The Active-Site Cysteines of the Periplasmic Thioredoxin-Like Protein CcmG of Escherichia coli Are Important but Not Essential for Cytochrome c Maturation In Vivo

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Received 27 October 1997/Accepted 3 February 1998

A new member of the family of periplasmic protein thiol-disulfide oxidoreductases, CcmG (also called DsbE), was characterized with regard to its role in cytochrome c maturation in Escherichia coli. The CcmG protein was shown to be membrane bound, facing the periplasm with its C-terminal, hydrophilic domain. A chromosomal, nonpolar in-frame deletion in ccmG resulted in the complete absence of all c-type cytochromes. Replacement of either one or both of the two cysteine residues of the predicted active site in CcmG (WCPTC) led to low but detectable levels of Bradyrhizobium japonicum holocytochrome c550 expressed in E. coli. This defect, but not that of the ccmG null mutant, could be complemented by adding low-molecular-weight thiol compounds to growing cells, which is in agreement with a reducing function for CcmG.

Covalent ligation of heme to apocytochrome c is a common feature of all c-type cytochromes. The binding occurs via two thioether bonds formed between the vinyl groups of heme and the cysteine residues of the conserved motif CXYCH of apocytochrome c. Although maturation of c-type cytochromes takes place in the oxidizing environment of the periplasm, the cysteines of apocytochrome c are expected to be in the reduced dithiol form before heme is attached.

Studies devoted to cytochrome c biogenesis in Escherichia coli led to the identification of the ccm (cytochrome c maturation) genes (8, 27), which are located in the aeg-46.5 operon (19, 21) together with the nap genes encoding the periplasmic nitrate reductase (Fig. 1A). The ccm genes encode eight proteins (CcmA, CcmB, CcmC, CcmD, CcmE, CcmF, CcmG, and CcmH) with high sequence similarity to other, previously identified bacterial proteins involved in cytochrome c biogenesis (1–7, 11, 16–18, 20, 23, 24, 30, 31). Their relevance for cytochrome c biogenesis in E. coli was shown by construction and analysis of a ccm null mutant that failed to produce mature cytochrome c (8, 27). The Ccm proteins include the subunits of an ATP-binding cassette transporter and of a putative cytochrome c-heme lyase complex and a thioredoxin-like protein. The E. coli thioredoxin-like protein is encoded by ccmG and has also been designated DsbE (14, 21). An in vivo complementation of a cytochrome c mutation has been shown experimentally for the homologous proteins HeIX of Rhodobacter capsulatus (2), CycY of Rhizobium leguminosarum (30), CycY of Bradyrhizobium japonicum (5), and CcmG of Pasteurella dentrificans (17).

Characteristic features of the CcmG subfamily of thioredoxin-like proteins are not only the WCXYC motif representing the active site of the protein but also a hydrophobic N-terminal and the well-conserved segment GVYGAPETF in the C-terminal part of the protein (residues 139 to 147 [5, 26]). Because of their thioredoxin-like active site and their involvement in cytochrome c maturation, CcmG homologs have been suggested to function as protein thiol-disulfide oxidoreductases in a cytochrome c-specific dithiol reduction pathway that reduces apocytochrome c for heme attachment.

In this work, we present direct biochemical evidence that ccmG codes for a periplasmic, membrane-bound thioredoxin. Having constructed a nonpolar ccmG deletion mutant, we show that CcmG is essential for cytochrome c maturation. We also report on the characterization of mutants containing replacements of the cysteines in the active-site motif WCXYC, which indicates a reducing function for the two cysteine residues in a pathway for maturation of c-type cytochromes.

Construction and characterization of a mutant carrying a nonpolar in-frame deletion in ccmG. It was shown previously that a deletion removing the genes ccmA to ccmH from the E. coli chromosome resulted in the loss of mature c-type cytochromes (8, 27). Here, we investigated the role of ccmG in cytochrome c biogenesis. A chromosomal deletion of 91 co-dons (L24 to S114) was constructed by gene replacement mutagenesis of the wild-type strain E. coli MC1061 (9), resulting in the nonpolar in-frame ccmG deletion mutant EC29 (Fig. 1B). The mutant failed to synthesize both indigenous and foreign c-type cytochromes (Fig. 2B, lane 1 [29]). Even overexpression of the soluble B. japonicum cytochrome c550 from plasmid pRJ3268 (28) did not allow detection of the holocytochrome in the ccmG mutant. The phenotype was complemented when ccmG was provided on plasmid pEC210 (Fig. 1B and 2B, lane 5), indicating that the ccmG deletion was the sole cause of the observed mutant phenotype. This is in contrast to the phenotype of a dsbE mutant that was found to be able to produce low levels of c-type cytochromes (12). However, the precise physical location of its Tn10 insertion is not known and may have led to incomplete inactivation of the gene.

Subcellular localization of CcmG. CcmG contains an N-terminal, hydrophobic sequence (residues 5 to 25) that might serve as a signal sequence. CcmG was detected in membranes of the wild type but not in those of the ΔccmG mutant by Western blot analysis with a purified, polyclonal antiserum against a CcmG-specific synthetic peptide (N103 to E118). Even if CcmG was expressed from the multicopy plasmid pEC210 under the control of the pACYC184-derived tetracycline promoter, CcmG could be detected only in the mem-
bran (Fig. 2A, lane 5), not in the soluble fraction (data not shown). Analysis of the activity of a CcmG143-PhoA fusion protein encoded by pEC229 (Fig. 1B) revealed the periplasmic location of the C-terminal CcmG domain. The alkaline phosphatase activity of strain CC118(phoA)/pEC229 was 56.31 ± 8.37 U as opposed to the control strain carrying ccmG without a phoA fusion (CC118/pEC210), for which 0.21 ± 0.09 U was obtained. These results show that CcmG is a periplasmic membrane-anchored protein and contradict the finding of Missiañas and Raina, who reported that DsbE (which is identical to CcmG) is a soluble, periplasmic protein (14).

**Phenotypic characterization of ccmG active-site mutants.**

The two cysteines at positions 80 and 83 of the putative CcmG active site were changed to serines, either individually (CcmG_C80S encoded by pEC207 and CcmG_C83S encoded by pEC208) or together (CcmG_C80S/C83S encoded by pEC209). The plasmids expressing the wild-type CcmG (pEC210) and the three mutant proteins (pEC207, pEC208, and pEC209 [Fig. 1B]) were used to complement the in-frame deletion mutant EC29, which in addition carried pRJ3268 for expression of the B. japonicum cytochrome c_550. Cells were grown anaerobically in the presence of fumarate and nitrite and were analyzed for the presence of holocytochrome c by heme staining. Although the signals produced by the mutant strains (Fig. 2B, lanes 2 to 4) were considerably weaker than those of cells complemented with the wild-type gene (Fig. 2B, lane 1), Western blot analysis showed that the point mutations did not affect the stability of the CcmG polypeptide (Fig. 2A).

The phenotypic effects of thiol compounds with respect to c-type cytochrome biogenesis in an E. coli mutant lacking the protein-disulfide reductase DsbD have been analyzed previously (25). Cytochrome c maturation was restored by supplementing the medium with l-cysteine or mercaptoethanesulfonic acid but not with other thiol compounds. Therefore, we tested the influence of various thiol or disulfide compounds on the formation of holocytochrome c. The effect of l-cysteine, for example, was investigated in the three active-site mutants and in the ΔccmG in-frame deletion mutant. Addition of 3 mM l-cysteine to the medium led to a proportional increase in maturation of cytochrome c in the active-site mutants. As was also found for untreated cells, l-cysteine-treated EC29/pRJ3268/pEC207 (CcmG_C80S) and EC29/pRJ3268/pEC209 (CcmG_C80S/C83S) produced higher amounts of holocytochrome c than did EC29/pRJ3268/pEC208 (CcmG_C83S) (Fig. 2C, lanes 2 to 4). By contrast, l-cysteine had no effect on cytochrome c maturation in the ΔccmG in-frame deletion mutant lacking the entire CcmG (Fig. 2C, lane 1). We conclude that, regardless of the redox state of the active site, the presence of the CcmG polypeptide is necessary for cytochrome c biogenesis. Interestingly, even the wild type could be stimulated for cytochrome c production when l-cysteine was added (Fig. 2C, lane 5).

An opposite effect was observed when the cells were treated with the corresponding, oxidized disulfide compound l-cystine. Upon addition of 3 mM l-cystine, the heme-stained bands of the wild type, and also of the active-site mutants, were much weaker than those of cells that had been grown without addition of any redox-active compound (Fig. 2D, lane 5; almost invisible in lanes 2 to 4). Again, no cytochrome c formation was observed in cells lacking the ccmG gene.

In addition, we analyzed the influence of various other thiol compounds with respect to c-type cytochrome biogenesis (Fig. 2E). The phenotype of the in-frame deletion mutant lacking the entire CcmG (Fig. 2E, lane 1) was restored by l-cysteine (Fig. 2E, lane 3) but not by other thiol compounds (Fig. 2E, lanes 2 to 4). As expected, no effect was observed when the cells were treated with l-cystine. The effect of l-cysteine, compared with other thiol compounds, is indicated in the right margin. The 16-kDa c-type cytochrome c is most likely the NapB protein (8).
and disulfide compounds on cytochrome c maturation in EC29/prJR3268/pECE208 (expressing CcmG_CSSS [data not shown]). The addition of the reducing agent mercaptoethanesulfonic acid or reduced glutathione resulted in cytochrome c formation increasing to a similar extent as was observed for the addition of l-cysteine. In contrast, the presence of oxidized glutathione and l-cysteine in cultures led to a decrease of mature cytochrome c. Moreover, we found that addition of the reducing agent d-cysteine or reduced dithiothreitol seemed to have an inhibitory rather than a stimulating effect, as these agents led to weakly heme-stainable bands.

**Conclusions.** The mutual analysis of the active-site cysteines revealed that, although they are not required for the stability of the CcmG protein, each of them is important for attachment of heme to apocytochrome c. Remarkably, cytochrome c biogenesis was drastically reduced, but not abolished completely, in these active-site mutants. Moreover, cells expressing the CcmG protein with the second cysteine of the active site exchanged (CcmG_CSSS) produced lower levels of mature cytochrome c than the CcmG_CSSS and CcmG_CCRSS_CCSS mutants (Fig. 2). This phenomenon might be explained by the reaction mechanism that has been proposed for thiorodoxins (10). The first active-site cysteine attacks the target protein to form a covalently linked mixed disulfide transition state, which is subsequently resolved by the attack of the second cysteine. This finally leads to a dithiol form of the target molecule and a disulfide form of the thiorodoxin. In mutants expressing either CcmG_CSSS or CcmG_CCRSS_CCSS, the formation of a mixed disulfide is presumably hampered. Thus, the target of CcmG remains accessible for reduction by other, perhaps less efficient reducing agents, whereby low levels of cytochrome c can still be formed. By contrast, the CcmG_CSSS protein might still be able to attack its target protein with C80, but it lacks the second cysteine (C83) that is necessary to resolve the mixed disulfide. In this case, the mixed disulfide is stabilized, and less CcmG target is released. Hence, less-mature cytochrome c might be produced.

Our finding that cytochrome c maturation in ccmG active-site mutants can be restored by certain thiol compounds such as l-cysteine and glutathione, but not by their oxidized counterparts l-cystine and glutathione, is consistent with the observations made in E. coli (15). We speculate that the CcmG protein has a dual function. On the one hand, it is a periplasmic disulfide reductase, reducing apoapocytochrome c, CcmH, or an as-yet-unidentified component of the cytochrome c maturation pathway. On the other hand, it appears to stabilize one or several proteins involved in cytochrome c formation. Lack of the reducing function, as it occurs in the active-site mutants, can be compensated for by the addition of certain thiol compounds, whereas the stabilizing effect requires the presence of the CcmG polyepitope itself. Suggesting two CcmG-specific functions might also explain why thiol compounds can restore only cytochrome c oxidase activity and not cytochrome c biosynthesis in ΔccmG mutants of *P. denitrificans* (17).

Having proposed a reducing function of CcmG for cytochrome c maturation, we can speculate that CcmG receives reduction equivalents from a donor that might be involved more generally in redox control of the periplasm, such as DsbD (13, 22). Future experiments will address the question of which are the proteins that interact directly with CcmG.

We are grateful to F. Fischer, P. Künzler, and R. Zülfreyer for their help with the construction of plasmids and characterization of the ΔccmG mutant and to A. Hungerbücher for excellent technical assistance.

This work was supported by grants from the Swiss National Foundation for Scientific Research and from the Federal Institute of Technology, Zurich, Switzerland.

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**JOURNAL OF BACTERIOLOGY**

*Vol. 180, 1998 NOTES 1949*


