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Subunit CcoQ is involved in the assembly of the Cbb_3-type cytochrome *c* oxidases from *Pseudomonas stutzeri* ZoBell but not required for their activity

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ABSTRACT

The Cbb_3-type cytochrome *c* oxidases (Cbb_3-CcOs), the second most abundant CcOs, catalyze the reduction of molecular oxygen to water, even at micromolar oxygen concentrations. In *Pseudomonas stutzeri* ZoBell, two tandemly organized *cbb*$_3$-operons encode the isoforms Cbb_3-1 and Cbb_3-2 both possessing subunits CcoN, CcoO and CcoP. However, only the *cbb*$_3$-2 operon contains an additional *ccoQ* gene. CcoQ consists of 62 amino acids and is predicted to possess one transmembrane spanning helix. The physiological role of CcoQ was investigated based on a CcoQ-deletion mutant and wild-type Cbb_3-2 crystals not containing subunit CcoQ. Cbb_3-2 isolated from the deletion mutant is inactive and appears as a dispersed band on blue native-PAGE gels. Surprisingly, in the absence of *ccoQ*, Cbb_3-1 also shows a strongly reduced activity. Our data suggest that CcoQ primarily functions as an assembly factor for Cbb_3-2 but is also required for correct assembly of Cbb_3-1. In contrast, once correctly assembled, Cbb_3-1 and Cbb_3-2 possess a full enzymatic activity even in the absence of CcoQ.

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1. Introduction

Cytochrome *c* oxidases (CcOs) are the terminal enzymes of the aerobic respiratory chains in eukaryotes and many prokaryotes. CcOs catalyze the four electron reduction of molecular oxygen to water and couple proton pumping across the biological membrane to this exergonic reaction process, thus contributing to the generation of the electrochemical proton gradient across the membrane.

CcOs belong to the superfamily of heme-copper containing terminal oxidases (HCOs), which can be classified into three families: A, B and C [1]. The crystal structure of at least one member of each family has been determined [2–5]. The Cbb_3-type CcOs (Cbb_3-CcO) form the C-family and are predominantly found in proteobacteria [6]. The structural architecture of the homologous central catalytic subunits is the only common feature of *cbb*$_3$-CcO and A- and B-family HCOs. In the genome of many *Pseudomonades* two tandemly arranged *cbb*$_3$-operons are present and encode two isoforms of *cbb*$_3$-CcO (Cbb_3-1 and Cbb_3-2) [7]. In *Pseudomonas stutzeri* ZoBell, both *cbb*$_3$-operons comprise the structural genes *ccoN*, *ccoO* and *ccoP*. An additional fourth gene (*ccoQ*) is present in *cbb*$_3$-2 only. Subunits CcoO and CcoP contain one and two heme *c* molecules, respectively. The catalytic subunit CcoN comprises a low-spin heme *b* and the active center consisting of a high-spin heme b_3 and Cu_B [5]. CcoQ is not present in the crystallized Cbb_3-1 from *P. stutzeri* [5,8]. To date, the physiological role of CcoQ was studied in the *cbb*$_3$-CcOs from *Bradyrhizobium japonicum* [9], *Rhodobacter capsulatus* [10] and *Rhodobacter sphaeroides* [11]. However, the functional significance of subunit CcoQ in the Cbb_3-complex is still under debate (see Section 4).

In this work, we set out to characterize the physiological role of CcoQ in Cbb_3-2 of *P. stutzeri*. We created a CcoQ deletion mutant (Cbb_3-2-ΔCcoQ), and compared the resulting variant and the wild-type Cbb_3-2 using poly acrylamide electrophoresis (PAGE), ultraviolet-visible light (UV-vis) spectroscopy and oxygen reductase activity measurements. Furthermore, Western blotting and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) measurements were applied to assess the presence of CcoQ in crystals of wild-type Cbb_3-2. Interestingly, dissolved wild-type Cbb_3-2 crystals do not contain CcoQ, yet retain full oxygen reductase activity. These results suggest that CcoQ is mainly involved in protein assembly. Surprisingly, in the absence of *ccoQ*, also Cbb_3-1 showed significantly reduced enzymatic activity demonstrating that CcoQ additionally contributes to the correct assembly of Cbb_3-1.

Abbreviations: CcO, Cytochrome *c* oxidase; UV-vis, ultraviolet-visible light; PAGE, poly acrylamide electrophoresis; IGR, intergenic-region; rec., recombinant; TMH, transmembrane-spanning helix; TMPD, N,N,N′,N′-tetramethyl-phenyl-enediamine dihydrochloride.

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2. Material and methods

2.1. Bacterial strains, media, and oligonucleotides

Wild-type *Pseudomonas stutzeri* strain ZoBell (ATCC 14405) and a *P. stutzeri* Cbb_3-2 knock-out strain (ΔCbb_3-2) were used throughout this work (Table S1). *P. stutzeri* cells were grown on lysogeny broth (LB) agar plates or in L-asparagine minimal medium at 32 °C [12,13]. Antibiotics were added in concentrations of 100 μg/ml for kanamycin and 170 μg/ml for chloramphenicol. *Escherichia coli* strain DH5α was used for general cloning procedures [14]. DNA sequencing was performed by Eurofins MWG Operon (Ebersberg, Germany). Plasmids and synthetic oligonucleotides used in this study are listed in Table S1.

2.2. Construction of ΔccoQ variants

The vectors pXH-22 and pXH-39 contain the *cbb*$_3$-1 and *cbb*$_3$-2 operons, respectively [15]. Fig. S1 illustrates the intergenic-region (IGR) between the *ccoO* and *ccoP* genes. The oligonucleotides Cbb_3-2-ΔCcoQ_Fw and Cbb_3-2-ΔCcoQ_Rev were used to delete *ccoQ* in pXH-39. PCR-amplified vector DNA was blunt-end ligated resulting in pMK-Cbb_3-2-ΔCcoQ. In order to retain a ribosomal binding site for *ccoP*-2, the plasmid pMK- Cbb_3-2-ΔCcoQ + IGR was subsequently constructed. For this purpose, the IGR between *ccoO*-1 and *ccoP*-1 was amplified from the vector pXH-22 using oligonucleotides Cbb_3-2-ΔCcoQ + -IGR_IF_Fw and Cbb_3-2-ΔCcoQ + IGR_IF_Rev, and ligated between the *ccoO* and *ccoP* genes of pMK- Cbb_3-2-ΔCcoQ (In-Fusion cloning kit; Clontech, Montain View, CA, USA).

2.3. P. stutzeri cultivation and Cbb_3-CcO purification

P. stutzeri ZoBell cells were cultured under microaerobic conditions [12]. Cell harvesting, membrane preparation and protein solubilization were performed as described previously [12,13]. Genomically encoded true wild-type Cbb_3-1 was purified from the membranes prepared from wild-type *P. stutzeri* strain using a four-step purification procedure [13]. If not mentioned otherwise, plasmid encoded recombinant (rec.) wild-type Cbb_3-1 and Cbb_3-2 were produced in the *P. stutzeri* ΔCbb_3-1 using the expression vectors pXH-22 and pXH-39, respectively. The CcoQ-deletion variant (Cbb_3-2-ΔCcoQ) was produced in the *P. stutzeri* ΔCbb_3-2 strain using the pMK-Cbb_3-2-ΔCcoQ vector. Purification of (Strep)-tagged rec. Cbb_3-CcOs was performed as previously described [15]. The purified protein was concentrated and stored in a buffer containing 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 μM EDTA, 10% (v/v) glycerol and 0.02% (w/v) n-dodecyl-β-D-maltoside (DDM). Crystallization of Cbb_3-2 was performed for potential subsequent X-ray structure determination. Rec. wild-type Cbb_3-2 intended for crystallization was isolated using four chromatographic steps consisting of Strep-Tactin affinity chromatography, chromoatofocusing, buffer exchange on a PD-10 column and detergent exchange on DEAE Sepharose FF.

2.4. Immunoprecipitation of CcoQ

A customized polyclonal anti-CcoQ antibody, which binds to an epitope in the C-terminal hydrophilic domain of CcoQ (DDETDAKKREEEASRSKK-COOH), was raised in rabbit (Thermo Fisher Scientific, Waltham, MA, USA). For the pull-down assay, we incubated the purified Cbb_3-2 enzyme with anti-CcoQ antibody cross-linked Protein A Sepharose High Performance matrix according to the manufacturer's protocol (GE Healthcare, Little Chalfont, UK). Binding was achieved in a buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl,. The isolation of CcoQ-depleted CcoNOP-2 complexes was achieved using a spin-down protocol provided by the manufacturer. The CcoNOP-2 complex without subunit CcoQ was found in the flow through buffer (50 mM Tris-HCl, pH 7.5, 150 mM, 0.02% (w/v) DDM). CcoQ is bound to the anti-CcoQ antibody cross-linked to Protein A matrix. The bound CcoQ can be eluted using buffer containing 100 mM glycine, pH 2.5, 0.02% (w/v) DDM.

2.5. SDS-PAGE, BN-PAGE and Western blot

The purified Cbb_3-CcOs were analyzed using sodium dodecyl sulfate (SDS)-PAGE on self-cast 12.5% Tris-glycine gels [16] stained with silver (SilverQuest Staining Kit, Invitrogen, Carlsbad, CA, USA). Blue native (BN)-PAGE [17] was performed on precast 4–16% Bis-Tris gels as described by the manufacturer (Novex, Life Technologies, Darmstadt, Germany). For the detection of CcoQ by Western blot, proteins (1 μg Cbb_3-CcO/lane) were transferred from precast 12% Bis-Tris SDS-PAGE gels onto polyvinylidene difluoride (PVDF) membrane using a semi-dry Blotter (Invitrogen). Subsequently, the membrane was incubated with anti-CcoQ antibody. Monoclonal anti-rabbit alkaline phosphatase conjugated antibody (Sigma-Aldrich, St. Louis, MO, USA) was used as secondary antibody, and the color reaction was developed using 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT).

2.6. UV-vis spectroscopy, pyridine hemochrome assay and ligand binding spectra

A Lambda 35 UV-vis spectrophotometer (PerkinElmer, Waltham, MA, USA) was employed to record absorption spectra of Cbb_3-CcO at protein concentrations of 1–2 μM. The concentration of air oxidized Cbb_3-CcO was determined using a molar extinction coefficient of $5.85 \times 10^5\ M^{-1}\ cm^{-1}$ at 411 nm [18]. Absorption spectra of reduced Cbb_3-CcO were obtained after addition of small amounts of dithionite. The concentration of Cbb_3-2 obtained from crystals dissolved in low amounts of buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.02% [w/v] DDM) was analyzed using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). The calculated concentrations were compared and subsequently normalized to concentrations of *cbb*$_3$-CcO preparations pre-determined via NanoDrop 1000 and UV-vis spectroscopy.

The content of *b*- and *c*-type hemes in Cbb_3-CcO was determined using the pyridine hemochrome assay. Briefly, 5–10 μM of purified Cbb_3-CcO were added to a solution of 100 mM NaOH, 20% (v/v) pyridine and 3 μl of 100 mM potassium hexacyanoferrate (III). After recording a steady absorbance of oxidized pyridine hemochromes, the samples were reduced by addition of a few grains of solid dithionite. The reduced-minus-oxidized difference absorbance spectra were used to quantify the concentrations of *b*- and *c*-type hemes according to Berry and Trumpower [19]. To calculate the heme *c* and heme *b* molecules per oxidase, the heme *c* and heme *b* content was normalized to the protein concentration used.

For the binding of CO, 1–3 μM Cbb_3-CcO was reduced using 2 mM sodium-ascorbate and 2 μM phenazine methosulfate, followed by changing to an N_2 atmosphere. Subsequently, N_2 was replaced by CO. The reduced-CO *minus* reduced difference absorbance spectra were analyzed in the Soret region for the presence of heme b_3-CO adduct.

2.7. Oxygen reductase activity measurement

A Clark-type oxygen electrode linked to a PA 2000 picoammeter (Unisense, Aarhus, Denmark) was used to measure the oxygen consumption rates of the purified Cbb_3-CcO. Oxygen consumption measurements were performed with samples stirred in a 2 ml glass vial (reaction volume: 600 μl) in a water bath at room temperature. The oxygen consumption was initiated by adding 8.3 nM of purified Cbb_3-CcO to buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.02% [w/v] DDM) containing 3 mM sodium-ascorbate and 1 mM N,N,N′,N′-tetramethyl-phenyl-enediamine dihydrochloride (TMPD) [15]. Oxygen reductase activity of Cbb_3-CcO was calculated in electrons/second.

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