Molecular interaction studies revealed the bifunctional behavior of triheme cytochrome PpcA from *Geobacter sulfurreducens* toward the redox active analog of humic substances

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**Abstract**

Humic substances (HS) constitute a significant fraction of natural organic matter in terrestrial and aquatic environments and can act as terminal electron acceptors in anaerobic microbial respiration. *Geobacter sulfurreducens* has a remarkable respiratory versatility and can utilize the HS analog anthraquinone-2,6-disulfonate (AQDS) as a terminal electron acceptor or its reduced form (AH2QDS) as an electron donor. Previous studies set the triheme cytochrome PpcA as a key component for HS respiration in *G. sulfurreducens*, but the process is far from fully understood. In this work, NMR chemical shift perturbation measurements were used to map the interaction region between PpcA and AH2QDS, and to measure their binding affinity. The results showed that the AH2QDS binds reversibly to the more solvent exposed edge of PpcA heme IV. The NMR and visible spectroscopy coupled to redox measurements were used to determine the thermodynamic parameters of the PpcA:quinol complex. The higher reduction potential of heme IV (−127 mV) compared to that of AH2QDS (−184 mV) explains why the electron transfer is more favorable in the case of reduction of the cytochrome by the quinol. The clear evidence obtained for the formation of an electron transfer complex between AH2QDS and PpcA, combined with the fact that the protein also formed a redox complex with AQDS, revealed for the first time the bifunctional behavior of PpcA toward an analog of the HS. Such behavior might confer selective advantage to *G. sulfurreducens*, which can utilize the HS in any redox state available in the environment for its metabolic needs.

**Keywords:**
- Geobacter
- Humics
- Multiheme cytochromes
- NMR
- Electron transfer

1. Introduction

Humic substances (HS) are the most abundant form of organic matter produced from the decomposition of plant, animal and microbial tissues in sedimentary environments [1]. HS can be divided into three main fractions according to their solubility in water at different pH values: humic acids (soluble at pH > 2), fulvic acids (soluble at any pH value) and humin (not soluble in the entire pH range) [1,2]. The redox-active anthraquinone-2,6-disulfonate (AQDS), used as the humic acid model compound, can act as an electron-transfer agent in the anaerobic respiration of microorganisms, such as *Geobacter* species [3,4]. Indeed, it was demonstrated that these bacteria can use AQDS as an electron acceptor and its reduced form, the anthrahydroquinone-2,6-disulfonate molecule (hereafter AH2QDS), as an electron donor [4,5]. It was also shown that the AH2QDS accelerates the dissimilatory reduction of Fe(III) by these microorganisms [4]. In this process, AH2QDS reduces Fe(III) to Fe(II) abiotically and restores the oxidized form of the quinone [4,6]. The electron transfer mechanisms between humic substances and *Geobacter* species are particularly important because these bacteria are the predominant Fe(III)-reducing microorganisms in aquatic sediments and sub-surface environments [7].

The bacterium *Geobacter sulfurreducens* is commonly used to study *Geobacter* species because the genome sequence, the genetic system and the genome-scale metabolic model are currently available for this microorganism [8–10]. The genome sequence of *G. sulfurreducens* encodes for 111 c-type cytochromes, out of which 73 contain two or more heme groups. Several genetic studies aiming to identify *G. sulfurreducens* cytochromes participating in the HS respiration have been reported in the literature [11,12]. From these studies evidence was obtained for the involvement of several outer membrane and periplasmic cytochromes in the reduction of AQDS [11,12]. Particularly, a knockout mutant of the gene encoding periplasmic triheme cytochrome PpcA showed significant inhibition of acetate-dependent reduction of both Fe(III) and AQDS by *G. sulfurreducens* [11]. Taken these studies...
together, it was proposed that PpcA can function as an electron carrier in the electron transport from acetate to the outer membrane cytochromes and it may also transfer electrons directly to AQDS or humic materials that are able to traverse the outer membrane [11]. However, the detailed mechanisms of HS respiration by *G. sulfurreducens* are still poorly understood.

PpcA is the best characterized protein from *G. sulfurreducens* [13–18]. It contains three c-type low spin heme groups with negative and distinct reduction potentials that are modulated by redox networks determined by the oxidation state of neighboring hemes (redox interactions) and by the solution pH (redox-Bohr interactions). These redox networks provide the protein the necessary properties to couple e−/H+ transfer in the pH range from 6.5 to 8.5 [16]. The crystal and the solution structures of PpcA were determined in the fully oxidized and fully reduced forms, respectively [14,15]. Also the NMR NH signals of the polypeptide backbone and amino acid side chains of PpcA, in both reduced and oxidized forms, were determined [18,19]. These data can now be used to identify interacting regions between putative redox partners, and to rationally investigate the role of this cytochrome in HS respiration.

In a previous work, we provided evidence, for the first time, for formation of an electron transfer complex between PpcA and AQDS from the combined use of kinetic and NMR experiments [18]. The NMR chemical shift perturbation measurements on PpcA samples at increasing concentrations of AQDS were used to map the interaction region in the complex formed to the vicinity of heme IV. However, the UV–Visible and stopped-flow kinetic measurements suggested that electron transfer between the humic substance analog and the cytochrome occurs preferentially from the *AH*2QDS to the oxidized cytochrome. Therefore, in the present work, we used NMR spectroscopy to investigate the interaction region(s) between *AH*2QDS and PpcA. In addition, since the redox properties of PpcA in the redox complex can differ from those previously determined for the free protein, the detailed thermodynamic properties of the cytochrome redox centers in the presence of the quinol, were also determined in the present work. The respiration of *G. sulfurreducens* using humic substances is rationalized in the light of the results obtained.

### 2. Materials and methods

#### 2.1. Expression and purification of triheme cytochrome PpcA

Uniformly 15N-labeled and unlabeled PpcA were heterologously expressed in *Escherichia coli* BL21(DE3) cells carrying the plasmid pEc86, which contains the *ccm* genes essential for the maturation of c-type cytochromes in *E. coli*, as previously described [20]. *E. coli* BL21(DE3) cells were then co-transformed with the plasmid pCK32 containing the gene for the cytochrome PpcA production. Bacteria were grown at 30 °C in 2x yeast–tryptone medium containing 34 mg/mL chloramphenicol and 100 mg/mL ampicillin, since the plasmids pEc86 and pCK32 are resistant to these antibiotics, respectively. Uniformly 15N-labeled and unlabeled protein expression was induced with 40 μM and 10 μM isopropyl β-D-thiogalactoside (IPTG), respectively. In both cases, PpcA was purified in two steps: (i) cation exchange chromatography, using two 5 mL Econo-Pac HighS cartridges (BioRad) connected together equilibrated with 10 mM Tris–HCl (pH 8.5) and eluted with a NaCl gradient from 0 to 300 mM in 10 mM Tris–HCl (pH 8.5) and (ii) gel filtration chromatography using a Superdex 75 (GE Healthcare) equilibrated with 100 mM phosphate buffer pH 8. The purity of the samples was confirmed by SDS-PAGE stained with Coomassie blue (Sigma).

#### 2.2. NMR studies

##### 2.2.1. Preparation of NMR samples

For interaction NMR studies, 15N labeled PpcA samples were prepared in 45 mM phosphate buffer pH 7.1 with NaCl (100 mM final ionic strength) to a final protein concentration of approximately 0.5 mM. The natural abundance samples used in the NMR redox titrations were prepared in the same buffer, as previously described [16]. The pH was measured using a glass microelectrode without correction for isotope effects. The 15N labeled PpcA samples were prepared in 92% H2O/8% D2O. The unlabeled PpcA samples were prepared in D2O (99.9 at.%). To avoid oxidation of the samples, the NMR tubes were sealed with a gas-tight septum cap and the air was flushed out from the sample. The reduced samples were obtained by adding gaseous hydrogen in the presence of catalytic amounts of Fe-hydrogenase isolated from *Desulfovibrio vulgaris* (Hildenborough), as previously described [21]. To prepare partially oxidized samples the hydrogen was replaced by argon, followed by the addition of controlled amounts of air into the NMR tube. For the reduced and the intermediate stages of oxidation, the pH was adjusted inside an anaerobic glove chamber with argon circulation to avoid sample reoxidation.

##### 2.2.2. NMR experiments and assignment of the signals

All the NMR experiments were acquired in a Bruker Avance III 600 spectrometer equipped with a triple-resonance cryoprobe (TCI). The 1H and 15N chemical shifts were calibrated using the water signal as an internal reference and through indirect referencing, respectively [22]. All the spectra were processed using the software TOPSPIN (Bruker Biospin, Karlsruhe, Germany) and analyzed with the program Sparky (TD Goddard and DG Kneller, Sparky 3, University of California, San Francisco, USA).

For the NMR binding studies the experimental conditions matched exactly with those used in the study of the interaction between PpcA in the oxidized state (PpcAox) and AQDS [18]. Briefly, the following set of experiments was acquired at 25 °C for cytochrome PpcA in the reduced state in presence of *AH*2QDS in a molar ratio of 1:5. 15N labeled sample: 2D–1H,15N HSQC (heteronuclear single quantum coherence); unlabeled sample in pure *H*2O: 2D–1H,13C (HSQC), 2D–1H,1H TOCSY (total correlation spectroscopy) with 45 ms mixing-time and 2D–1H,1H NOESY (nuclear Overhauser effect spectroscopy) with 80 ms mixing-time. The 1D–1H NMR spectra were obtained before and after each multidimensional spectrum to confirm protein integrity. The effect of humic substance analog on the chemical shifts of PpcAred backbone NH signals was monitored by the analysis of a series of 2D–1H,15N HSQC spectra in the presence of increasing amounts of *AH*2QDS. The pH of the sample was measured before and after each experiment to confirm that pH of the solution is maintained. To investigate the binding reversibility between PpcA and AQDS, 1D–1H NMR spectra were acquired for PpcA in the absence of AQDS and after removal of this molecule by ultrafiltration method (Amicon Ultra, 3 k). All 1D–1H NMR spectra were acquired by collecting 64 k data points with at least 64 scans. To determine the thermodynamic parameters of PpcA in the presence of the humic analog, the 2D–1H-exchange spectroscopy (EXSY) NMR spectra were also acquired in partially oxidized samples in the pH range of 6.0–9.0, as described for the free protein [16].

##### 2.2.3. Determination of the interaction regions between PpcAred and *AH*2QDS

The backbone NH and heme proton signals of PpcA were previously assigned in the reduced state at 25 °C and pH 7 [19,23]. In the present work, those signals were used to monitor the *AH*2QDS-induced chemical shift perturbations of PpcA. In order to map the binding site(s) between PpcA and *AH*2QDS we used the methodology previously described by Schumann and co-workers [24]. The weighted average chemical shift (Δδavg) of each NH signal was calculated as described by Eq. (1):

$$\Delta\delta_{\text{comb,j}} = \sqrt{(\Delta\delta_{\text{H}})^2 + (\Delta\delta_{\text{N}})^2}$$

where ΔδH is the chemical shift change in ppm in 1H dimension, ΔδN is the chemical shift change in ppm in 15N dimension and the term $w_i = [\text{I}^n\text{N}] / [\text{I}^n\text{H}]$ compensates for the scaling differences between $^{15}$N and $^1$H chemical.
shifts and is calculated from the ratio of the magnetogyrinic ratios (1.000 and 0.102, for $^1$H and $^{15}$N, respectively) [24].

The cut-off value was determined by an iterative procedure to calculate the correct standard deviation to zero value ($\sigma_{corr}$) as described by Schumann and co-workers [24]. The Eq. (2) (see below) was described for the following equilibrium: $P + L \leftrightarrow PL$, where $P$ and $L$ correspond to PpcA and AH2QDS, respectively. The dissociation constant for 1:1 binding equilibrium ($K_d$) can be estimated under fast exchange conditions by fitting the observed chemical shift changes to Eq. (2):

$$\Delta\delta_{\text{obs},i} = (\Delta\delta_{P-L} - \Delta\delta_{P}) \frac{(c_{P}^{\text{total}} + c_{L}^{\text{total}} + K_d)}{2c_{P}^{\text{total}} c_{L}^{\text{total}} + K_d} - 4 c_{P}^{\text{total}} c_{L}^{\text{total}}$$

(2)

where the $c_{P}^{\text{total}}$ and $c_{L}^{\text{total}}$ corresponds to the total concentration of protein and ligand, respectively. The $K_d$ values were estimated adjusting the calculated values to the experimental data, using Excel Solver tool.

2.2.4. Effect of quinol binding on the redox properties of PpcA

The detailed thermodynamic properties of the redox centers in PpcA in the presence of humic acid analog were determined as previously described for the free protein by means of a thermodynamic model that utilizes data obtained from NMR and visible redox titrations [16]. Briefly, the data obtained from 2D-1H EXSY NMR experiments allow the discrimination of the individual heme signals in the different oxidation stages (each containing the microstates with the same number of oxidized hemes) and, thus to monitoring the stepwise oxidation of each heme throughout the redox titration from the fully reduced to the fully oxidized state. By combining the NMR information, with data obtained from visible redox titrations at different pH values, it is possible to determine the thermodynamic parameters relative to reference state, which is the fully reduced and protonated protein (for a review see Refs. [16,21]). The thermodynamic parameters include the reduction potential of each heme group, the $pK_a$ of the redox-Bohr center plus six two-center redox interactions (three heme–heme and three redox-Bohr).

In the characterization of the free protein, the chemical shifts obtained for the heme methyls $^{12}$CH$_3$, $^{15}$CH$_{3}$, and $^{13}$CH$_{3}$ (labeled according to the IUPAC nomenclature [25]) in each oxidation stage in the pH range of 6.0–9.0 were fitted simultaneously with the visible redox titrations data obtained at pH 7.0 and 8.0 [26]. In order to obtain the thermodynamic parameters of PpcA in the presence of the quinol, the same set of methyls were used to monitor the stepwise oxidation of the heme groups and visible redox titrations were performed in identical experimental conditions. The experimental uncertainty of the NMR data was evaluated from the line width of each NMR signal at half height; the visible data points were given an uncertainty of 3% of the total optical signal.

2.3. Redox titrations followed by visible spectroscopy

The redox titrations of cytochrome PpcA were carried out inside an anaerobic chamber (MBraun) at 15 °C, as described previously [26]. PpcA samples with 18 μM protein concentration were prepared at pH 7.0 and 8.0 in NaCl/phosphate buffer solution at different ionic strength values. To check for hysteresis, each redox titration was performed in both oxidative and reductive directions. The reduced fraction of the proteins was determined by integrating the area of the α-peak (552 nm) above the line connecting the flaming isosbestic points (543 and 559 nm) to subtract the optical contribution of the redox mediators, as previously described [21]. The experiments were performed at least two times, and the reduction potentials (relative to standard hydrogen electrode, SHE) were found to be reproducible within ± 5 mV.

2.4. Molecular docking simulations

Molecular docking simulations between PpcA and AH2QDS were performed in two steps. In the first step, the docking was performed with rigid protein and flexible ligand using Anchor-and-Grow algorithm as implemented in DOCK 6.7 [27]. The solution structure of PpcA (PDB ID: 2LDO) was used and atomic coordinates for AH2QDS were taken from LUPBUT entry of the Cambridge Structural Database. The structures of the ligand and the protein were both prepared for docking calculations with UCSF Chimera 1.5.2 [28]. Hydrogens were added or modified when needed, to have the protonation pattern reflecting the neutral pH. The atomic charges of the ferrous form of heme were based on AMBER force field [29].

In the second docking step, the top binding configurations matching NMR data from rigid protein–flexible ligand docking simulations were used as input for flexible protein–flexible ligand simulations using AMBER scoring function as implemented in DOCK 6.7 with the default molecular dynamics settings. The positions of residues with non-hydrogen atoms located greater than 4 Å from non-hydrogen atoms of the residues with the biggest NMR shifts ($\text{Asn}^{10}$, $\text{Cys}^{65}$, $\text{Gly}^{66}$, $\text{His}^{69}$, heme IV) were constrained (fixed) whereas heme IV and 25 out of 71 PpcA amino acids (residues 6, 7, 8, 9, 10, 11, 12, 13, 41, 43, 46, 47, 51, 52, 55, 62, 63, 64, 65, 66, 67, 68, 69, 70 and 71) were allowed to be fully flexible. 500 step energy minimization was performed as the initial step. It was followed by a 3000 step molecular dynamic run and concluded with another 500 step energy minimization.

3. Results and discussion

3.1. Interaction between PpcA and AH2QDS probed by NMR

The NMR chemical shifts of the nuclei are very sensitive to changes in their chemical environment and can be explored to map protein–protein or protein–ligand interactions. The 2D-1H,15N HSQC NMR spectra are largely used for this purpose since they provide information on the NH signals of the polypeptide backbone and amino acid side chains. In the case of electron transfer proteins these “fingerprints” can be explored to map the docking regions between redox partners. In the present study, a series of 2D-1H,15N HSQC NMR spectra were used to monitor the molecular interactions between PpcA and AH2QDS (Fig. 1). The NH signals of PpcA have been previously assigned, except for the first two residues, Ala$^1$ and Asp$^2$ [19], and were used to monitor the chemical shift perturbations caused by the AH$_2$QDS. The inset in Fig. 1 shows the combined chemical shift changes averaged for each NH signal. The backbone NH signals of Cys$^{65}$ and Gly$^{66}$ and the side chain NH signals of Asn$^{10}_{O_{21}}$ and His$^{69}_{O_{61}}$ show the highest chemical shift perturbation. The backbone NH signal of Gly$^{66}$ appears slightly above the cut off line. However, as previously shown, the proton signal of Gly$^{66}$ is strongly up-field shifted due to the large ring current shifts caused by heme I [19]. In fact, this signal is observed at 3.90 ppm (see Fig. 1), which clearly contrasts with the typical positions for these signals in non-heme proteins and with the other PpcA glycine NH signals in the range of 9 to 6 ppm. Therefore, any marginal reorganization on the polypeptide chain in the neighborhood of Gly$^{66}$ NH group is expected to affect its chemical shift.

For electron transfer to occur between PpcA and any redox partner at physiologically relevant rates it is expected that at least one of the heme groups of the protein would be in close proximity to the interacting partner. This sets the heme substituents as excellent targets for monitoring relevant interactions for electron transfer [18,30,31]. Therefore, in the present work the interactions between AH$_2$QDS and PpcA were further evaluated by monitoring the chemical shift perturbation on the heme substituents by 2D-1H NOESY experiments. The analysis of the chemical shift perturbation clearly shows that the signals of heme IV are clearly the most affected and constitute additional evidence for a specific interaction between the quinol and the protein.
in the neighborhood of this heme (Fig. 2). Particularly, the most perturbed heme signals are located in the more solvent exposed heme edge and include the heme methyls $2^1\text{CH}_3$ and $18^1\text{CH}_3$, and the meso-proton $20\text{H}$. Moreover, the chemical shifts of these methyls in the absence of AH$_2$QDS are 3.59 ppm and 3.33 ppm for $2^1\text{CH}_3$ and $18^1\text{CH}_3$, respectively. On the other hand, in the presence of AH$_2$QDS the chemical shifts are nearly identical (3.20 ppm and 3.18 ppm for $2^1\text{CH}_3$ and $18^1\text{CH}_3$, respectively) indicating that the chemical environment in the vicinity of these protons is similar in the presence of the quinol (Fig. 3).

To further confirm the interaction between the quinol and PpcA we also monitored the NMR signals of AH$_2$QDS. Small molecules, such as AH$_2$QDS, have short correlation times ($\tau_c$) due to their fast tumbling in solution and, consequently, the intramolecular NOE connectivities have different sign relative to the diagonal signals (positive NOEs). On
the other hand, when bound to a protein a small molecule acquires the motional properties of the macromolecule and will have larger \( \tau_c \), and the NOE connectivities will have the same sign as the diagonal signals (negative NOEs). Therefore, the interaction of AH₂QDS with PpcA was also evaluated by the analysis of the 2D-¹H NOESY spectra of PpcA acquired in the presence and absence of the ligand (Fig. 4). The region of the 2D-¹H NOESY spectrum of PpcA containing the AH₂QDS signals is indicated in Fig. 4A. The sharp peaks corresponding to the quinol signals are easily identified at 8.84, 8.43, 7.79 ppm, which correspond to the three pairs of equivalent protons (H1/H5; H3/H7; H4/H8). The singlet at 8.84 ppm corresponds to the H1/H5 pair. The two doublets centered at 8.43 and 7.79 ppm correspond to the non-specifically assigned pairs H3/H7 and H4/H8. The cross-peaks between these two pairs have negative NOEs, a feature only observable if the \( \tau_c \) of the small molecule increases upon binding to high molecular weight entity (Fig. 4A). In the region of the 2D-¹H NOESY spectra indicated in Fig. 3, the presence of transfer NOE (tr-NOE) connectivities between the quinol protons and the heme methyls 2\(^{13}\)CH\(_3\) and 18\(^{13}\)CH\(_3\) are illustrated [see black squares in Fig. 3]. These signals are also in phase with the diagonal of the spectrum, which constitutes additional evidence for interaction between the protein and the AH₂QDS at the most solvent exposed edge of heme IV.

The residues showing the highest chemical shift perturbations caused by the AH₂QDS including heme IV are highlighted on the three-dimensional structure of PpcA (Fig. 5). The comparison of 1D-¹H NMR the NMR data obtained for PpcA in the presence and absence of AH₂QDS showed that the interaction is not permanent (data not shown). After identifying that the region containing heme IV is involved in the formation of the complex between PpcA and AH₂QDS, we then analyzed the chemical shift perturbation of the most affected NH signals, as a function of quinol concentration. The increasing chemical shift perturbations indicated that the free and bound forms of PpcA\textsuperscript{red} are in fast exchange on the NMR time scale. The average size of the chemical shift perturbation for PpcA:AH₂QDS complex is higher than the one previously reported for PpcA:AQDS [18]. This indicates that PpcA and AQDS form an encounter complex consisting of a dynamic ensemble of orientations (very small perturbation, for a review see Ref. [32]). On the other hand, the higher average size of the chemical shift perturbation and the smaller number of affected residues suggest that complex formed by PpcA and AH₂QDS exists in fewer orientations, which indicates the formation of a more specific complex in solution.

The dependence of the chemical shift perturbation as a function of AH₂QDS concentration yielded hyperbolic binding curves, which were fitted to a 1:1 model considering fast exchange conditions (Fig. 6). The \( K_d \) value obtained from the fitting of the ligand-induced chemical shift perturbation curves are in the millimolar range and suggest the formation of a low affinity complex (Fig. 6). In addition, the average \( K_d \) value obtained in the present work (8 ± 1 mM) is also comparable to the one obtained for PpcA\textsuperscript{ox} interacting with AQDS (17.8 ± 5 mM [18]) reinforcing the evidence obtained from the previous UV–Visible and stopped-flow studies, which suggested that the electron transfer proceeds in both directions at similar rates and that the thermodynamic equilibrium is reached in a short time scale (\( k_{obs} \) 1 s\(^{-1}\) for reductive experiments and \( k_{obs} \) 3 s\(^{-1}\) in the oxidative ones). Compared to AQDS, the smaller \( K_d \) value obtained for binding of AH₂QDS also corroborates with the previous studies indicating that the extent of electron transfer is under thermodynamic control, i.e., it is more favorable from AH₂QDS to PpcA\textsuperscript{ox}, than in the opposite direction [18]. The low-binding affinity complex and the specific interaction in the proximity of heme IV warrant a rapid and selective electron transfer between AH₂QDS and PpcA, a typical feature in electron transfer reactions between redox partners [32–34].

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Inspection of the PpcA solution structure showed that the region close to heme IV has a highly positive electrostatic surface conferred by a large number of lysine residues [15]. These positively charged residues are expected to form an anion binding region and drive the negatively charged AH₂QDS molecule toward heme IV, thus contributing favorably to the
binding affinity between the cytochrome and the quinol. Recently, molecular dynamics simulation of an anionic porphyrin, free base TPPS [meso-tetrakis(4-sulfonatophenyl)porphyrin], binding to PpcA also implicated the same positively charged surface near the heme IV [35].

The binding of quinol near heme IV is also supported by the docking calculations, which show that top 10 modeled complex structures obtained with rigid protein–flexible ligand docking were clustered in the vicinity of this heme (shown in Fig. 7A). One of those two sites corresponded to the area with the strongest observed NMR shifts. Top four docking complex structures with ligands located in this area were further refined with flexible protein–flexible ligand docking simulations. They revealed that in addition to a number of non-polar interactions between the protein and the quinol ligand, lysine residues of the protein were reorienting toward sulfonates and hydroxyls of the bound quinol. In the best docking model of the complex (Fig. 7B), the quinol is stacked between the side chain of Lys71 and the main chain near Cys65 and Gly66 (Fig. 7). One of the quinol hydroxyl groups hydrogen bonds with the main chain O of His69 and with the side chain NZ of Lys71. One of the sulfonate groups forms hydrogen bonds with NZ of Lys70 and main chain N of Lys71. Side chain of Asn10 forms hydrogen bond with NZ of Lys71. Side chain of Asn10 is in van der Waals contact with the heme IV atoms CHB (20H) and CMA (181CH3IV). The other sulfonate group is exposed to solvent and is in close proximity to CMB of heme IV (21CH3IV).

3.1.1. Effect of binding of AQDS on the thermodynamic properties of the redox centers in the triheme cytochrome PpcA

Electron transfer between redox partners at rates compatible with metabolic processes requires bringing the redox centers of the donor and acceptor molecules to close proximity and in proper orientation. In addition, the reduction potentials have to be such that they ensure favorable driving force, which is one of the main determinants of the electron transfer rates [36]. Therefore, to properly interpret the data obtained for PpcA in the presence of the HS analog, it is important to evaluate the impact on the redox properties of PpcA upon formation of the complex with this compound. To achieve this, we have
undertaken a detailed thermodynamic characterization of PpcA in the presence of the quinol.

The thermodynamic parameters of the free protein were previously determined by fitting the pH dependence of the chemical shifts of heme methyls $12^1$CH$_3$, $7^1$CH$_3$ and $12^2$CH$_3$, measured in different stages of oxidation, together with data from visible redox titrations obtained at pH 7 and 8 [16]. In the present work, the same set of heme methyl groups were used to characterize the redox properties of PpcA heme groups in the presence of the HS analog. 2D-1H EXSY NMR spectra were collected at different pH values and the chemical shifts of heme methyls $12^1$CH$_3$, $7^1$CH$_3$ and $12^2$CH$_3$ were measured for the different oxidation stages. Examples of these spectra are given in Fig. S1. The similarity between the spectra obtained for PpcA in the absence and presence of the quinol indicates that the formation of the complex did not decrease the spectral quality. Thus, as for the free protein, in the presence of quinol the following NMR features were observed: (i) slow inter- and fast intramolecular electron transfer among the redox equilibria are maintained allowing discrimination of the individual heme signals in different oxidation stages and (ii) fast exchange in the NMR time scale for the bound and unbound states so that a single signal is observed at a position that is weighted by their relative populations. Therefore, it was possible to monitor the oxidation profiles of the hemes by 2D-1H EXSY NMR in the presence of the quinol molecule. The chemical shifts of heme methyls $12^1$CH$_3$, $7^1$CH$_3$ and $12^2$CH$_3$ measured for oxidation stages 1–3 in the pH range of 6.0–9.0 are indicated in Fig. S2.

In order to measure the impact of the bound quinol on the redox properties of the heme groups in PpcA a thermodynamic model was fit to the pH dependence of the observed chemical shift of heme methyls, together with the data from visible redox titrations (see Materials and methods). The thermodynamic parameters obtained from the fitting are indicated in Table 1. The quality of the fittings obtained for the pH dependence of the NMR paramagnetic chemical shifts clearly shows that the experimental data is well described by the model (see solid lines in Fig. S2).

Overall, the results obtained showed that the quinol binding to PpcA had little effect on the reduction potential of the hemes in the fully reduced and protonated protein (Table 1). Also the redox interactions remain essentially unaffected compared to the free cytochrome, indicating that the intramolecular dielectric environment and the heme core structure is essentially undisturbed by complex formation. On the other hand, as a result of the complex formation, the properties of the PpcA redox-Bohr center, previously assigned to heme IV propionate $P_{13}$ [15,16,21] are more affected. However, the strongest redox-Bohr interactions with heme IV and the similar pH dependence of the heme methyl signals in the presence of the quinol indicate that the redox-Bohr center is the same (heme IV propionate $P_{13}$).

To evaluate the effect of the quinol interaction on the heme redox properties at physiological pH, the oxidation curves of each heme and the molar fractions of each microstate were computed from the thermodynamic parameters listed in Table 1 and are represented in Fig. 8. From the analysis of the individual heme oxidation curves, it is clear that the $e_{app}$ values of heme IV slightly decrease in the presence of the quinol and its oxidation occurs at earlier oxidation stages compared to that of heme III. As a consequence, the positive redox interaction between hemes IV and III has a larger contribution in the midpoint reduction potential of the latter, which increases proportionally the heme IV oxidation fraction. As a result, the last step of oxidation is even more dominated by the oxidation of heme III and the order of oxidation of the hemes is thus I–IV–III (see upper panels in Fig. 8). As discussed above, this order of oxidation of the hemes is crucial to establish the preferred $e^-$/H$^+$ transfer pathway, which is a key feature of the PpcA functional mechanism [17,37,38]. Therefore, to evaluate the effect of the presence of the quinol on the PpcA functional mechanism, the relative contribution of each of the 16 possible microstates was determined as a function of the solution potential (Fig. 8 lower panels). Such study was previously undertaken for the free protein and a coherent electron transfer pathway coupled to proton transfer was identified [16]. The results obtained in the presence of the quinol showed that despite the changes observed in the heme oxidation profiles that are reflected in the higher contribution of the microstate $P_{14}$ (see below), the protein is still able to perform a concerted $e^-$/H$^+$ transfer between oxidation stages 1 and 2. In fact, the oxidation stage 0 is dominated by the fully reduced and protonated microstate $P_{0H}$ and stage 1 is dominated by the oxidation of heme I ($P_{1I}$) while keeping the redox-Bohr center protonated. Oxidation stage 2 is dominated by the oxidation of hemes I and IV and deprotonation of the acid–base center ($P_{14}$), that remains deprotonated in stage 3 upon full oxidation of heme III ($P_{34}$). Therefore, a route is defined for electron transfer in PpcA:quinol complex: $P_{0H}$ $\rightarrow$ $P_{1I}$ $\rightarrow$ $P_{14}$ $\rightarrow$ $P_{34}$ (same as in PpcA in the absence of the ligand [18,26]) and the cytochrome retains the essential features of its functional mechanism.
In the previous section, it was shown that the heme IV region is involved in the complex formation between the PpcA and the quinol. Therefore, the reduction potential value of heme IV (−127 mV) determined in the presence of the quinol thermodynamically favors the reduction of the former and is in agreement with the previous UV–Visible and stopped-flow kinetic experiments that showed that electron transfer proceeds in both directions at a similar rate but its extent is thermodynamically controlled, favoring the reduction of the cytochrome by the AH2QDS [18].

4. Conclusions

In the present work, we probed the molecular interaction between the triheme cytochrome PpcA from G. sulfurreducens and AH2QDS, the reduced form of the anthraquinone-2,6-disulfonate (AQDS) by NMR chemical shift perturbation experiments. The results showed that the more solvent exposed edge of heme IV, located in a region containing a large number of positively charged lysines, is involved in the interaction between PpcA and AH2QDS, a feature also supported by docking calculations.

Previous studies showed that the AQDS also interacts with PpcA in the same region [18]. Therefore, the results obtained in the present
work revealed for the first time the bifunctional behavior of PpcA toward an analog of the redox active components of humic substances and is consistent with the previous UV–Visible and stopped-flow studies, which suggested that PpcA can reduce AQDS, but the protein can also be reduced by AH₂QDS. In this study, the kinetic data also indicated that the latter process is more favorable, despite the similar observed rates for both reactions, suggesting that the extent of electron transfer is thermodynamically controlled. In order to validate this hypothesis, in the present work we also undertook the detailed thermodynamic characterization of PpcA redox centers when bound to the quinol. The results showed that the binding of the negatively charged ligand partially shields the positive residues in the vicinity of heme IV and explains the decrease in the reduction potential of this heme compared to the free protein. As a consequence, the difference between the reduction potential of heme III and its predecessor in the heme oxidation order I–IV–III is increased, maintaining the preferential electron e⁻/H⁺ transfer pathway, which is a key feature of the PpcA functional mechanism. The higher reduction potential of heme IV (−127 mV) compared to that of AH₂QDS (−184 mV [39]) explains why the electron transfer is thermodynamically controlled and more favorable in the case of the reduction of the cytochrome by the quinol.

Overall, the results obtained in the present work, together with those obtained in previous studies, provide a clear picture of the molecular interaction between PpcA and the humic substance analog and constitute an important step toward better understanding of the G. sulfurreducens respiratory chains. In fact, the triheme cytochrome PpcA can interact with either the reduced or oxidized forms of the humic analog but the reduction of the protein is thermodynamically favored. The similar rates observed for reduction of AQDS by the cytochrome also make this process physiologically feasible, as long as the diffusion and regeneration of the quinone molecules is compatible with the flow of electrons through the metabolism of the organism. This provides a rationalization at the molecular level for versatility of G. sulfurreducens, which can utilize reduced or oxidized humic analog substances depending on whatever is available in the environment.

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Appendix A. Supplementary data

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References


