insight into where heme is delivered to and thus an idea of the translocation pathway of heme. However, preliminary experiments could not confirm CcmB or CcmC as the possible interaction partners of ferrochelatase (data not shown).
Attempts to identify the substrate of CcmAB

Are redox-active compounds transported?

Another possible substrate for CcmAB tested in this work was a redox-active substrate. A possible substrate could be a reductant that keeps heme in the periplasm reduced for heme ligation, despite the oxidizing environment of the periplasm. Alternatively, oxidizing substances were considered because CcmE preferably binds oxidized heme in vitro (Daltrop et al., 2002). Nicholson and Neupert reported a dependence of holo-cytochrome c formation in the mitochondria on NADH and either FAD or FMN by the reduction of heme (Nicholson and Neupert, 1989). Thus, FAD and FMN were also tested, but no stimulation was observed. Several redox-active substances present in the cell were tested for their ability to stimulate the ATP hydrolysis, but none of these revealed any specific stimulation. This result is in agreement with the failure of complementation of a ΔccmA mutant for holo-cytochrome c formation by adding reductants to the medium (Schulz et al., 1999).

The possibility of CcmAB being a transporter for a redox-active substance should not be excluded completely as not all possible substrates were tested. Moreover, the coupled hydrolysis assay used in this work is not ideal to address this issue, as it is dependent on the oxidation of NADH. Redox-active substances could directly interfere with this assay, even though they were used at a much lower concentration than NADH, making it unlikely that they influence NADH oxidation. For determining stimulation by redox-active substances, an assay using radioactively-labeled ATP would be the method of choice (Walter et al., 1992).

No substrate?

Last, it should be mentioned that CcmAB might not transport a substrate at all, but rather have a different role in cytochrome c maturation. Several examples of proteins are described, which contain the common features of ABC transporters but do not show transport activity. For example, the SapABCDF proteins encode an ATP-binding cassette (ABC) transporter of unknown function which regulates the potassium uptake (Trk) system in E. coli, but not by the transport of a substrate (Harms et al., 2001). In addition, the cystic fibrosis conductance regulator
(CFTR) is a chloride channel, but not a transporter (Cotten et al., 1996). Finally, the UvrA protein resembles all the conserved motifs of an ABC transporter but is involved in DNA repair (Goosen and Moolenaar, 2001). Thus, the possibility of CcmAB being involved in a process different from transport activities should not be ruled out. Perhaps CcmAB simply transduces energy provided by ATP hydrolysis across the membrane via conformational changes, which triggers heme delivery on the periplasmic side of the membrane.

As CcmC strongly interacts with CcmB, one has to assume that functional ABC transporter activity can only be obtained, when CcmC and CcmE are present in the reaction mixture. If it can be shown, that CcmE indeed interacts with CcmB in vivo, which can be tested by co-immunoprecipitation or pull down experiments, then the next step would be to purify CcmA and CcmB together with CcmC and CcmE. Therefore, a reasonable attempt would be the copurification of CcmABCDE expressed from the same plasmid, thereby also avoiding any problems that may arise due to improper protein expression ratios. The purified CcmABCDE complex could then be used as a tool in search for the substrate of CcmAB, and maybe even for in vitro studies of holo-CcmE formation.
CcmAB are involved in CcmE dimer formation

CcmA and CcmB were previously reported to be involved not only in the maturation steps before heme incorporation to CcmE, but also afterwards (Schulz et al., 1999). Furthermore, a fraction of CcmE was observed to run as dimers in a denaturing SDS-polyacrylamide gel. This observation was particularly interesting in view of the fact that CcmE as well as CcmAB are hallmarks of system I cytochrome c maturation. Genomic analysis indicates that both the ABC transporter CcmAB and the heme chaperone CcmE are found exclusively in system I, whereas homologues of other Ccm proteins, for example CcmC, can also be found in the maturation system II. Thus, a physiological connection between CcmAB and CcmE seems likely.

When purified holo-CcmE was added to the ATPase assay, it stimulated ATP hydrolysis activity of purified CcmAB. The ATP hydrolysis activity was also slightly stimulated by the soluble CcmE protein, indicating that this stimulation is not dependent on the membrane-anchored part. As CcmAB activity was stimulated by an apo/holo CcmE mixture, it is of interest to know whether the ATP hydrolysis stimulation is due to the apo-form, the holo-form or the dimer form. Dimer formation was investigated, and it could be shown that this dimer formation is not an artifact produced by overexpressed proteins, but was dependent on the activity of the ABC transporter. This observation provided a new phenotype involving the ABC transporter and CcmE. It was speculated that this might give a hint to the substrate of the ABC transporter. The presence of CcmC, but also heme incorporation into CcmE were needed to obtain CcmE dimer. This is in agreement with the previous observation that CcmC was needed for heme incorporation into CcmE (Schulz et al., 1999). Purified apo-CcmE from cells lacking the other ccm genes did not contain even traces of dimers. Interestingly, cytochrome c formation was only observed when dimer formation of CcmE was present. All tested CcmE point mutants exhibited not only cytochrome c formation, but also CcmE dimer. The only mutant that was defective in cytochrome c maturation was also unable to form CcmE dimers. This mutant, CcmE H130A, was unable to bind heme. Thus, the presence of CcmE dimer might be a prerequisite of cytochrome c formation.

An interesting finding was the observation that the mutant CcmA(K40D), unable to hydrolyze ATP, was not only deficient in cytochrome c maturation but was also deficient in CcmE dimer.
formation. This suggests that the ABC transporter either directly interacts with CcmE or transports a substance needed for dimer formation.

What is the chemical bond of the dimer? As dimer formation was dependent on heme incorporation, and heme staining was observed for the dimers under denaturing conditions, the binding most likely occurred via the heme. However, it was not clear whether two hemes (Fig. 52 B) or one heme (Fig. 52 A) is involved in this dimer formation. The mass spectrum of purified dimers revealed a mass corresponding to the sum of two CcmE polypeptides lacking the first amino acid plus two hemes. This can be explained using the following models: the first model proposes a heme:heme stacking. Two holo-CcmE molecules are linked to each other via strong interactions between the two covalently bound hemes, which are flat hydrophobic molecules and may be placed on top of each other (Fig. 57 A). The preference of heme to aggregate in aqueous solution speaks for this model. However, it is not clear why such stacked molecules would not dissociate in a denaturing SDS polyacrylamide gel, at high pH, where heme is known to be soluble or during MALDI-TOF experiments.

The second model involves the covalent binding between the hemes of each holo-CcmE polypeptide (Fig. 57 B). The inability to dissociate the dimer and the dependence of an ABC transporter transporting a factor required to form this bond would be in agreement with such a model. The only possible reactive group in heme bound to CcmE is the free vinyl group of the heme. This hypothesis is attractive, as both vinyl groups have to be activated to get bound to the CXXCH motif of the apo-cytochrome c. However, there is no precedent in the literature known for this kind of heme-heme linkage.

The third model involves a covalent bond between two amino acid residues of each CcmE polypeptide (Fig. 57 C). Such a crosslink is not obvious, but could occur via specific amino acid side chains other than cysteine (as none of those are present in CcmE) or via a yet unknown crosslinking molecule. For both model B and C, a role of the ABC transporter is feasible.
CcmAB are involved in CcmE dimer formation

Figure 57. CcmE dimer models. A, two holo-CcmE proteins are linked to each other via stacking of their covalently bound hemes. B, the two holo-CcmE monomer are linked via covalent bonds between the free vinyl group of each heme. C, the two holo-CcmE monomer are linked via their amino acid residues. H130 forms the covalent bond between CcmE with one of the vinyl groups of heme (Lee et al., 2005). Squares represent the heme molecule with iron indicated in the center. The dashed line indicates a covalent bond between the amino acid residues of CcmE, perhaps with the help of an unknown cross-linker. No cysteines are present in the CcmE polypeptide.

To elucidate whether CcmE dimers are crosslinked via heme or via the amino acid residues, a tryptic digest of the isolated CcmE dimer was performed, followed by mass spectroscopy (data not shown). Among the identified peptides, none corresponded in mass to the expected heme-peptide of a dimer or to that of a heme-bound monomer.

Interestingly, in vitro studies with the monomeric holo-CcmE showed a release from CcmE and subsequent transfer to the apo-cytochrome c under reducing conditions, after at least 18 hours (Daltrop et al., 2002). The slow velocity of this in vitro reaction may indicate that not the monomer is the needed form for further transfer of heme to apo-cytochrome c, but rather the dimer. Since this work has shown that CcmE monomer and dimer can be separated on a preparative scale, the rate of heme transfer from the CcmE dimer to the apo-cytochrome c should now be compared with that of the monomer.

Understanding the chemical properties of the CcmE dimer may be the key to answer the question of the substrate of CcmAB. The tryptic digest of the CcmE dimer should be repeated with more protein, whereby the tryptic peptides should be purified by HPLC before characterization by mass spectroscopy. In addition, a full comparison of all tryptic peptides derived from pure
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monomer versus those derived from pure dimer might reveal an extra peptide in the dimer fraction, which contains the intermolecular crosslink.

Conclusion

What is the substrate of the ABC transporter encoded by ccmAB? It appears from these studies that besides the compounds heme and apo-cytochrome c transported during cytochrome c maturation, there may be a requirement for an additional component transported by CcmAB that has not yet been identified. This component is directly involved in heme attachment to and from CcmE and/or dimer formation. At present, the question of what kind of molecule, if any at all, is transported by CcmAB remains open.
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