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Stability, redox parameters and electrocatalytic activity of a cytochrome domain from a new subfamily

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ABSTRACT

We report a spectroscopic, electrochemical and spectroelectrochemical characterization of the soluble cytochrome *c* domain (Cyt-D) from the *Rhodothermus marinus* caa_3 terminal oxygen reductase and its putative electron donor, a high potential [4Fe–4S] protein (HiPIP). Cyt-D exhibits superior stability, particularly at the level of the heme pocket, compared to archetypical cytochromes in terms of thermal and chemical denaturation, alkaline transition and oxidative bleaching of the heme, which is further increased upon adsorption on biomimetic electrodes. Therefore, this protein is proposed as a suitable building block for electrochemical biosensing. As a proof of concept, we show that the immobilized Cyt-D exhibits good electrocatalytic activity towards H_2O_2 reduction. Relevant thermodynamic and kinetic electron transfer parameters for Cyt-D and HiPIP are also reported, including reorganization energies of 0.33 eV and 0.42 eV, respectively.

1. Introduction

C-type monoheme cytochromes are mostly small soluble proteins involved in respiratory and photosynthetic electron transport chains [1–3]. In eukaryotes cytochrome *c* (Cyt-c) shuttles electrons from the bc_1 complex to the terminal respiratory enzyme in the inner mitochondrial membrane, a heme–copper oxygen reductase and, in addition, it plays a crucial role in apoptosis [4–6].

A common feature to all cytochromes *c* is that the heme prosthetic group is covalently attached to the protein via two thioether bonds to cysteine residues that usually present a CXXCH motif, with the histidine residue acting as one of the axial ligands of the heme iron. The other axial position is most often occupied by a methionyl ligand, leading to a six-coordinated low spin complex, but can also remain either vacant or coordinated by other amino acids such as histidine, cysteine or lysine [1,3,7].

Class I cytochromes represent a large group of single domain low-spin C-type heme proteins that, according to sequence, phylogeny and function, have been classified into 16 subclasses. All members of the family present a fold that includes a minimum of three α helices arranged around the heme group, and further less conserved structural elements [7,8].

In addition to the soluble electron shuttles, C-type cytochromes may also be part of larger redox enzymes and, moreover, heme *c* containing protein domains can also be found fused to other domains. This is the case of the caa_3 terminal oxygen reductase from *Rhodothermus marinus*, a Gram-negative strict aerobe thermophile bacterium [9–14]. This enzyme belongs to the A2 subfamily of oxygen reductases. As in the mammalian counterparts, the catalytic site of the *R. marinus* enzyme is constituted by a binuclear heme a_3–Cu_B center located in the subunit I. The main difference refers to subunit II which, in addition to the canonical Cu_A site, contains a cytochrome *c* domain (Cyt-D) in the C-terminus that has been suggested as the primary acceptor of electrons transported by the putative donor, a periplasmic high-potential iron–sulfur protein (HiPIP) [9–14]. Cyt-D has been overexpressed in *Escherichia coli*, yielding a well folded and stable soluble protein that retains the spectroscopic and redox properties of the domain in the holoenzyme [15–17].

The structure of Cyt-D has been solved at 1.3 Å resolution, revealing a heme group covalently linked to cysteines 16 and 19 and buried into the protein matrix, exposing only partially the substituent of ring C to the solvent. Methionine 74 and histidine 20 are the axial ligands of the six-coordinated low spin heme iron. The secondary structure comprises three relatively long and two short α-helices, two antiparallel β-strands that are connected by random coil segments forming a small β-sheet, and two extended loops. A comparison of the amino acid sequences of Cyt-D and other monoheme cytochromes from all known families shows similarities only with the C-fragments of caa_3 oxygen reductases, thus revealing a new subfamily of cytochromes distinct from the archetypical C-type proteins [15].

Abbreviations: Cyt-D, soluble cytochrome *c* domain of the caa_3 O_2-reductase from *R. marinus*; HiPIP, high potential iron–sulfur protein; TR-SERR, time-resolved surface-enhanced resonance Raman; GuHCl, guanidine hydrochloride.

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In recent work we have investigated the electron transfer (ET) dynamics of Cyt-D in biomimetic complexes by employing a novel combination of experimental and computational methods that allowed for the identification of the most likely electron entry and exit points of Cyt-D, as well as for the interaction domains with the redox partner proteins [17].

Here we report crucial thermodynamic and kinetic redox parameters of Cyt-D and its putative electron donor HiPIP [18]. We also assess the stability of Cyt-D towards thermal and chemical denaturation, alkaline transitions and bleaching by reactive oxygen species. It is shown that, in general terms, this protein is characterized by higher stability than canonical counterparts such as horse heart Cyt-c and, therefore, it appears as a promising building block for the construction of bioelectronics devices. As a proof of concept, we tested the electrocatalytic activity of Cyt-D immobilized on biocompatible electrodes towards hydrogen peroxide reduction (pseudoperoxidase activity).

2. Experimental section

2.1. Chemicals

4-Mercapto-1-butanol (C_4-OH), 1-butanethiol (C_3-CH_3), 6-mercapto-1-hexanol (C_6-OH), 1-hexanethiol (C_5-CH_3), 8-mercapto-1-octanol (C_8-OH), 1-octanethiol (C_7-CH_3), 11-mercapto-1-undecanol (C_{11}-OH), 1-undecanethiol (C_{10}-CH_3), 16-mercapto-1-hexadecanol (C_{16}-OH), 1-hexadecanethiol (C_{15}-CH_3), 16-mercaptohexadecanoic acid (C_{15}-COOH), N-cyclohexyl-N′-(2-morpholinoethyl) carbodiimide metho-p-toluenesulfonate (CMC) and N-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich. Eicosane-1-thiol (C_{19}-CH_3) and 20-mercaptoeicosan-1-ol (C_{20}-OH) were synthesized and purified as described before [17]. Hydrogen peroxide was purchased from Mallinckrodt Baker Inc. All reagents were of the highest available purity and used without further purification. Solutions were prepared with deionized water (R ≥ 18 MΩ; Millipore).

2.2. Proteins

The protocols for protein expression and purification have been described in detail elsewhere. Briefly, Cyt-D and HiPIP were obtained by expression in *E. coli* cells, followed by cell breaking in a French press, ultracentrifugation and thorough chromatographic purification [15,18].

2.3. Raman experiments

Resonance Raman (RR) spectra were acquired employing a confocal microscope coupled to a single stage spectrograph (Jobin Yvon XY) equipped with a 1800 L/mm grating and liquid nitrogen cooled back illuminated CCD detector. Elastic scattering was rejected with Notch filters. The 413 nm line of a cw. krypton ion laser (Spectra Physics BeamLok 2060) was focused onto the surface of a quartz rotating cuvette containing the sample by means of a long working distance objective (20×; N.A. 0.35). Typically, experiments were performed with laser powers of ca. 2 mW at the sample and acquisition times between 2 and 10 s.

Silver ring working electrodes for surface-enhanced resonance Raman (SERR) spectroelectrochemistry were polished and subjected to oxidation–reduction cycles to create a SERR-active surface, as previously described [19]. The electrodes were incubated into ethanolic solutions of the corresponding alkanethiol mixtures (ca. 1 mM total concentration) overnight to produce self-assembled monolayers (SAMs), rinsed and transferred to the spectroelectrochemical cell. The SERR spectroelectrochemical cell has been described before [19]. It contains a Pt wire and a Ag/AgCl (3.5 M KCl) electrode as counter and reference electrodes, respectively. During SERR measurements the working electrode was rotated at ca. 5 Hz to avoid laser-induced sample degradation. The electrolyte solution was 12.5 mM phosphate buffer (PBS), pH 7.0; 12.5 mM K_2SO_4. Protein adsorption was achieved by 15 minute incubation of the SAM-coated electrode in the SERR cell containing approximately 0.1 μM Cyt-D in the working electrolyte, at −400 mV. SERR spectra were measured in back-scattering geometry.

For time-resolved (TR-SERR) experiments, potential jumps of variable height and duration were applied to trigger the reaction. The SERR spectra were measured at variable delay times after each jump [20]. Synchronization of potential jumps and measuring laser pulses was achieved with a four channel pulse-delay generator (BNC-565) and a home-made pulse amplifier. The measuring pulses were generated by passing the cw laser beam through two consecutive laser intensity modulators (Linos Photonics). After background subtraction the spectra were treated by component analysis in which the spectra of the individual species were fitted to the measured spectra using home-made analysis software. The time resolution of the set-up is determined by the time constant of the electrochemical cell, which has been determined ca. 100 μs for the typical experimental conditions reported throughout this work.

2.4. Electrochemical measurements

Cyclic voltammetry (CV) and chronoamperometric experiments were performed either with a potentiostat/galvanostat PAR model 263A or with a Gamry REF600 electrochemical workstation, using a jacketed single compartment electrochemical cell (Dr. Bob's cell, Gamry) under Ar and contained inside a Faraday cage (Vista Shield, Gamry). As working electrodes we employed either home-made gold bead electrodes (1 mm diameter) or 3 mm diameter alumina-polished Au disk electrodes (Gamry). The system is completed with a Pt wire counter electrode and an Ag/AgCl (3.5 M KCl) reference electrode to which all potentials reported in this work are referred. The home-made working electrodes were cleaned by first annealing, then performing an oxidation at 2 V in 1 M $HClO_4$ and were finally treated in an ultrasonic pool for about 30 min. To remove residual adsorbed impurities, all electrodes were subjected to 30 voltammetric cycles between −0.2 and 1.6 V at 0.3 V/s in 1 M $HClO_4$. The surfaces of the clean electrodes were modified by overnight incubation into 1 mM ethanolic solutions of the desired alkanethiols. The SAM-coated electrodes were thoroughly washed with ethanol and water, subjected to 20 voltammetric cycles between −0.3 and 0.3 V at 0.3 V/s in PBS pH 7 and then incubated overnight into 200 μM solutions of Cyt-D for protein adsorption (PBS pH 7).

2.5. UV–vis absorption

UV–visible absorbance spectra were recorded on a Thermo Scientific Evolution Array spectrophotometer using 1.0 nm spectral bandwidth. Unless stated otherwise, Cyt-c and Cyt-D solutions were prepared in 10 mM PBS, pH 7.0, at 25 °C.

2.6. Circular dichroism

CD spectra measurements were conducted with a Jasco J-815 spectropolarimeter and were collected in the far-UV (260–200 nm) region. The experiments were performed on Cyt-D samples prepared in 20 mM Tris/HCl buffer, and placed in a 1 mm cell at 25 °C.

3. Results and discussion

3.1. Cyt-D stability

R. marinus is a thermophile bacterium with ca. 65–70 °C optimal growth temperature; hence implying that its terminal caa_3 oxygen reductase complex is thermally stable within this temperature range in the native environment. Cyt-D, however, is only a soluble domain