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Recognition and binding of apocytochrome c to *P. aeruginosa* CcmI, a component of cytochrome c maturation machinery

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ABSTRACT

The biogenesis of c-type cytochromes (Cytc) is a process that in Gram-negative bacteria demands the coordinated action of different periplasmic proteins (CcmA-I), whose specific roles are still being investigated. Activities of Ccm proteins span from the chaperoning of heme *b* in the periplasm to the specific reduction of oxidized apocytochrome (apoCyt) cysteine residues and to chaperoning and recognition of the unfolded apoCyt before covalent attachment of the heme to the cysteine thiols can occur. We present here the functional characterization of the periplasmic domain of CcmI from the pathogen *Pseudomonas aeruginosa* (Pa-CcmI*). Pa-CcmI* is composed of a TPR domain and a peculiar C-terminal domain. Pa-CcmI* fulfills both the ability to recognize and bind to *P. aeruginosa* apo-cytochrome c551 (Pa-apoCyt) and a chaperoning activity towards unfolded proteins, as it prevents citrate synthase aggregation in a concentration-dependent manner. Equilibrium and kinetic experiments with Pa-CcmI*, or its isolated domains, with peptides mimicking portions of Pa-apoCyt sequence allow us to quantify the molecular details of the interaction between Pa-apoCyt and Pa-CcmI*. Binding experiments show that the interaction occurs at the level of the TPR domain and that the recognition is mediated mainly by the C-terminal sequence of Pa-apoCyt. The affinity of Pa-CcmI* to full-length Pa-apoCyt or to its C-terminal sequence is in the range expected for a component of a multi-protein complex, whose task is to receive the apoCyt and to deliver it to other components of the apoCyt:heme *b* ligation protein machinery.

1. Introduction

Cellular life relies on the function of a large number of proteins acting in highly regulated and intricate metabolic networks, which often do not perform their functions freely diffusing in the cytoplasm; different complex processes, such as protein synthesis, degradation, transport and many others are carried out by a battery of proteins acting in a concerted manner so that the cell should ultimately be considered as "a collection of protein machines" [1]. Understanding how the different proteins composing these "machines" act synergistically is a major challenge, which may be tackled *via* a detailed structural and functional characterization on each individual protein. The biogenesis of c-type cytochromes (Cytc), ubiquitous heme-containing proteins involved in cellular respiration, is an intricate post-translational process that in prokaryotes demands dedicated multi-protein complexes such as System I in Gram-negative bacteria and plant mitochondria and System II in Gram-positive bacteria and in the thylakoids of plants and algae [2,3]. These evolutionary conserved heme-containing proteins, being synthesized as unfolded apo-proteins, must bind stereospecifically Fe-protoporphyrin IX (heme *b*) in order to fold and to become functional. In gram-negative bacteria this process occurs in the periplasm after the newly synthesized apoCytochrome (apoCyt) has been translocated by the Sec system [4]. While in the mitochondria of eukaryotic cells Cytc biogenesis requires only one protein, the Cytc heme lyase CCHL [5,6], the bacterial System I is composed of a set of eight/nine proteins either spanning or anchored to the membrane, known as Cytochrome c maturation proteins (CcmA-I) [2,3]. This observation suggests the intriguing hypothesis of the involvement of Ccm proteins in other functions critical for bacterial physiology, such as growth and virulence [7], providing a rationale to explain why bacteria have evolved a metabolically expensive operon to accomplish an apparently simple task such as heme *b* ligation to apoCyt.

The functioning of the Ccm protein machinery is still to be understood at the molecular level, but evidences indicate that each Ccm protein plays a particular role in the assembly line needed to synthesize functional Cytc. It is yet to be unveiled how the heme *b* is translocated from the cytoplasm to the periplasm. Although it was initially hypothesized that CcmABC proteins were involved in heme translocation as they have the features of an ABC transporter, other studies have shown that this is not the case [8,9]. CcmE is a heme binding protein crucial for

Abbreviations: Pa-apoCyt, Pa-apocytochrome; Cytc, cytochrome c; Ccm, cytochrome c maturation; FRET, fluorescence resonance energy transfer; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; TPR, tetratricopeptide repeats; CS, citrate synthase

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the heme delivery to the other components of the Ccm apparatus; it has been studied in detail and structurally characterized [10,11]. As the newly synthesized apoCyt is translocated in the oxidizing environment of the periplasm [12], the two Cys residues in the heme-binding motif (CXXCH) must be reduced for the thioether bonds with the heme to be formed. Two redox-active Ccm proteins anchored to the membrane and facing their active site to the periplasm, i.e. CcmG and CcmH, carry out this highly specific thio-reduction process. While the former is a canonical thioredoxin-like protein [13–15], CcmH has been recognized, both from a structural and functional point-of-view, an atypical thiol-oxidoreductases specific to System I [16,17]. How the unfolded apoCyt is specifically recognized by the different components of System I and, above all, how the Ccm machinery catalyzes or promotes the formation of the heme-apoCyt covalent bonds, remains to be unveiled. Past observations and recent experiments indicate that CcmF and CcmI, possibly together with CcmH, are involved in these final steps [2,18–20]. CcmF is a large integral membrane protein containing a conserved periplasmic WWD domain [3] and conserved His residues probably involved in heme binding [21]. Surprisingly, it has been shown that CcmF from *E. coli* contains heme *b* as cofactor which led to the hypothesis that, in addition to its heme lyase function, it may act as a quinole: heme oxidoreductase, responsible for heme iron reduction [22].CcmI is a membrane bound protein; it has been shown that in *Rhodobacter capsulatus* it is composed of an N-terminal membrane-spanning domain including two transmembrane (TM) helices and a cytoplasmic loop, followed by a periplasmic tetratricopeptide repeat (TPR) domain [23]. The TPR repeat is a degenerate 34 amino acid sequence with a consensus pattern of conserved residues that bind specific peptide ligands, thought to mediate protein–protein interactions in a variety of biological systems [24]. Genetic studies have shown that inactivation of the ccmI gene in *R. capsulatus* and *Bradyrhizobium japonicum* leads to inability to synthesize functional c-type cytochromes [25–27]; however the exact role played by the CcmI protein in the Cytc maturation is still unclear. In plant mitochondria [2,3] and probably in some reducing bacteria such as *Desulfovibrio desulfuricans* [28], Cyt c maturation occurs in the absence of CcmI. In plants however, Cyt c maturation occurs via a highly divergent System I lacking CcmD and CcmG in addition to CcmI and showing the CcmF protein separated in three separate proteins ($CcmF_{N1}$, $CcmF_{N2}$, $CcmF_{C}$). In *D. desulfuricans* it has been shown that the presence of the CcmI protein is not essential for Cyt c maturation, although the protein is needed to enhance Cyt c production [29]. It has been proposed that CcmI may provide a platform for the unfolded apoCyt, chaperoning it to its heme *b* attachment site, probably located on CcmF. CcmI may thus form a membrane-integral multi subunit heme ligation complex together with CcmF and CcmH [18]. Moreover, very recently it has been shown that CcmI from *R. capsulatus* (Rc-CcmI), over and above the recognition of the apo form of cytochrome c2 (Rc-apoCyt), can interact also with the apo form of the heme binding protein CcmE [20,27].

In an effort to shed light on the final steps of Cytc biogenesis, we present here the characterization of the soluble periplasmic domain of CcmI from the γ-proteobacterium *Pseudomonas aeruginosa* (Pa-CcmI*; Fig. 1). We show here that Pa-CcmI*, as other CcmI proteins from different bacteria, is predicted to contain a TPR region; however, it is also endowed with a C-terminal region devoid of recognizable sequence features. We present a biochemical characterization of Pa-CcmI* and of its N-terminal TPR domain and C-terminal region as well as a quantitative characterization of the interaction between Pa-CcmI* and its substrate apo-cytochrome c551 (Pa-apoCyt); our equilibrium and kinetic FRET binding experiments carried out with Pa-CcmI* and its constituent TPR and C-terminal domains with appropriate labeled peptides, allowed us to provide for the first time a measure of the K_D for this interaction together with the relative association/dissociation rate constants. Moreover we report on the ability of Pa-CcmI* to act as a molecular chaperon by preventing protein aggregation in model systems. Our findings suggest that both the ability to bind Pa-apoCyt as well as the chaperone activity can be ascribed only to the TPR domain of Pa-CcmI*.

2. Materials and methods

2.1. Cloning, expression and purification of Pa-CcmI*, TPR and α-β domain

The soluble periplasmic domain of CcmI from *P. aeruginosa* (Pa-CcmI*, residues 116–407) was cloned into *NdeI*/*BamHI* digested pET28 b+ vector (Gene Art/Life Technologies). Expression of the His-Tag fusion protein in *E. coli BL21* was induced with 1 mM isopropyl-1-thio-D-galactopyranoside (IPTG); after induction cells were grown in Luria–Bertani (LB) broth supplemented with Kanamicin (30 μg/ml) at 25 °C for 15 hours. Cells were lysed in 50 mM sodium phosphate buffer pH 7.2, 200 mM NaCl, 50 mM Imidazole, 1 mM phenylmethanesulfonylfluoride (PMSF), 1 tablet of Complete Protease Inhibitor cocktail (Roche). Cell's lysate was loaded on a nickel(II)-charged chelating Sepharose FF column (Amersham Biosciences) and eluted with 0.2–1 M Imidazole gradient. The purified protein was buffer-exchanged in Tris–HCl 50 mM pH 8.0 with Sephadex G-25 Medium prepacked Columns (GE Healthcare). The yield of purified protein was ~30 mg/liter. The purity of the protein was confirmed by SDS–PAGE and its integrity was confirmed by mass spectrometry. The TPR domain (residues 116–279 of Pa-CcmI*) was obtained from Pa-CcmI* by replacing Proline residue (Pro 280) with a stop codon using Quick Change Mutagenesis Kit (Stratagene, La Jolla, CA). The α-β domain (residues 290–407 of Pa-CcmI*) was cloned into *NdeI*/*BamHI* digested pET28 b+ vector (Gene Art/Life Technologies). Both constructs were expressed and purified as described for Pa-CcmI*.

2.2. Cloning, expression and purification of Pa-apocytochrome c551 (Pa-apoCyt)

A synthetic gene coding for Pa-apoCyt was designed and successfully cloned into *NdeI*/*BamHI* digested pET28 b+ vector (Gene Art/Life Technologies). This gene lacks the N-terminal signal sequence for periplasmic translocation and carries the substitutions C12A and C15A to avoid cytochrome c maturation in the periplasm and aspecific heme attachment in the cytoplasm. Expression of the His-Tag fusion Pa-apoCyt in *E. coli* (BL21) was induced with 1 mM IPTG. After induction cells were grown in LB broth at 37 °C for 4 hours. Cells were lysed in 50 mM sodium phosphate buffer pH 7.2, 1 mM PMSF. Cell's lysate was loaded on a nickel(II)-charged chelating Sepharose FF column (Amersham Biosciences) and eluted with an Imidazole gradient (0–1 M imidazole) in 50 mM sodium phosphate buffer pH 7.2. Pa-apoCyt was further purified with an ion-exchange column (Q-Sepharose, Amersham Biosciences) in Tris–HCl 50 mM, pH 8.0 buffer. The purity of the eluted protein was confirmed by SDS–PAGE.

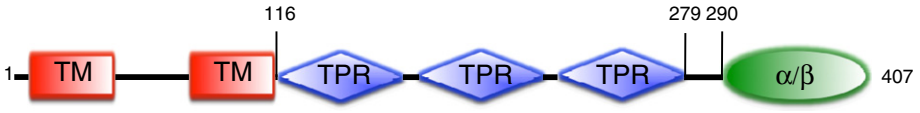

Fig. 1. Domain organization of CcmI from *P. aeruginosa*. Pa-CcmI is anchored to the inner membrane by two TM helices. Starting from the N-terminal region, the soluble periplasmic domain Pa-CcmI* (residues 116–407) is composed by a domain containing three TPR repeats (residues 116–279), followed by an additional α-helix and a linker region, and a C-terminal domain, predicted to contain α and β segments (residues 290–407).