Doctoral Thesis

New insights into the redox pathway of bacterial cytochrome c maturation
CycY/CcmG and CcmH

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New insights into the redox pathway of bacterial cytochrome c maturation: CycY/CcmG and CcmH

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Abstract

The characteristic feature of c-type cytochromes is the covalent attachment of the prosthetic heme group to the polypeptide; the vinyl groups of heme are added via two thioether bonds to the cysteine residues in the conserved motif C-X-X-C-H of apocytochrome c. The key step of the cytochrome c biogenesis pathway, i.e., the covalent attachment of heme, takes place in the periplasm. This requires that apocytochrome c and heme are transported to the periplasm and that both the cysteiny1 side chains of apocytochrome c and heme are in the reduced state for ligation.

In various Gram-negative bacteria genes have been identified that are essential for the maturation of c-type cytochromes: ccmABCDEEGHI. The gene products of two of them, CcmG and CcmH, participate in the redox pathway of cytochrome c maturation. Both proteins possess the conserved motif C-X-X-C, which is in common with many known protein thiol:disulfide oxidoreductases. CcmG, in particular, shows extended similarity to the thioredoxin-like proteins, whereas the sequence of CcmH displays no further similarity.

The thioredoxin-like protein with the conserved motif W-C-X-X-C has been analyzed in two organisms, Bradyrhizobium japonicum and Escherichia coli. The protein in E. coli is called CcmG, whereas for historical reasons the homologue in B. japonicum is called CycY. It was demonstrated by Western blot analysis, that both proteins are anchored to the membrane via their N-terminal signal sequence. Translational phoA fusions to the genes demonstrated that, when expressed, their hydrophilic C-terminal domain with the conserved motif is exposed to the periplasm.

CycY of B. japonicum was analyzed biochemically. A soluble version of the protein devoid of its N-terminal membrane anchor (CycY*) was expressed in E. coli and purified to homogeneity from the periplasmic fraction. The protein showed a redox-dependent migration difference when separated by SDS-PAGE under reducing and non-reducing conditions. However, it failed to react in vitro with model substrates such as insulin or DsbA, indicating that it may possess a high degree of substrate specificity. Like other thioredoxin-like proteins, oxidized CycY is able to quench partially the tryptophan fluorescence. This property was used to determine its equilibrium constant with glutathione from which a redox potential of −0.217 mV was calculated. This redox
potential was closer to that of proteins acting as reductases (e.g., thioredoxin) than to that of proteins acting as oxidases (e.g., DsbA). Therefore, it was suggested that the protein might play a reducing role during cytochrome c biogenesis by keeping - directly or indirectly - the heme-binding motif of apocytochrome c in the reduced state.

This assumption was confirmed by the genetic analysis of the ccmG gene in E. coli. The active-site cysteine residues were exchanged against serine residues by site-directed mutagenesis, and the resulting effect on cytochrome c maturation was tested. Surprisingly, the active-site mutants were still able to form some holoprotein, yet, they were clearly affected in this ability. The addition of reducing agents such as L-cysteine, 2-mercapto-ethanesulfonic acid (MESA) or glutathione could restore the formation of mature protein in the active-site mutants. Remarkably, no restoration was observed for the nonpolar in-frame ccmG deletion mutant. Thus, it was suggested that CcmG is involved in the redox pathway of cytochrome c maturation and that it has a reducing function. The active site of the protein is important for this process but not essential. By contrast, the presence of the CcmG polypeptide proved to be essential, indicating that CcmG might have a further function in cytochrome c maturation in addition to the reducing one.

Finally, CcmH of E. coli was characterized. The protein is supposed to have two transmembrane helices. Translational phoA fusion analysis showed that the N-terminal domain with the conserved motif L-R-C-X-X-C is oriented to the periplasm. Several ccmH deletion mutants were analyzed for their ability to form holocytochrome c. It was found that the hydrophilic C-terminal domain, which is assumed to be also located in the periplasm, is not required for cytochrome c maturation. However, deleting more of the ccmH gene (leading to a truncated CcmH product consisting of the N-terminal soluble domain) strongly affected cytochrome c biogenesis. The cysteine residues of the conserved motif were exchanged by site-directed mutagenesis. In contrast to the ccmH deletion mutant, the ability to produce holocytochrome c was not completely destroyed in the active-site mutants, but strongly affected. Analysis of the active-site mutants under different growth conditions revealed that both cysteine residues are required for cytochrome c maturation during aerobic growth, whereas only the more C-terminal cysteine residue is required for cytochrome c maturation during anaerobic growth. The only reducing substance that could restore cytochrome c maturation in the active-site
mutants was MESA. It was suggested that CcmH also plays a reducing role in cytochrome c maturation.

It was proposed that reduction of the heme-binding site of apocytochrome c occurs via a redox cascade involving CcmG, CcmH and further thiol:disulfide oxidoreductases such as the DsbD protein. Two alternative models for the redox pathway of cytochrome c biogenesis may be formulated. Unfortunately, the attempt to confirm one of them by identifying the target proteins of CcmG and CcmH has failed so far.
Zusammenfassung

Die charakteristische Eigenschaft von c-Typ Cytochromen ist die kovalente Bindung der prosthetischen Hämgruppe an das Polypeptid; die Vinylgruppen des Häms sind über zwei Thioetherbindungen an die Cysteinreste im konservierten Motiv C-X-X-C-H des Apocytochroms c geknüpft. Der entscheidende Schritt während der Cytochrom c-Biogenese, d.h. die kovalente Verknüpfung von Häm, erfolgt im Periplasma. Daher müssen Apocytochrom c und Häm ins Periplasma transportiert werden und sowohl die Cysteinylseitenketten von Apocytochrom c also auch Häm in der reduzierten Form vorliegen.


CycY von B. japonicum wurde biochemisch analysiert. Dazu wurde eine lösliche Domäne ohne den N-terminalen Membrananker (CycY*) in E. coli exprimiert und aus der periplasmatischen Fraktion gereinigt. Das Protein zeigte ein unterschiedliches Auftrennungsverhalten im SDS-PAGE je nach reduzierenden bzw. oxidierenden Bedingungen. Jedoch reagierte es in vitro nicht mit Modellsubstraten wie zum Beispiel Insulin oder DsbA, was auf eine hohe Substratspezifität hindeutet. Wie in anderen Thioredoxin-ähnlichen Proteinen wird im oxidierten CycY die Tryptophanfluoreszenz teilweise gelöscht. Diese Eigenschaft wurde für die Bestimmung
Zusammenfassung


Desweiteren wurde CcmH von E. coli charakterisiert. Das Protein hat vermutlich zwei transmembrane Helices. Eine translationelle phoA Fusion zeigte, daß die N-terminale Domäne mit dem konservierten Motiv L-R-C-X-X-C ins Periplasma ragt. Verschiedene ccmH-Deletionsmutanten wurden auf ihre Eigenschaften während der Cytochrom c-Biogenese untersucht. Es wurde gefunden, daß die C-terminale Domäne, die wahrscheinlich ebenfalls im Periplasma liegt, nicht für die Cytochrom c-Reifung erforderlich ist. Hingegen führte die zusätzliche Deletion von weiterer 3'-Region des ccmH Gens (es wurde nur noch die lösliche N-terminale Domäne von CcmH hergestellt) zur Unterbrechung der Cytochrom c-Biogenese. Durch ortsspezifische Mutagenese wurden die Cysteinstellen im konservierten Motiv ausgetauscht. Im Unterschied zur ccmH Deletionsmutante war in den Punktmutanten die Fähigkeit zur Holocytochrom c Herstellung nicht vollständig zerstört, aber stark beeinträchtigt. Analyse der Punktmutanten unter unterschiedlichen
Zusammenfassung

Wachstumsbedingungen zeigte, daß beide Reste unter aeroben Wachstumsbedingungen für die Cytochrom c-Biogenese wichtig sind, während unter anaeroben Bedingungen nur der C-terminale Cysteinrest des Motivs erforderlich ist. Als einzige reduzierende Substanz konnte MESA in den Punktmutanten die Fähigkeit zur Cytochrom c-Biogenese wiederherstellen. CcmH spielt daher wahrscheinlich auch eine reduzierende Rolle während der Cytochrom c-Biogenese.

Die erzielten Resultate legen nahe, daß die Reduktion der Häm-Bindungsstelle von Apocytochrom c über eine Redoxkaskade erfolgt, die CcmG, CcmH und weitere Protein Thiol:Disulfid Oxidoreduktasen wie das DsbD Protein einschließt. Zwei alternative Modelle wurden für den Redoxweg der Cytochrom c-Biogenese formuliert. Bisher war es jedoch nicht möglich, die Targetproteine von CcmG oder CcmH zu identifizieren und so eine der beiden Alternativen zu bestätigen.