A Strategic Protein in Cytochrome c Maturation
THREE-DIMENSIONAL STRUCTURE OF CcmH AND BINDING TO APOCYTOCHROME c

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CcmH (cytochromes c maturation protein H) is an essential component of the assembly line necessary for the maturation of c-type cytochromes in the periplasm of Gram-negative bacteria. The protein is a membrane-anchored thiol-oxidoreductase that has been hypothesized to be involved in the recognition and reduction of apocytochrome c, a prerequisite for covalent heme attachment. Here, we present the 1.7 Å crystal structure of the soluble periplasmic domain of CcmH from the opportunistic pathogen Pseudomonas aeruginosa (Pa-CcmH). The protein contains a three-helix bundle, i.e. a fold that is different from that of all other thiol-oxidoreductases reported so far. The catalytic Cys residues of the conserved LRXXC motif (Cys25 and Cys28), located in a long loop connecting the first two helices, form a disulfide bond in the oxidized enzyme. We have determined the pK_a values of these 2 Cys residues of Pa-CcmH^2 (both >8) and propose a possible mechanistic role for a conserved Ser^36 and a water molecule in the active site. The interaction between Pa-CcmH and Pa-apocyt c551 (where cyt c551 represents cytochrome c551) was characterized in vitro following the binding kinetics by stopped-flow using a Trp-containing fluorescent variant of Pa-CcmH and a dansylated peptide, mimicking the apocytochrome c551 heme binding motif. The kinetic results show that the protein has a moderate affinity to its apocyt substrate, consistent with the role of Pa-CcmH as an intermediate component of the assembly line for c-type cytochrome biogenesis.

What is the mechanism whereby the heme is covalently attached to c-type cytochromes? Despite the crucial role played by these proteins in many cellular processes, this question remains largely unanswered. In c-type cytochromes, the heme is covalently bound to the apoprotein via two thioether bonds between the heme vinyls and the thiol groups occurring in the conserved CXXCH motif. The apoprotein and the heme group have to be in the correct stereochemical orientation for promotion of thioether bond formation; however, the chemistry of such a process is poorly understood, and only recently, in vitro formation of a c-type cytochrome has been reported (1, 2). In the cell, the necessary stereospecific insertion and attachment of heme to apocytochrome c (apocyt)^2 occurs on the p-side of the membrane, i.e. the intermembrane space of mitochondria, the chloroplast lumen, or the periplasm of bacteria. This process requires an elaborate post-translational modification machinery that must also ensure translocation of both apocyt and heme from the n-side of the membrane where they are synthesized.

It is known that three different multienzymatic systems have evolved in different organisms to accomplish this task. Although System III consists of a single cyt c heme lyase (3) and is adopted exclusively by eukaryotes, Systems I and II consist of different proteins that have been discovered in Gram-negative bacteria, in plant or protozoal mitochondria (System I), and in Gram-positive bacteria, plant chloroplasts, and cyanobacteria (System II) (4–6). Both systems comprise different membrane-anchored proteins that expose their active site to the periplasm (System I) or to the exterior (System II), each performing a particular function. The CcmABC components of System I have been recently recognized as subunits of an ATP binding cassette transporter that, together with CcmE and CcmD, acts as a heme release complex (7, 8). On the other hand, since in the periplasm the 2 Cys residues of the heme binding motif of apocyt (CXXCH) are oxidized by DsbA (9), a crucial bottleneck in the maturation of apocyt is the required reduction of these cysteines before covalent heme attachment can occur. In System II, this reductive step is carried out by the thioredoxin-like (TRX-like) ResA, a membrane-associated thiol-oxidoreductase (10–12), whereas in System I, the reduction of the apocyt disulfide bond was proposed to be carried out by CcmH and/or CcmG via the formation of a mixed disulfide complex (13–15). According to this view, the mixed disulfide complex between CcmH and apocyt is subsequently resolved by CcmG, releasing reduced apocyt and oxidized CcmH (15, 16). Although CcmG is a structurally well characterized TRX-like protein that receives electrons from DsbD (17–19), no structural information is available for any CcmH protein. Previous studies indicated that

The abbreviations used are: apocyt, apocytochrome c; cyt c, cytochrome c; Ccm, cytochromes c maturation; TRX, thioredoxin; DTNB, dithio-bis (2-nitrobenzoic acid); MAD, multiwavelength anomalous dispersion; MR, molecular replacement; FRET, fluorescence resonance energy transfer; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.

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The atomic coordinates and structure factors (code 2HL7) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

The on-line version of this article (available at http://www.jbc.org) contains two supplemental figures.

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2 The abbreviations used are: apocyt, apocytochrome c; cyt c, cytochrome c; Ccm, cytochromes c maturation; TRX, thioredoxin; DTNB, dithio-bis (2-nitrobenzoic acid); MAD, multiwavelength anomalous dispersion; MR, molecular replacement; FRET, fluorescence resonance energy transfer; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.
CcmH is a membrane-bound protein exposing the conserved redox-active LRCXXCQ motif to the periplasm; its essential role in the maturation of apocyt was suggested by the observation that loss of CcmH impairs the synthesis of c-type cytochromes in vivo (13, 14, 20). Moreover, in vitro evidence indicates that Rhodobacter capsulatus and Arabidopsis thaliana CcmH homologues are able to reduce the CXXCH motif of an apocyt-mimicking peptide (13, 21). Taken together, these observations suggest that CcmH is redox-active and is specifically involved in the thioether pathway of cytochrome c maturation.

To shed light on the extracytoplasmic-heme ligation process occurring in System 1 organisms, we expressed, crystallized, and characterized the soluble periplasmic domain of CcmH from the opportunistic pathogen Pseudomonas aeruginosa (hereafter named Pa-CcmH*). The three-dimensional structure of Pa-CcmH* solved at 1.7 Å resolution reveals that the periplasmic domain adopts a peculiar three-helix bundle fold that is different from that of canonical thiol-oxidoreductases and provides a basis for understanding its specific reductant role 1 in the maturation process, using a Trp-containing fluorescent variant of Pa-CcmH* and a dansylated nonapeptide mimicking the heme binding motif of P. aeruginosa cytochromes c551.

**EXPERIMENTAL PROCEDURES**

Cloning, Expression, and Purification—The soluble N-terminal domain of CcmH from P. aeruginosa (residues 21–100) was obtained by removing the signal peptide, the membrane-anchoring helix, and the cytoplasmic C-terminal stretch. The domain was amplified from the genomic DNA (strain PA01) and cloned in pET28b (Novagen). Site-directed mutants were produced by using a QuikChange mutagenesis kit (Stratagene, La Jolla, CA). Expression of the His tag fusion protein in Escherichia coli BL21 (DE3) was induced with 1 mM isopropyl-1-thio-β-d-galactopyranoside; cells were grown in LB broth at 27 °C. Pa-CcmH* was purified in two steps with nickel affinity and ion-exchange chromatography. The yield of purified protein was ~25 mg/liter. After tag cleavage with thrombin, Pa-CcmH* was again subjected to ion-exchange purification. Selenomethionine-substituted Pa-CcmH* was expressed in BL21 (DE3) E. coli cells in minimal medium supplemented with 50 mg/liter D-L-selenomethionine following standard procedures and was purified under the same conditions as the wild type protein.

Protein Crystallization—Crystallization conditions were 0.1 M Tris-HCl buffer (pH 8.5) containing 20% polyethylene glycol 1000 at room temperature. Drops were prepared by mixing 2.5 μl of precipitant solution and 1.0 μl of protein (13 mg/ml) and allowed to equilibrate against 0.5 ml. High quality crystals for the Se-Met protein were obtained by mixing 1 μl of protein (9 mg/ml) and 1 μl of the reservoir solution consisting of 38% polyethylene glycol 6000, 0.8 M NaCl, and 0.1 M Tris-HCl, pH 8.0. Crystals appeared in 2 days and grew to full size in 1 week. Diffraction data for both native and Se-Met crystals were collected at cryogenic temperature (100 K) at the European Synchrotron Radiation Facility (ESRF) Synchrotron (Grenoble, France). Data were indexed and integrated with DENZO and SCALEPACK (22). One molecule was found in the asymmetric unit for both the native and the Se-Met derivative crystals.

Crystal Structure Determination and Refinement—Pa-CcmH* structure was solved by a combination of MAD and MR procedures. MAD was carried out on the Se-Met derivative data. The program SOLVE (23) identified one selenium site. Initial phase calculation led to an overall figure of merit of 0.61 at 35-3.5 Å resolution. Solvent flattening with RESOLVE (24) improved the quality of the map, yielding a figure of merit of 0.8 at 2.5 Å resolution. The ARP-WARP (25) automatic building procedure provided a partially built model. This model was then used as a template to perform an MR with the native data set using AMORE (26) and gave a complete and easily interpretable electron density map at 1.7 Å resolution. The model (residue 1 in the structure corresponds to Ala21 in the full-length protein) was completely built, and water molecules were added using COOT (27). Several cycles of refinement (REFMAC5 (28)), visual inspection, and manual rebuilding led to a final R-factor of 21.0% and Rfree of 23.5% at 1.7 Å resolution. The average atomic B-factor for the structure is 31.16 Å². The model quality was checked with PROCHECK (29); all residues lie in the most favored and additionally allowed regions of the Ramachandran plot. The atomic coordinates have been deposited in the Protein Data Bank (Protein Data Bank ID code 2HL7). Analysis of the cavities was performed with the program CASTp (30).

Biochemical Analyses—Pa-apocyt was obtained from purified Pa-cyt c551 (prepared as described in Ref. 31) following the original protocol of Anfinsen and co-workers (32). Standard redox potentials (E0′) of Pa-CcmH* and Pa-apocyt were determined as described previously (33).

pKa Determination of the Active Site Cysteines—Reduced Pa-CcmH* (5 mM) was mixed with an excess of 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB; 50–100 μM) in the pH range 4.0–9.5 in the Pi-Star stopped-flow instrument (Applied Photophysics, Leatherhead, UK), and the reaction was monitored following the absorbance at 412 nm. Absorbance data were fitted to a single exponential function to obtain an observed, pseudo-first order rate constant (kobs). pKa values were determined by plotting kobs values as a function of pH and fitting to an appropriate equation. For single pKa processes, Equation 1 was used,

\[ k_{obs} = \frac{k_{SH} + k_\infty \cdot 10^{pH - pK_a}}{1 + 10^{pH - pK_a}} \]  

(Eq. 1)

where kSH and k_\infty are the rate constants for the protonated and deprotonated forms, respectively (34). For two proton dissociation events, an equation that takes into account two pK_a values (35) was used.

Binding Kinetics—Binding kinetics of a Trp-containing variant of Pa-CcmH* (Y64W) with dansylated nonapeptide (dansyl-KGCVACHAI; JPT Peptide Technologies, Berlin, Germany) was measured in 50 mM sodium phosphate buffer, pH 7.0, at 25 °C. Reduced Pa-CcmH* (1 mM Tris (2-carboxymethyl)
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**TABLE 1**

| Data collection, phasing and refinement statistics | Native* | Se-Met Crystal |
|--------------------------------------------------|---------|---------------|
| **Space group** | P2₁,2₁ | P2₁,2₁ |
| **α, β, γ (°)** | 40.07, 45.41, 48.13 | 32.30, 41.10, 65.02 |
| **Completeness (%)** | 97.7 (96.5) | 99.2 (99.7) |
| **Redundancy** | 7.4 (5.9) | 6.5 (6.1) |

**Refinement**

| Resolution (Å) | 30.1-1.7 | 50-2.5 (2.59-2.50) |
|----------------|-----------|------------------|
| Rwork (%)     | 21.0/23.5 | 5.0 (18.1) |
| Rfree (%)     | 21.0/23.5 | 25.6 |
| Protein       | 653       | 98.7 (99.7) |
| Water         | 70        | 98.6 (93.0) |
| Bond lengths (Å) | 0.015  | 0.015 |
| Bond angles (°) | 1.358   | 1.358 |

*Highest resolution shell is shown in parentheses.

**RESULTS**

Overall Structure of CcmH—The soluble redox-active N-terminal domain of *Pa*-CcmH*⁺* (*Pa*-CcmH*), obtained by removing the N-terminal signal peptide and the C-terminal membrane-anchoring helix, is composed of 80 residues. This domain was expressed in *E. coli*, purified, and crystallized, and its three-dimensional structure was solved by a combination of MAD and MR (see “Experimental Procedures”). The high quality electron density map obtained at 2.5 Å resolution by MAD using a Se-Met derivative enabled us to build a partial model of the protein. MR was employed to position the model in a different crystal form, obtaining a complete model at higher resolution. The final structural model consisting of 4 residues derived from the His tag cleavage site (numbered from −4 to −1) plus 78 residues of the protein domain (Val⁹⁹ and Asn¹⁰⁰ were excluded because they were undetectable in the electron density) was refined at 1.7 Å resolution with \( R_{work} = 21\% \) and \( R_{free} = 23.5\% \). The model presents a good stereochemistry with all residues located in the allowed regions of the Ramachandran plot (see “Experimental Procedures” and Table 1).

The fold of *Pa*-CcmH*⁺* (Fig. 1A) consists of a three-helix bundle stabilized by hydrophobic interactions, with a short N-terminal extension and a C-terminal tail connecting the bundle to the transmembrane helix in the full-length protein. Helices \( \alpha_1 \) (residues 10–22) and \( \alpha_2 \) (residues 39–54) are connected by a long loop (residues 23–38), whereas helices \( \alpha_2 \) and \( \alpha_3 \) are linked by a short loop (residues 58–69). A search using DALI (37) returned significant hits only with unrelated \( \alpha \)-helical proteins, indicating that the three-helix bundle fold of *Pa*-CcmH*⁺* is unique among redox-active proteins.

In *Pa*-CcmH*⁺*, the redox-active motif Cys²⁵-Pro²⁶-Lys²⁷-Cys²⁸ is part of the loop connecting helices \( \alpha_1 \) and \( \alpha_2 \) (Fig. 1, A and B). The electron density for the side chains of these 2 Cys residues is continuous, indicating that the protein is in the oxidized state (supplemental Fig. 1). Attempts to crystallize the reduced form were unsuccessful. The conformation of the disulfide bond, albeit located in a peculiar fold, resembles that of the TRX and TRX-like proteins, displaying the typical right-hand hook conformation with the \( \chi_\text{s} \)-\( \chi_\text{d} \) dihedral angle = 81.1°, the Sy-Sy distance = 2.02 Å, and the Ca-Ca distance = 5.34 Å (38, 39). The accepted catalytic mechanism of TRX proteins (40) assumes that the solvent-exposed N-terminal cysteine of the CXXC motif performs a nucleophilic attack on the disulfide bridge of the target substrate. This process leads to a mixed disulfide complex that is subsequently resolved by the buried C-terminal Cys activated by a nearby conserved acidic residue (Asp or Glu) (41–43). Interestingly, analysis of the accessible surface area reveals that in *Pa*-CcmH*⁺*, the N-terminal Cys²⁵ residue is buried, whereas the C-terminal Cys²⁸ is solvent-exposed, at variance with the canonical arrangement observed in the TRX superfamily where the N-terminal Cys of the CXXC motif is always solvent-exposed. Moreover, neither the acidic...
Asp/Glu residue nor the conserved and functionally important cis-Pro residue (44) are present in the active site, suggesting that Pa-CcmH* may catalyze thiol-disulfide exchange by a different mechanism.

Multiple sequence alignment of CcmH homologues from various organisms (Fig. 2) shows that, in addition to the catalytic residues Cys25 and Cys28, 10 other residues are fully conserved, mostly located near the active site (Fig. 1B). Gly55 and Gly70 act as helix-breaking residues and are located at the end of the α2 and α3 helices, respectively; Leu24 and Val72 side chains point to the hydrophobic core of the bundle and presumably fulfill a structural role; Arg24 and Gln29 are located close to the catalytic Cys and may be involved in the binding to the redox partners of CcmH, apocyt, and/or CcmG. The hydroxyl group of the fully conserved Ser36 is at 6.2 Å distance from the Sγ of Cys28 and H-bonded to the –OH group of Ser36 (2.59 Å). This H-bonding network involving a water molecule seems to be highly conserved in the CcmH proteins since the position of Ser36 is secured by the presence of the fully conserved Arg45, whose guanidinium group is H-bonded to the backbone oxygen of Ser36 (2.70 and 3.09 Å, respectively) (Fig. 1C). Taken together, evidence suggests a possible role for Ser36 in the activation of Cys28 through a water molecule (see below).

The side chain of Ser36 is also found to line a small cavity (volume = 89.8 Å³). This pocket, located behind the catalytic cysteines, is surrounded by conserved or conservatively mutated polar and hydrophobic residues (Cys25, Cys28, Gln29, Asn30, Gln31, Ile33, Ser36, Ala38, Ile40, Ala41, Leu44). Although the overall hydrophobic character of the pocket is suitable for the interaction with an unfolded substrate, such as the heme binding motif of apocyt, the presence of conserved polar residues may be strategic in the recognition of the conserved His of the CXXCH motif of c-type cytochromes.

Determination of the Redox Potential of Pa-CcmH* and Pa-apocyt—To provide information about the thermodynamic driving force for the flow of reducing equivalents in the redox pathway involving CcmH and apocyt in P. aeruginosa, we determined the redox potential ($E_0'$) of Pa-CcmH* and Pa-apocyt. The difference in electrophoretic mobilities of the reduced-modified form and the oxidized form was used to estimate the $E_0'$ values of Pa-CcmH* and Pa-apocyt following Inaba and Ito (33). Fit of the data (supplemental Fig. 2) to the Nernst equation yields $E_0' = -215$ mV for Pa-CcmH* and $E_0' = -190$ mV for Pa-apocyt. These values are in agreement with the estimates previously reported for the homologous protein Ccl2 from...
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![Image](https://example.com/image1)

**FIGURE 2.** Multiple sequence alignment of the periplasmic soluble domains of CcmH/Ccl2/CycL proteins from different organisms, PSEAE, P. aeruginosa; PSEFL, Pseudomonas fluorescens; ECOLI, E. coli; HAEIF, Haemophilus influenzae; RHIME, Rhodobacter meliloti; RHODA, R. capsulatus; RHOCA, R. capsulatus; RHIFA, R. capsulatus; NSSF, E. coli; NSSFparalogue of CcmH. Identical residues (*), conservative substitutions (:), and semiconservative substitutions (.) are indicated. The three α-helices of Pa-CcmH* are represented by cylinders.

![Image](https://example.com/image2)

**FIGURE 3.** pH dependence of the reaction of DTNB with Pa-CcmH*. A, data on the wild type protein are best fit to a single proton dissociation event. Inset, representative time course of the reaction of Pa-CcmH* with DTNB (Pa-CcmH*, −4 μM, DTNB, 90 μM after mixing). B, pH dependence of the reaction of DTNB with single-site variants C25S (open symbols) and C28S (closed symbols). The line is the best fit of the data to a one-proton dissociation process (C25S, pKₐ = 8.6 ± 0.1; C28S, pKₐ = 8.4 ± 0.1).

R. capsulatus and for a model peptide of apocyt c₂ (−210 and 170 mV, respectively) (45). The slightly more positive E₀' value of Pa-apocyt is consistent with its reduction by Pa-CcmH*. Therefore we conclude that the difference in redox potential, although small, is thermodynamically favorable.

**The pKₐ of the Active Site Cysteines**—The pH dependence of the rate of reaction between DTNB and Pa-CcmH* was used to determine the pKₐ of the Cys residues. Control experiments carried out under pseudo-first order conditions at selected pH values confirmed that the reaction is second order (data not shown). At variance with what was observed in the case of TRX and TRX-like proteins (43), the time course was always monophasic (Fig. 3A, inset), suggesting a one-step bimolecular reaction mechanism. The pH dependence of the pseudo-first order rate constant for DTNB reduction by Pa-CcmH* (Fig. 3A) displayed a minor systematic deviation from a model accounting for a single titratable group, showing a Hill coefficient of about 0.8 (data analysis not shown). Thus, to assess the pKₐ of the active site thiols individually, two single variants of Pa-CcmH* (C25S and C28S) were generated and purified. The pseudo-first order rate constant of the reaction with DTNB of these variants at various pH values (Fig. 3B) can be fitted to a one-proton dissociation process, with very similar pKₐ values (8.4 ± 0.1 for Cys25 and 8.6 ± 0.1 for Cys28; see also Ref. (45). In the case of TRX and TRX-like proteins, the pKₐ values of the catalytic Cys residues are widely separated, being in the range of 6.7–7.5 for the N-terminal reactive cysteine and >9 for the C-terminal one (42, 46). Thus, the active site Cys residues of Pa-CcmH* have unusual pKₐ values when compared with canonical TRX proteins. Recently however, also for the TRX-like ResA protein from Bacillus subtilis, the two Cys residues were reported to have similarly close pKₐ values (12); in this case, the crystal structure of the reduced state of the protein, responsible for the reduction of apocyt in System II, reveals an unusually large separation between the two thiols, which may account for the result.

Comparison of the pH dependence of the reduction of DTNB by Pa-CcmH* (Fig. 3A) with that of the two single Cys variants (Fig. 3B) highlights faster pseudo-first order rate constants in the former case under the same conditions. This unpredicted observation can be explained on the basis of the kinetic mechanism if both Cys residues were able to reduce DTNB. Although each Cys residue can react individually with DTNB because of their similar pKₐ values, their close proximity in the three-dimensional structure of Pa-CcmH* prevents them from reacting simultaneously, giving rise to a partitioning effect between two alternative parallel pathways (involving either Cys25 or Cys28, respectively). Such a partitioning process is clearly not available in the case of the single Cys variants, which therefore react, under the same conditions, with slower rate constants. These effects may account for the low Hill coefficient calculated for the wild type Pa-CcmH*, whose titration results from a complex combination of two different processes (36).

**Interaction with Pa-apocyt**—As outlined above, it is generally accepted that CcmH may act as a specific thiol-oxidoreductase...
in the redox pathway of the c-type cytochromes biogenesis (4, 6). As a control, we observed that, similarly to its homologues Ccl2 (13) and AtCCMH (21), Pa-CcmH* is unable to reduce insulin in vitro (data not shown).

The ability of Pa-CcmH* to interact with apocyt was initially tested in an experiment whereby reduced Pa-CcmH* was labeled with DTNB and then either mixed with E. coli TRX or mixed with a nonapeptide corresponding to the heme binding motif of Pa-apocyt (KGCVACHAI). The results (data not shown) indicate that only the peptide releases thionitrobenzoic acid and thus can interact with the labeled cysteines of Pa-CcmH*.

To further characterize the interaction in solution between Pa-CcmH* and the same Pa-apocyt nonapeptide, we carried out fluorescence stopped-flow binding experiments. We followed the binding kinetics by FRET between a fluorescence donor (an engineered variant of Pa-CcmH* with the substitution Tyr64 to Trp; Y64W) and an acceptor (a dansyl group) under pseudo-first order conditions matched single exponentials at all concentrations of Pa-CcmH* (always in excess) (Fig. 4A). A plot of the observed rate constant against Pa-CcmH* concentration (Fig. 4B) shows a minor deviation from second-order kinetics; fitting the data to Equation 2 yields the two microscopic rate constants for the binding of Pa-CcmH* to the dansylated nonapeptide (κon = 0.05 μM⁻¹ s⁻¹ and κoff = 0.5 s⁻¹), from which an equilibrium dissociation constant (KD) of ~10 μM was calculated. A similar KD value was calculated from the amplitudes of the kinetic traces (data not shown). Nevertheless, we should point out that this experiment demands that the two catalytic partners are in opposite redox states; since we were unable to isolate fully reduced fractions of Pa-CcmH*, the actual concentration of reduced Pa-CcmH* is uncertain, and thus the KD value is very likely to be underestimated. When the same experiment was carried out using the two single Cys variants (Y64W-C25S and Y64W-C28S), no binding was observed by FRET (data not shown); apparently in vitro, both cysteines are required for the recognition/binding reaction of Pa-CcmH* to the Pa-apocyt-mimicking nonapeptide. These results imply that a more complex scenario may be more appropriate to describe the binding reaction of Pa-CcmH* to the Pa-apocyt, as mirrored by the systematic deviation from linearity of the observed pseudo-first order rate constant, and given that in vivo, both cysteines of CcmH are necessary for cytochrome c maturation during anaerobic growth (14).

DISCUSSION

The maturation of c-type cytochromes in Gram-negative bacteria occurs in the periplasm; it is in this oxidizing compartment that the newly synthesized polypeptide chain is translocated and covalently linked to the heme group. Although it is still not known how this process is carried out, it is clear that attachment of the heme to the (presumably unfolded) apocy...
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exposed; this suggested to us that, at variance with TRX proteins, the Cys28 is the one presumed to perform the nucleophilic attack on the apocyt disulfide bond. Although this hypothesis is based on the crystal structure of oxidized Pa-CcmH+, it would imply a mechanism different from that described in TRX and TRX-like proteins where the reactive cysteine is the N-terminal one, as substantiated by a model previously proposed for the reduction of apocyt by CcmH (14).

This peculiarity of the active site cysteines of Pa-CcmH+ is also supported by their pKa values that we found to be quite similar (8.4 ± 0.1 for Cys25 and 8.6 ± 0.1 for Cys28). In TRX proteins characterized by aspecific thiol-oxidoreductase activity, the pKa value of the cysteine performing the initial nucleophilic attack on its target substrate is significantly lower (i.e. ∼7); as recently discussed for ResA, a TRX-like protein involved in System II (12), the higher pKa values of the Pa-CcmH+ active site thiols may ensure that this component of the Ccm apparatus is unreactive toward nonspecific substrates.

Although a detailed description of the catalytic mechanism of Pa-CcmH+ would profit from the determination of the structure of the reduced state, we propose that Ser256, which in Pa-CcmH+ is close to the active site (Fig. 1, B and C) and strictly conserved in all CcmH homologues (Fig. 2), may play a role in either one or both of two crucial events, i.e. (i) recognition of the conserved His residue in the heme binding motif of apocyt and/or (ii) activation of Cys28. Indeed, on the basis of its pKa values of the Pa-CcmH+ active site thiols may ensure that this component of the Ccm apparatus is unreactive toward nonspecific substrates.

In TRX and TRX-like proteins, resolution of the mixed disulfide complex is subsequently carried out via a nucleophilic attack by the C-terminal Cys residue on the intermolecular disulfide (40). In the case of Pa-CcmH+, however, the higher pKa of the N-terminal Cys25 and the absence of activating acidic residues in the active site region support a different mechanism. Rupture of the mixed disulfide and release of reduced Pa-apocyt should involve a second redox partner of the Ccm maturase complex, such as CcmG (14, 15); indeed, previous in vitro experiments with the R. capsulatus homologue CcI support an interaction with the CcmG homologue HelI X (13).

How can Pa-CcmH+ cope with the requirement to recognize and interact with two different redox partners such as Pa-apocyt and Pa-CcmG? Although it cannot be excluded that reduced Pa-CcmH+ may adopt a different conformation, the crystal structure reported here highlights the presence of a small pocket on the surface of Pa-CcmH+, close to the active site disulfide bond; it is tempting to speculate that this cavity, surrounded by conserved hydrophobic and polar residues, could represent the recognition site for the heme binding motif of apocyt. This possibility is made more likely since a small cavity with similar features has been recently identified in the structure of reduced ResA and implicated in apocyt recognition (11). On the other hand, it is plausible that Pa-CcmG recognition may be mediated by some conserved basic/polar residues located near the active site of Pa-CcmH+ (i.e. Arg24 and Gln29); indeed, the unusually acidic nature of the redox-active center of E. coli CcmG has been hypothesized to be important in the interaction with its CcmH partner (17, 18).

Finally, we want to highlight that our kinetic data on Pa-CcmH+ provide the first quantitative in vitro evidence for the recognition and binding of a Ccm component to its apocyt substrate; this result was obtained by mixing with a nonapeptide (dansyl-KGCVACHAI), which is identical to the heme binding motif of Pa cyt c551, the physiological partner. The measured off-rate constant (in the 0.2–1 s−1 range) combines an adequate affinity (low μM) with the need to release reduced apocyt to other component(s) of the System I maturase complex.

On the basis of the structural and functional data on Pa-CcmH+ presented here, it is conceivable to envisage an assembly line of holo cytochrome c in which reduced CcmH (a non-TRX-like thiol-oxidoreductase) specifically recognizes, binds, and reduces oxidized apocyt via the formation of a mixed disulfide complex, subsequently resolved by CcmG, a TRX-like thiol-oxidoreductase. This view supports the general picture whereby redox cascades are mediated by the interaction between proteins alternating a TRX-like and a non-TRX-like fold (19, 47). Further characterization of the multienzymatic redox pathway involved in the maturation of c-type cytochromes will require structural and biochemical investigations of the recognition processes within the ternary complex involving CcmH, apocyt, and CcmG.

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