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Thermodynamic and kinetic characterization of PccH, a key protein in microbial electrosynthesis processes in *Geobacter sulfurreducens*

Telma C. Santos [1], André R. de Oliveira [1], Joana M. Dantas, Carlos A. Salgueiro *, Cristina M. Cordas *

UCIBIO-REQUIMTE, Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Campus Caparica, 2829-516 Caparica, Portugal

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ABSTRACT

The monoheme *c*-type cytochrome PccH from *Geobacter sulfurreducens*, involved in the pathway of current-consumption in biofilms, was electrochemically characterized in detail. Cyclic voltammetry was used to determine the kinetics and thermodynamics properties of PccH redox behavior. Entropy, enthalpy and Gibbs free energy changes associated with the redox center transition between the ferric and the ferrous state were determined, indicating an enhanced solvent exposure. The midpoint redox potential is considerably low for a monoheme *c*-type cytochrome and the heterogeneous electron transfer constant rate reflects a high efficiency of electron transfer process in PccH. The midpoint redox potential dependence on the pH (redox-Bohr effect) was investigated, over the range of 2.5 to 9.1, and is described by the protonation/deprotonation events of two distinct centers in the vicinity of the heme group with *pKa* values of 2.7 (pK_{ox1}); 4.1 (pK_{red1}) and 5.9 (pK_{ox2}); 6.4 (pK_{red2}). Based on the inspection of PccH structure, these centers were assigned to heme propionic acids P_{13} and P_{17}, respectively. The observed redox-Bohr effect indicates that PccH is able to thermodynamically couple electron and proton transfer in the *G. sulfurreducens* physiological pH range.

1. Introduction

The cytochrome PccH from *Geobacter sulfurreducens* (*Gs*) is a 15 kDa protein containing 129 amino acids with one *c*-type heme-binding sequence CXXCH close to the N-terminus. The heme Fe ion is low-spin, both in the reduced and oxidized forms, and axially coordinated by a histidine and a methionine residues (Fig. 1) [1,2]. The genome of *G. sulfurreducens* encodes for a total of 14 small monoheme cytochromes (10–15 kDa). However, a BLAST search with the PccH sequence did not show any significant homology with other monoheme cytochromes from *Gs* [2]. On the other hand, this search revealed a relatively high homology with a monoheme *c*-type cytochrome from *Pelobacter propionicus* (70% identity), but modest homology (~50%) with six cytochromes from other organisms [1]. A dendogram produced with these sequences and those of Ambler's class I cytochromes showed that PccH and related proteins form a separate group, suggesting that they are representatives of a new subclass within the class I cytochromes [2]. This was further supported by the structural features of PccH [2]. In fact, the structure of PccH is unique among the monoheme cytochromes of class I known to date. The structural fold of PccH can be described as forming two lobes, one N-lobe consisting of residues 1–63 and 125–129; and one C-lobe consisting of residues 64–124 (Fig. 1). The two lobes have their own hydrophobic cores and the heme is sandwiched in a cleft between the two lobes. This contrasts with the other cytochromes, which usually form one globular domain, with the polypeptide chain wrapping around the heme placed in the central part of the hydrophobic core of the protein. Consequently, in the latter cytochromes the heme group is more buried in the protein core. The unique location of PccH heme group in the cleft between the two lobes increases its solvent exposure and might explain why this cytochrome displays the lowest reduction potential observed to date for a monoheme cytochrome (−35 mV vs NHE at pH 8) [1].

In addition to the structural and functional features described above, the biological context of cytochrome PccH is also quite unique. The protein plays a crucial role in *Gs* current-consuming biofilms, as shown by comparative microarray analysis of gene transcript abundance in current-consuming versus current-producing biofilms [3]. The gene encoding for PccH showed clearly the largest transcript abundance and its deletion completely inhibited the electron transfer from electrode surfaces [3]. Thus, cytochrome PccH is the first protein identified as crucial for *Geobacter*-based microbial electrosynthesis, a process that can be used by cells to reduce CO_2 to methane or multi-carbon fuels, thus opening new perspectives in the field of bioremediation and biofuel production [4,5]. To develop these applications, understanding the molecular mechanisms by which microorganisms can accept electrons from electrodes is essential. Therefore, it is crucial to investigate the kinetics and thermodynamics properties of the electron transfer components that ultimately regulate the cellular redox balance. So far, reports in literature focus mainly on the electrochemical behavior

* Corresponding authors.
E-mail addresses: csalgueiro@fct.unl.pt (C.A. Salgueiro), c.cordas@fct.unl.pt (C.M. Cordas).
[1] These authors contributed equally to the work.

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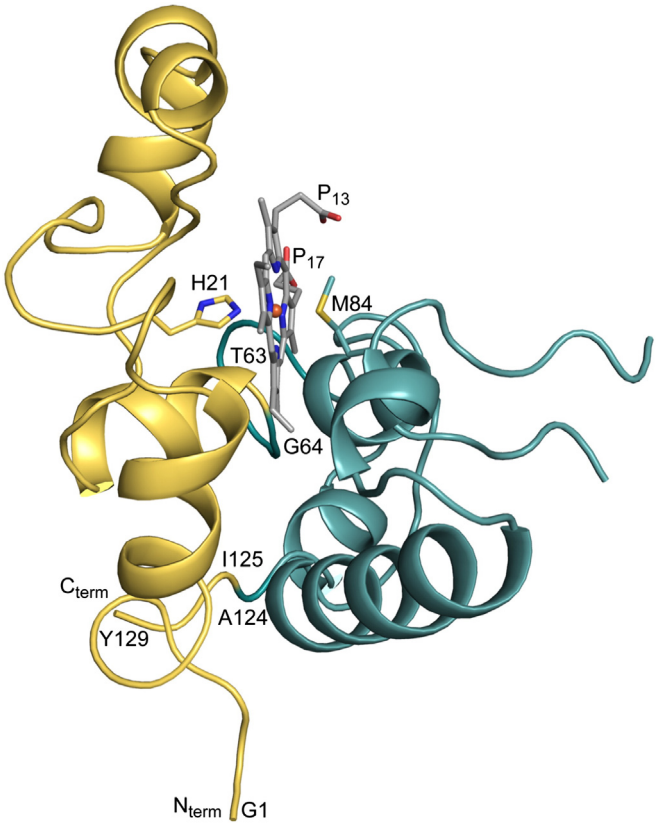

Fig. 1. Crystal structure of cytochrome PccH from *G. sulfurreducens* (PDB code, 4DWL [2]). The N-lobe (residues 1–63 and 125–129) and C-lobe (residues 64–124) are colored gold and blue, respectively. The heme is colored gray and the side chains of the axial ligands (His^{21} and Met^{84}) are shown in stick representation with atom type colors. Figure was produced using the program PyMOL [39].

of *Gs* cells, particularly within biofilms (for a review see [6–8]), with only a few describing the individual protein features [9]. An efficient electronic transfer occurs when the electrode substitutes the physiological redox partner and the analysis of the obtained response contributes to the understanding of the protein structure, function and reaction mechanisms [10–12]. Several electrochemical methods have been used to study the electronic transfer between proteins and electrodes, from classical bulk electrochemistry approach, in which the biomolecules are in solution, with or without the use of chemical mediators, to other innovative methods such as immobilization of the biological material on electrode surfaces [13,14]. In the last years enormous advances have been accomplished in the study of electrochemical reactions in biomolecules through direct electron transfer. The immobilization of proteins on electrode surfaces has demonstrated to be an efficient method to achieve high transfer rates between proteins and the electrodes. Among the possible methods, physical immobilization by adsorption or by entrapment on cellulose membranes has been extensively used by the inherent advantages such as the easiness of preparation, low cost and simplification of the theoretical analysis [11,14–17].

Here we present the first direct electrochemical studies of *Gs* cytochrome PccH, by cyclic voltammetry, using a thin-layer regime obtained with the entrapment of the protein in a membrane pyrolytic graphite electrode. Kinetic and thermodynamic data on the PccH redox behavior were attained. The effect of the pH on the redox center midpoint potential was also investigated and the structural information recently obtained for PccH was used to fingerprint the responsible centers for the redox-Bohr effect, a crucial property that allows PccH to couple electron and proton transfer.

2. Experimental

2.1. Expression and purification of cytochrome PccH

Cytochrome PccH was produced and purified as previously described [1]. In a few words, competent cells of *Escherichia coli* strain BL21(DE3) (Novagen) harboring plasmid pEC86, containing the cytochrome *c* maturation gene cluster, *ccmABCDEFGH*, were co-transformed with plasmid pCS3274 that encodes for mature PccH. Cells were grown in 2xYT medium at 303 K and supplemented with 34 μg/mL chloramphenicol (Sigma) and 100 μg/mL ampicillin (Sigma). Protein expression was induced with 100 μM isopropyl β-D-thiogalactopyranoside (IPTG). After 16–18 h, cells were harvested by centrifugation at 6400 ×*g* for 20 min and then lysed by osmotic shock in the presence of lysozyme. The periplasmic fraction was recovered by two centrifugation steps at 15,000 ×*g* for 20 min and at 225,000 ×*g* for 1 h 30 min, both at 277 K. The supernatant constitutes the periplasmic fraction, which was dialyzed twice against 10 mM Tris–HCl pH 8 and then purified by cation exchange and gel filtration chromatography. The purity of the protein was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) stained with Coomassie blue.

2.2. Electrochemical studies

The electrochemical assays were performed using a μAUTOLAB type III or a CH instruments, model CHI 440b potentiostat, in a single compartment electrochemical cell (Metrohm) and three electrodes configuration. Pyrolytic graphite (PG) disk was used as working electrode (diameter 4 mm). A platinum wire and a saturated calomel electrode (SCE) were the counter and reference electrodes, respectively. The working electrode was polished with alumina with different grades, then immersed in Millipore water in an ultra-sound bath and finally thoroughly rinsed with Millipore water. A small amount of protein (7 μL, 100 μM) was immobilized on the electrode using a cellulose membrane (spectra/Por) with a 3500 Da cut-off that was fitted to the electrode by an O-ring, forming a uniform thin layer. For the kinetics and thermodynamics studies the supporting electrolyte was 10 mM phosphate buffer, 0.1 M KNO_3, pH 8. The experiments were carried out at 293 K and, when indicated, in the temperature range of 298 to 313 K. In the temperature dependence assays, a single compartment isothermal cell (Metrohm) was used. The assays were performed after the cell reach thermic equilibrium and the obtained redox potential values were corrected for the SCE reference temperature dependence, according to the established procedure for isothermal cells [18,19], applying Eq. (1) [20].

$$E(V) = 0.2412 - 6.61 \times 10^{-4}(T - 25\ ^\circ\mathrm{C}) - 1.75 \times 10^{-6}(T - 25\ ^\circ\mathrm{C})^2 - 9 \times 10^{-10}(T - 25\ ^\circ\mathrm{C})^3. \quad (1)$$

A mix of Tris/citrate/phosphate buffers with NaCl (100 mM final ionic strength) in the pH range of 2.5 to 9.1 was used for the pH dependence study.

The cyclic voltammetry (CV) assays were performed at different scan rates (from 1 to 150 mV s^{-1}). Before the electrochemical experiments all electrolytes were degassed for 15 min using a continuous flow of high purity argon (Gasin). The potential values were converted and are presented in reference to the normal hydrogen electrode (NHE).

3. Results and discussion

3.1. Electrochemistry of cytochrome PccH

Cytochrome PccH was immobilized on the PG electrode membrane and its direct electrochemical response was observed by cyclic voltammetry. Typical voltammograms are presented in Fig. 2. A well-