JPP JOURNAL OF PROTEINS AND PROTEOMICS
3(1), January-June 2012, pp. 1-7

THE REDOX PATHWAY OF *Pseudomonas aeruginosa* CYTOCHROME C BIOGENESIS

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Abstract: Cytochrome c contains heme covalently bound to the polypeptide chain through two thioether bonds between the heme vinyl groups and the two cysteines of the conserved heme- binding motif of the apoprotein. Surprisingly, the biochemical events leading to the synthesis of the functional holoprotein in the cell are largely unknown. In the human pathogen *Pseudomonas aeruginosa*, the biogenesis of Cytc is mediated by a group of membrane or membrane-anchored proteins (CcmABCDEFGHI), exposing their active site to the periplasm. The Ccm proteins involved in the necessary reduction of apoCyt disulfide bond are CcmG and CcmH. Here we present the structural and functional characterization of these two redox-active proteins. We determined the crystal structure of CcmG, both in the oxidized and the reduced state. CcmG is a membrane-anchored thioredoxin-like protein acting as a mild reductant in the redox pathway of Cytc biogenesis. The 3D structure of the soluble periplasmic domain of CcmH revealed that it adopts a peculiar three-helix bundle fold that is different from that of canonical thiol-oxidoreductases. Moreover, we present protein-protein interaction experiments aiming at elucidating the molecular mechanism of the reduction of apoCyt disulfide bond for heme attachment *in vivo*. On the basis of the structural and functional data on CcmG, CcmH and their interactions, we propose an assembly line for Cytc biogenesis in *P. aeruginosa* in which reduced CcmH specifically recognizes, binds and reduces oxidized apoCyt via the formation of a mixed disulfide complex, which is subsequently resolved by CcmG.

***Keywords*:** cytochrome c; heme; Ccm proteins; disulfide exchange reaction; *Pseudomonas aeruginosa.*

Introduction

Cytochrome c (Cytc) is a ubiquitous protein crucial for cellular life, well known to act as an electron carrier in energy transduction processes in both aerobic and anaerobic respiration. Cytc proteins, however, are also involved in other fundamental biochemical processes such as detoxification and redox homeostasis (Bertini *et al.*, 2006). All c-type cytochromes are characterized by a covalently bound heme (Fe-protoporphyrin IX) linked to the polypeptide chain through two thioether bonds between the heme vinyl groups and the two cysteines of the conserved heme-binding motif CXXCH of the apoprotein. Surprisingly, although Cytc is considered a textbook protein, the biochemical events leading to the synthesis of the functional holoprotein in the cell (Cytc biogenesis) are largely unknown (Sanders *et al.*, 2010; Ferguson *et al.* 2008).

Despite the high sequence and structural homology shared by Cytc proteins from different organisms, three main systems composed of different proteins, have been described to be responsible for the maturation of Cytc in gram-negative bacteria (System I), in gram-positive bacteria (System II) and in eukaryotic cells (System III). In the human pathogen *Pseudomonas aeruginosa*, the biogenesis of Cytc is mediated by System I, composed of nine membrane or membrane anchored proteins (also known as CcmABCDEFGHI, from Cytochrome c maturation), facing their active site to the periplasm.

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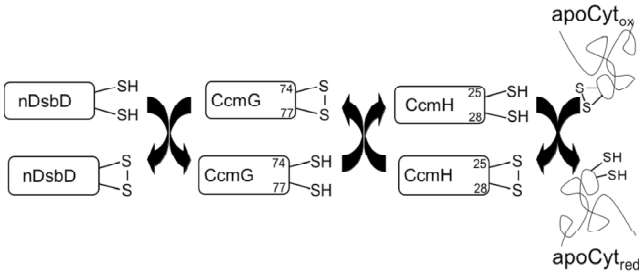
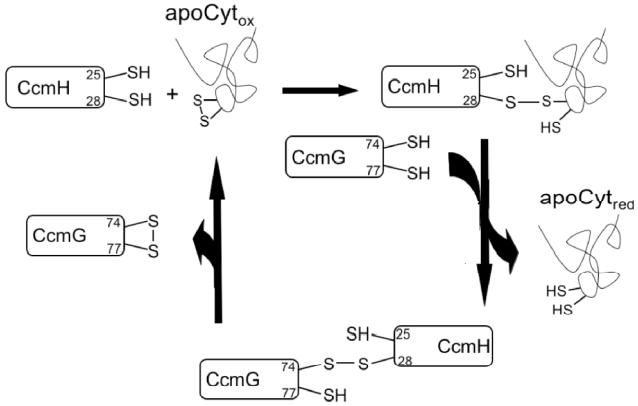
Although our view of the molecular mechanism whereby the System I apparatus is able to attach the heme to the unfolded apocytochrome (apoCyt) is still far from complete, three main functions have been ascribed to different Ccm proteins: 1) CcmABCDE: transport of heme from the cytoplasm to the periplasm and its delivery to other components of System I, 2) CcmGH: reduction of the disulfide bond of apoCyt, 3) CcmFI: chaperoning of the apoCyt and formation of the covalent bonds between the heme vinyls and the thiol groups of the conserved heme binding motif of apoCyt. Among the Ccm proteins, only CcmE, CcmG and CcmH have been structurally characterized at atomic level. (Ahuja *et al.*, 2008; Harvat *et al.* 2009; Di Matteo *et al.*, 2007; Di Matteo *et al.*, 2010).

Notably, over-and-above its role in Cytc biogenesis, System I is attracting increasing interest because there is now evidence that mutations on some ccm genes induce phenotypes that cannot be explained only in terms of impaired Cytc biogenesis (Cianciotto *et al.*, 2005). These phenotypes include loss of siderophore production/utilization and reduced abilities to grow in low-iron conditions in eukaryotic host cells leading to the possibility to interfere with the pathogen's viability and/or virulence by employing Ccm proteins as drug targets.

Although we know the Ccm proteins involved in the thioreductive branch of Cytc biogenesis, CcmG and CcmH, our view of the mechanism leading to the necessary reduction of apoCyt disulfide bond is still limited. At least two mechanisms have been hypothesized, which refer to a linear redox cascade of disulfide bonds formation and reduction (Scheme 1), or a non-linear redox process involving transient formation of a mixed-disulfide complex (Scheme 2), respectively. Additional open questions concern the mechanism(s) whereby these two proteins are able to specifically recognize different redox partners and an unfolded protein such as apoCyt.



Scheme 1



Scheme 2

Materials and Methods

Cloning, protein expression and purification - *Pa*-CcmH and *Pa*-CcmG were cloned into the vector pET28b+ (Novagen) and all mutants were obtained by using the QuickChange mutagenesis kit (Stratagene) according to the manufacturer's instructions. All of the mutations were confirmed by DNA sequencing (PRIMM, Italy). Proteins were expressed and purified as described previously (Di Matteo *et al.*, 2007; Di Matteo *et al.*, 2010).

Formation of the mixed-disulfide complex and analytical gel-filtration purification - *Pa*-CcmH C25S (20mg/mL) was reduced with DTT (40mM) for 2 hours at 25 °C; after this incubation time the excess of DTT was removed by dialysis. DTNB-activated *Pa*-CcmH C25S was obtained by incubating reduced protein with excess of DTNB (1mM) in 20 mM TrisHCl pH 8.0, 1mM EDTA, 200 mM NaCl buffer at 25 °C for 1 hour. The excess TNB^- was removed by dialysis using a PD10 Desalting Columns (GE Healthcare). TNB-activated *Pa*-CcmH C25S was mixed with *Pa*-CcmG C77S in 20 mM TrisHCl pH 8.0, 1 mM EDTA, 200 mM NaCl buffer overnight at 25 °C. Formation of the complex between *Pa*-CcmG C77S and *Pa*-CcmH C25S was monitored following the absorbance of the released TNB^- at 412 nm. In order to purify the complex between *Pa*-CcmH and *Pa*-CcmG, the sample was applied onto a Superdex 75 10/300 GL column

equilibrated with 20 mM TrisHCl pH 8.0, 200 mM NaCl, and eluted at 0.5 mL/min using a Lab Flow 4000 apparatus (Lab Service Analytica) HPLC pump. Two consecutive gel-filtration runs were carried out to obtain > 98% purified complex.

Crystallization - The purified *Pa*-CcmH C25S-*Pa*-CcmG C77S mixed-disulfide complex was crystallized by vapour diffusion using the hanging drop technique. Drops were prepared by mixing 1uL of protein solution (20mg/mL) and 1µL of reservoir solution (22-24% PEG 3350 w/v, 0.2M Sodium Chloride, 0.1M Tris-HCl pH 8.5). Mixed solutions were equilibrated at 21°C against the reservoir solution; crystals appeared in 3 days and grew to their final dimensions in 1 week.

FRET Fluorescence measurements - Steady state fluorescence measurements were carried out with Jobin-Yvon spectrofluorimeter. The cuvette holder was thermostated with a circulating bath and the fluorescence experiments were performed at 25°C in 10 mm path length quartz cuvette with maximum volume of 0.5mL. Dansylated peptide (dans-KGCVASHAI) was synthesized by JPT (Germany). *Pa*-CcmH Y64W-C25S (5µM) was mixed with the dans-KGCVASHAI-OH (5µM) in 20 mM TrisHCl pH 8.0, 200 mM NaCl buffer. Trp was excited at 280 nm and the emission spectra recorded from 300 to 600 nm before and after addition of reduced *Pa*-CcmG (15µM).

Results and Discussion

Specific thiol-oxidoreductases involved in bacterial Cytc biogenesis

The periplasm is a highly reducing environment in which proteins with cysteines in their thiol (-SH) or thiolate ($-S^-$) state are present. (Inaba and Ito, 2008). The presence of an efficient oxidative system in the bacterial periplasm composed of the DsbAB proteins (Messens and Collet, 2006; Kadokura *et al.*, 2003) is responsible for the formation of a disulfide bond between the two Cys residues of apoCyt. This disulfide bond has to be specifically reduced in order to make the apoCyt competent for subsequent heme ligation. In gram-negative bacteria, such as the human pathogen *Pseudomonas aeruginosa*, this specialized thio-reduction pathway includes the membrane proteins CcmG and CcmH (Di Matteo *et al.*, 2010). The necessary reducing power is transferred from cytoplasmic thioredoxin (TRX) to CcmG *via* DsbD, a membrane protein with an N-terminal periplasmic domain with a IgG-like fold (nDsbD), a C-terminal periplasmic domain with a thioredoxin-like (TRX-like) fold (cDsbD) and a central transmembrane domain (Stirnimann *et al.*, 2005).

CcmG

CcmG is a membrane-anchored TRX-like protein. The overall structure of the soluble periplasmic domain of CcmG from *E. coli* (Ouyang *et al.*, 2006), *B. japonicum* (Edeling *et al.*, 2001), and *P. aeruginosa* (Di Matteo *et al.*, 2010) is well conserved (Rsmd=0.8Å between *Pa*-CcmG and *Ec*-CcmG; Rsmd=1.35Å between *Pa*-CcmG and *Bj*-CcmG).

Although all these proteins adopt a TRX-like fold and contain the redox-active motif CXXC, they are inactive in the classic insulin reduction assay (Monika *et al.*, 1997) (Fabianek *et al.*, 1997); they are therefore considered specific thiol-oxidoreductase able to recognize and selectively interact only with their upstream and downstream binding partners in the assembly line leading to mature Cytc.

The crystal structure of CcmG from *P. aeruginosa* (*Pa*-CcmG) was determined by us both in the oxidized (2.2Å resolution) and the reduced state (1.8 Å resolution) (Di Matteo *et al.*, 2010). The TRX domain of *Pa*-CcmG is composed of the canonical βαβ and ββα structural motifs linked by a short helix and forming a four-stranded β-sheet surrounded by three helices. It contains an additional N-terminal extension (residues 26–62) and a central insert (residues 102–123) (Figure 1). Structural superposition of the two redox states shows no substantial structural rearrangement (Rmsd of the Cα atoms in the two redox forms is 0.19 Å), therefore excluding it as a mechanism used by *Pa*-CcmG to discriminate between reduced (DsbD) and oxidized partners (*Pa*-CcmH or apoCyt).

Characterization of the functional properties of *Pa*-CcmG indicated that it might act as a mild reductant in the thioreductive branch of Cytc biogenesis. First of all, we determined its standard redox potential and noticed that its value ($E^{0'}$ = 0.213 V at pH 7.0) is similar to that obtained

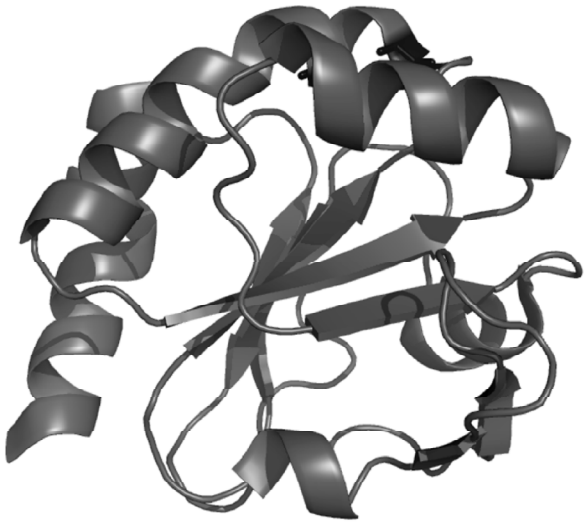


Figure 1: **Ribbon representation of the reduced *Pa*-CcmG structure (pdb code: 3KH7). The overall structure resembles a TRX fold with an N-terminal extension and a central insert.**

for *Pa*-CcmH ($E^{0'}$ = 0.215 V) (Di Matteo *et al.*, 2007). This finding is clearly against a linear redox cascade hypothesis, as depicted in Scheme 1. To gain further insights into the role played by *Pa*-CcmG in the thio-reductive branch of Cytc biogenesis, we determined the pK_a of active site thiols both in the wild-type protein and in the two single Cys-containing mutants, C74S and C77S. Contrary to the wild-type (showing two pH-dependent transitions), the ionization equilibria obtained for the two mutants showed a single transition with a pK_a = 6.13 ± 0.05 (C74S) and pK_a = 10.5 ± 0.07 (C77S). These results are in agreement with the microscopic Cys pK_a values measured in different TRXs, where the active N-terminal Cys residue has a pK_a close to pH 7.0, whereas the C-terminal Cys has a much higher pK_a (Kallis and Holmgren, 1980; Chivers and Raines, 1997). It has been shown that such a large difference between the two pK_a values in the TRX family is of functional significance (Chivers and Raines, 1997): it allows the N-terminal Cys (C74 in *Pa*-CcmG) to perform the nucleophilic attack on the target disulfide, while the C-terminal Cys (C77 in *Pa*-CcmG) is involved in the resolution of the resulting mixed-disulfide.

CcmH: an atypical thiol-oxidoreductase

CcmH is a membrane-anchored protein containing a redox-active CXXC motif, involved in the reduction of apoCyt. Surprisingly, when we solved the 3D structure of its soluble periplasmic domain in the oxidized redox state, we revealed that it adopts a peculiar three-helix bundle fold that is different from that of canonical thiol-oxidoreductases (Figure 2). This fold is unique among redox-active proteins, and indeed a search using DALI (Holm and Sander, 1996) returned significant hits only with unrelated α-helical proteins. At variance with the canonical arrangement of the redox-active CXXC motif observed in the TRX superfamily where the N-terminal Cys is always solvent-exposed, analysis of the accessible surface area reveals that in *Pa*-CcmH, the N-terminal C25 residue is buried, whereas the C-terminal C28 is solvent-exposed.

This finding suggested to us that, at variance with TRX proteins, C28 is the one presumed to perform the nucleophilic attack on the apoCyt disulfide, implying a mechanism different from that described in TRX and TRX-like proteins where the reactive cysteine is the N-terminal one, as proposed earlier (Fabianek *et al.*, 1999). Furthermore, functional characterization of *Pa*-CcmH highlighted peculiar pK_a values of the active site cysteines; indeed, we found similar pK_a values for both C25 and C28 (8.4 ± 0.1 and 8.6 ± 0.1). As recalled above, in TRX proteins characterized by aspecific thiol-oxidoreductase activity, the pK_a value of the cysteine performing

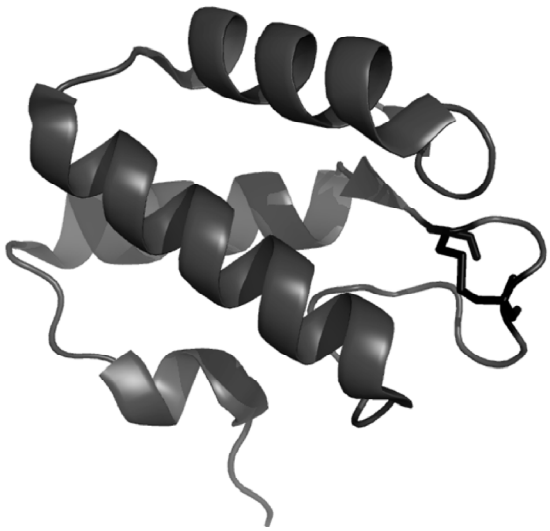


Figure 2: **Ribbon representation of the oxidized *Pa*-CcmH structure (pdb code: 2HL7). The figure shows the three-helix bundle forming the peculiar fold of *Pa*-CcmH. The active site disulfide bond between residues Cys25 and Cys28 in the long loop connecting helices 1 and 2 is high-lighted in black.**

the initial nucleophilic attack on its target substrate is significantly lower (i.e. 7) than the pK_a value of the Cys residue responsible for the resolution of the intermediate mixed-disulfide. It is tempting to speculate that the peculiar pK_a values of the *Pa*-CcmH active site thiols may ensure the necessary specificity of this component of the Ccm apparatus toward the apoCyt substrate.

Both the thiol-disulfide exchange mechanisms depicted in Scheme 1 and 2 above suggest that CcmH is the direct reductant of the apoCytc disulfide; *in vitro* experimental evidences for this hypothesis come from yeast two-hybrid experiments on the CcmH homologue from *A. thaliana* (Meyer *et al.*, 2005) and from FRET kinetic experiments, using a Trp-containing fluorescent variant of *Pa*-CcmH (Tyr64Trp) and a dansylated nonapeptide mimicking the heme binding motif of *P. aeruginosa* cytochromes c551 (dans-KGCVACHAI) (Di Matteo *et al.*, 2007). This experiment allowed measuring an off-rate constant (in the 0.2–1 s^{-1} range) which combines an adequate affinity (low μM) with the need to release reduced apoCyt to other component(s) of the System I maturase complex Di Matteo *et al.* (2007).

On the basis of the structural and functional data on *Pa*-CcmG and *Pa*-CcmH that we obtained, we hypothesized an assembly line for Cytc biogenesis in *P. aeruginosa* in which reduced *Pa*-CcmH (a non-thioredoxin-like thiol-oxidoreductase) specifically recognizes, binds and reduces oxidized apoCyt via the formation of a mixed disulfide complex, which is subsequently resolved by *Pa*-CcmG, as depicted in Scheme 2.

The interaction between CcmG and CcmH

As stated above, knowledge of the structural and functional properties of the proteins involved in the redox pathway of Cytc biogenesis is a necessary step, but it is insufficient to unveil the biochemical mechanism of this complex process. Indeed, although according to the linear redox cascade mechanism depicted in Scheme 1, *Pa*-CcmG is the direct reductant of oxidized *Pa*-CcmH, our attempts to monitor directly the formation of the mixed disulfide complex between these two redox-active proteins were unsuccessful (Di Matteo *et al.*, 2007). However, as shown in Scheme 2, *Pa*-CcmG may play a different role, being involved in the recognition and reduction of the mixed-disulfide complex between *Pa*-CcmH and apoCyt, as previously proposed also by others (Reid *et al.*, 2001). In an attempt to mimic the mixed-disulfide complex *Pa*-CcmH-apoCyt, we exploited the use of the Ellmann's reagent DTNB and of the single Cys containing mutant C25S of *Pa*-CcmH, which, upon mixing, form a mixed-disulfide complex (*Pa*-CcmH (C25S)-TNB). Specific reduction of this complex was observed by absorbance stopped-flow experiments when reduced *Pa*-CcmG was added to the solution (Di Matteo *et al.*, 2010). This result is in favour of the mechanism depicted in Scheme 2, and suggests that the target of the TRX-like *Pa*-CcmG is not oxidized *Pa*-CcmH, but rather an intermolecular disulfide bond formed between the C-terminal cysteine of *Pa*-CcmH and its substrate.

In an effort to reproduce the mixed-disulfide complex between *Pa*-CcmH and apoCyt occurring in *vivo*, here we present the result of a fluorescence resonance energy transfer (FRET) experiment carried out with the same dansylated nonapeptide (dansyl-KGCVASHAI) used for binding experiments (see above), and with the *Pa*-CcmH Y64W/C25S double variant. The result of this experiment is shown in Figure 3. By exciting the engineered donor Trp residue of the *Pa*-CcmH double variant, fluorescence emission at 530 nm by resonance energy transfer (FRET) was observed, indicating close proximity of the donor-acceptor pair and therefore the formation of the mixed-disulfide complex. Interestingly, addition of reduced *Pa*-CcmG determined the decrease of the FRET emission band, clearly indicating the *Pa*-CcmG mediated reduction of the disulfide bond between *Pa*-CcmH Y64W/C25S and the peptide. The same decrease of the FRET emission band was observed in a control experiment where the disulfide bond between *Pa*-CcmH Y64W/C25S and the peptide was reduced by the addition of DTT (data not shown). Although still qualitative, this observation adds further evidence that, in *vivo*, the physiological substrate of *Pa*-CcmG is the mixed-disulfide complex between *Pa*-CcmH and apoCyt, as previously proposed (Di Matteo *et al.*, 2010) (Fabianek *et al.*,

1999; Reid *et al.*, 2001). Furthermore, according to the mechanism we are beginning to delineate (see Scheme 2), it should be possible to trap the mixed disulfide complex between *Pa*-CcmH and *Pa*-CcmG, if the appropriate single Cys-containing mutant of the latter protein is used. Indeed, by employing the experimental strategy described above to activate *Pa*-CcmH (by DTNB) and using the mutant C77S of *Pa*-CcmG, unable to carry on the resolution of the resulting mixed-disulfide, we were able to trap the complex between DTNB-activated *Pa*-CcmH C25S and *Pa*-CcmG C77S. Figure 4A shows the SDS-PAGE under non-reducing and reducing conditions, highlighting the presence/absence of the mixed-disulfide complex and of the two isolated proteins, respectively. The purified complex (Figure 4B) has been crystallized and we are currently involved in solving its 3D structure. The results of these experiments will provide structural insights for understanding the molecular details of the *in vivo* reduction of apoCyt disulfide before heme attachment.

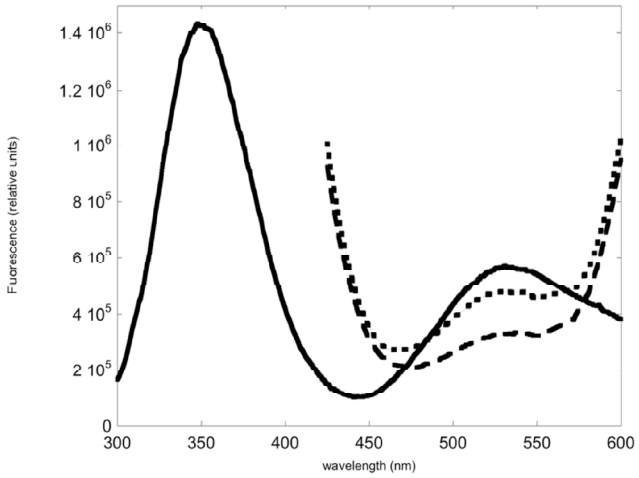


Figure 3: **Reduction by *Pa*-CcmG of the disulfide bond between *Pa*-CcmH and the dansylated peptide. Fluorescence emission spectrum of the mixed-disulfide complex between *Pa*-CcmH Y64W/C25S (5μM) and the peptide (dans-KGCVASHAI; 5μM) (solid line). Fluorescence emission spectrum of the mixed-disulfide complex reduced by *Pa*-CcmG (15μM) after 2 minutes (dotted line) and 30 minutes (dashed line).**

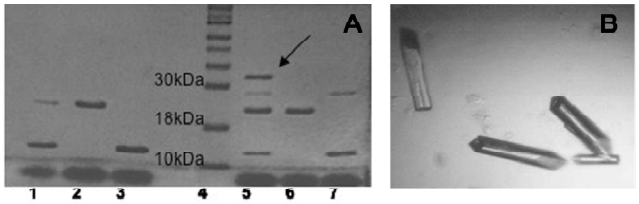


Figure 4: **Panel A. SDS-PAGE analysis of *Pa*-CcmG C77S, *Pa*-CcmH C25S, and their mixed-disulfide complex. Lanes 1-3: reducing conditions. Mixed-disulfide complex reduced by DTT yields the two single proteins *Pa*-CcmG C77S and *Pa*-CcmH C25S (lane 1); purified *Pa*-CcmG C77S (lane 2) and *Pa*-CcmH C25S (lane 3); Marker (lane 4). Lanes 5-7: non-reducing conditions. Lane 5: Mixed-disulfide complex (indicated by the arrow). The additional band below the complex is the Pa-CcmH dimer; purified *Pa*-CcmG C77S (lane 6) and *Pa*-CcmH C25S (lane7).**
Panel B. Typical crystals of the mixed-disulfide complex obtained by vapour diffusion using the hanging drop technique.

Acknowledgments

We thank Prof. Maurizio Brunori for helpful discussion and encouragement. Work partially supported by grant RBRN07BMCT_007 from MIUR (Italy).

Abbreviations

apoCyt, apocytochrome; Cytc, Cytochrome c; Ccm, cytochrome c maturation; TRX, thioredoxin; DTNB, dithiobis (2-nitrobenzoic acid); DTT, dithiothreitol; FRET, fluorescence resonance energy transfer.

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