Role of Met<sup>58</sup> in the regulation of electron/proton transfer in trihaem cytochrome PpcA from Geobacter sulfurreducens

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Synopsis

The bacterium Gs (Geobacter sulfurreducens) is capable of oxidizing a large variety of compounds relaying electrons out of the cytoplasm and across the membranes in a process designated as extracellular electron transfer. The trihaem cytochrome PpcA is highly abundant in Gs and is most probably the reservoir of electrons destined for the outer surface. In addition to its role in electron transfer pathways, we have previously shown that this protein could perform e<sup>−</sup>/H<sup>+</sup> energy transduction. This mechanism is achieved by selecting the specific redox states that the protein can access during the redox cycle and might be related to the formation of proton electrochemical potential gradient across the periplasmic membrane. The regulatory role of haem III in the functional mechanism of PpcA was probed by replacing Met<sup>58</sup>, a residue that controls the solvent accessibility of haem III, with serine, aspartic acid, asparagine or lysine. The data obtained from the mutants showed that the preferred e<sup>−</sup>/H<sup>+</sup> transfer pathway observed for PpcA is strongly dependent on the reduction potential of haem III. It is striking to note that one residue can fine tune the redox states that can be accessed by the trihaem cytochrome enough to alter the functional pathways.

Key words: electron transfer, Geobacter, multihaem cytochrome, NMR site-directed mutagenesis

INTRODUCTION

Extracellular electron transfer is one of the most remarkable features of the Geobacter species, by which they can reduce toxic or radioactive metals and convert renewable biomass into electricity [1]. These properties could conceivably be explored for practical applications by genetically engineering strains or by the functional optimization of key respiratory electron transfer chain components. Metal oxides such as those of Fe(III) and Mn(IV) are examples of extracellular electron acceptors whose reduction has an important role in the geochemistry of water-saturated soils and aquatic sediments through the release of dissolved Fe(II) and Mn(II) [2,3]. In addition, the ability of Geobacter to reduce U(VI) and other radionuclides can also have an important impact on the dissemination of these compounds in the environments [4–6].

The most emergent feature from the analysis of the six genomes of Geobacter species already sequenced is indubitably the large diversity of c-type cytochromes [7–10]. In fact, with the exception of G. lovleyi, other Geobacter species sequenced have approximately 100 c-type cytochrome genes per genome [7]. Despite this, the number of well-conserved c-type cytochrome families is small among the Geobacter species [7]. One of these well-conserved families is the PpcA family of trihaem periplasmic cytochromes. The Gs (Geobacter sulfurreducens) PpcA family is presently the only one studied in detail by genetic, functional and structural methods [11–21]. This family is composed by five 10-kDa cytochromes, designated PpcA–PpcE, that have a
conserved overall fold and haem core [14,21]. With the exception of PpcC, the functional electron transfer mechanisms of these proteins have been characterized [20,22]. The results obtained suggested that PpcA and PpcD can couple $e^-/H^+$ transfer and may be involved in the generation of a proton electrochemical potential gradient across the periplasmic membrane [20]. Studies carried out on Gs with knock-out mutations of the genes encoding the PpcA family cytochromes showed that cellular U(VI) and Fe(III) reduction activities are affected [19]. Interestingly, PpcA and PpcD are the only members of this family that, when deleted, showed marked alterations in Gs phenotypic characteristics in presence of iron oxides [18,19]. The solution structure of PpcA was recently determined [14] and can be used for rational design of PpcA mutants, an essential step towards engineering variants of Gs with increased respiratory rates and the concomitant improvement in the biotechnological applications.

In the present work, we aimed to probe the putative regulatory role of haem III in the functional mechanism of PpcA as a natural follow-up of the observations obtained from the functional characterization of Gs PpcA family [20]. In that work, it was proposed that the relative high reduction potential value of haem III was crucial in selecting the microscopic redox states that the protein can access during its redox cycle and, thus, in establishing the preferential pathways for electron transfer. In PpcA, haem III is located between the other two haem groups at the bottom of a cleft sandwiched by two $\alpha$-helices formed by residues Ala$^{19}$–Lys$^{72}$ and Lys$^{52}$–Met$^{58}$ (Figure 1). Given the location of residue Met$^{58}$ at the end of the latter $\alpha$-helix, pointing its side chain towards the interior of the cleft, it is conceivable that it might control the solvent accessibility of haem III and, concomitantly, regulate its reduction potential value. In the present study, this residue was replaced by serine (PpcAM58S), asparagine (PpcAM58N), aspartic acid (PpcAM58D) and lysine (PpcAM58K) to evaluate the effects of polarity and charge at this position. Each mutant was $^{15}$N-labelled by serine (PpcAM58S), asparagine (PpcAM58N), aspartic acid (PpcAM58D) and lysine (PpcAM58K). Transformed E. coli cells were grown in the $2\times$YT medium containing 34 $\mu$g/ml chloramphenicol and 100 $\mu$g/ml ampicillin. Protein expression was induced by adding IPTG (isopropyl $\beta$-D-thiogalactoside) to a final concentration of 10 $\mu$M. PpcA mutants were purified by cation exchange and gel filtration, as described for the wild-type cytochrome [17]. The purity of the proteins was evaluated by SDS/PAGE (15 % gel), stained with Coomassie Brilliant Blue. To confirm the correct haem incorporation ESI–MS (electrospray ionization MS) was performed in the HHMI Biopolymer Laboratory and W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University. Uniformly $^{15}$N-labelled PpcA mutants were also expressed in E. coli as previously described [25]. Purification of labelled proteins was carried out as described above for unlabelled proteins.

**EXPERIMENTAL**

**Site-directed mutagenesis**

For mutagenesis, the QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used in accordance with the manufacturer’s instructions. Oligonucleotides were synthesized by MWG Biotech. PpcA expression vector pCK32 [23] was used as a template. The presence of desired mutations was confirmed by DNA sequencing performed by MWG Biotech.

**Bacterial growth and purification of PpcA mutants**

*Escherichia coli* strain BL21(DE3) containing the plasmid pEC86 [24] was co-transformed with the expression vector containing the PpcA mutants (PpcAM58S, PpcAM58N, PpcAM58D or PpcAM58K). Transformed *E. coli* cells were grown in the $2\times$YT medium containing 34 $\mu$g/ml chloramphenicol and 100 $\mu$g/ml ampicillin. Protein expression was induced by adding IPTG (isopropyl $\beta$-D-thiogalactoside) to a final concentration of 10 $\mu$M. PpcA mutants were purified by cation exchange and gel filtration, as described for the wild-type cytochrome [17]. The purity of the proteins was evaluated by SDS/PAGE (15 % gel), stained with Coomassie Brilliant Blue. To confirm the correct haem incorporation ESI–MS (electrospray ionization MS) was performed in the HHMI Biopolymer Laboratory and W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University. Uniformly $^{15}$N-labelled PpcA mutants were also expressed in *E. coli* as previously described [25]. Purification of labelled proteins was carried out as described above for unlabelled proteins.

**NMR studies**

**Preparation of NMR samples**

NMR samples and experimental conditions matched exactly those used in the characterization of the wild-type protein in the reduced and intermediate stages [20] and are summarized below. PpcAM58 mutant samples of about 140 $\mu$M for fully-reduced studies and 70 $\mu$M for redox titrations were prepared in 80 mM phosphate buffer with NaCl (250 mM final ionic strength) in $^2$H$_2$O. Full reduction of the samples was achieved by the reaction with gaseous hydrogen in the presence of catalytic amounts of the enzyme Fe-hydrogenase, isolated from *Desulfovibrio vulgaris* (Hildenborough). Partially oxidized samples were obtained by first removing the hydrogen from the reduced sample with argon followed by the addition of controlled amounts of air into

![Figure 1 Location of residue Met$^{58}$ in PpcA solution structure (PDB code 2LDO) [14]](

The PpcA polypeptide chain (grey), haem groups (green) and the side chain of the methionine (blue) are represented. The haems are numbered I, III and IV, a designation that derives from the superimposition of the haems in cytochromes $c_1$ with those of the structurally homologous tetrahaem cytochromes $c_3$.}
using the assigned signals in the wild-type protein [27]. The specific assignment of the haem signals in different oxidation stages [30,31] was obtained from the NMR data in each oxidation stage in the pH range 6–9 were fitted simultaneously with visible redox titrations to monitor the stepwise oxidation of each haem using 2D-EXSY NMR experiments, which allow discriminating the individual haem signals in different oxidation stages [30,31]. The information obtained from the NMR data allows the determination of the thermodynamic parameters relative to the reference state (for a review see [29]).

In the characterization of the wild-type protein, the chemical shifts obtained for the haem methyls 12H1CH3I, 7H1CH3III and 12H1CH3IV (labelled according to the IUPAC nomenclature; see Supplementary Figure S2 at http://www.bioscierep.org/bsr/033/bsr033e002add.htm) in each oxidation stage in the pH range 6–9 were fitted simultaneously with visible redox titrations data obtained at pH 7 and 8.

In order to obtain thermodynamic parameters of the four PpcAM58 mutants the same set of haem methyls were used and visible redox titrations were performed under identical experimental conditions. The experimental uncertainty of the NMR data was evaluated from the linewidth of each NMR signal at half height; the visible data points were given an uncertainty of 3% of the total optical signal.

**RESULTS AND DISCUSSION**

**Impact of the mutations on the global fold and haem core**

The yields of the four PpcAM58 mutants were similar to that obtained for the wild-type protein (approximately 3 mg/l culture). The mutant proteins showed identical UV–visible and NMR spectroscopic features both in the reduced and oxidized states when compared with the wild-type protein (results not shown).
The most affected backbone amide signals are marked in the spectrum or in the expansion. The inset shows the comparison of the observed haem proton chemical shifts of reduced PpcAM58 mutants and those of PpcA. The symbols correspond to haem I (□), haem III (△) and haem IV (○). The colour code of the NMR spectra was used for each mutant. The rmsd between the chemical shifts measured for PpcAM58 mutants and those of PpcA are for haems I, III and IV, respectively 0.02; 0.03; 0.01 p.p.m. for PpcAM58S, 0.02; 0.03; 0.03 p.p.m. for PpcAM58D, 0.02; 0.07; 0.01 p.p.m. for PpcAM58K and 0.01; 0.05; 0.01 p.p.m. for PpcAM58N. The continuous line has a unit slope.

Chemical shifts are exquisitely sensitive probes of molecular structure and in the present work 1H–15N HSQC NMR experiments were used to fingerprint the overall structure of PpcAM58 mutants and to evaluate the impact of each mutation on the protein conformation. All backbone amides, except for the first two residues, were assigned for the wild-type protein [27] and the same methodology was used to assist in the assignment of PpcAM58 mutant signals. The comparison of the 1H–15N HSQC NMR spectra obtained for wild-type and each mutant show a similar dispersion of signals, indicating that the overall fold of the native protein is maintained (Figure 2). For all mutants the backbone amide signal of residue 58 is always the most affected one, showing the largest variation for mutant PpcAM58N. In general, the other affected amide signals, although to a smaller extent, correspond to residues located in the polypeptide segment His55–Gly61. Thus, it can be concluded that the replacement of Met58 residue led only to a small rearrangement of the neighbouring residues in this polypeptide segment without affecting the global fold of the protein. Depending on the mutation, the residues in this region are differently affected. For PpcAM58K, only backbone amide signals for residues Met58 and Gly61 are significantly affected. In case of PpcAM58D and PpcAM58S, the most affected residues are in the region Glu56–Gly61 and His55–Gly61, respectively. On the other hand for PpcAM58N, the changes extended to residue Gly63 (covering the region His55–Gly63), indicating that replacement of methionine at position 58 by an asparagine residue involves more conformational rearrangements.

The impact of the mutations on the haem core architecture of the four PpcAM58 mutants was also probed by 2D 1H-NMR; the haem proton resonances were assigned (Supplementary Table S1 at http://www.bioscirep.org/bsr/033/bsr033e002add.htm) and their chemical shifts were compared with those of the wild-type cytochrome (see inset in Figure 2) [13]. The rmsd (root mean square deviation) values between the chemical shifts for the wild-type and mutant cytochromes are low, although slightly higher for haem III signals in each case, as expected because of the close proximity of this haem to Met58. The good correlation obtained in the chemical shifts of haem protons in the native and mutant cytochromes are low, although slightly higher for haem III signals in each case, as expected because of the close proximity of this haem to Met58. The good correlation obtained in the chemical shifts of haem protons in the native and mutant cytochromes are low, although slightly higher for haem III signals in each case, as expected because of the close proximity of this haem to Met58.

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Role of Met58 in the regulation of e⁻/H⁺ transfer in PpcA

Figure 3  Fitting of the thermodynamic model to the experimental data for PpcAM58 mutants
The continuous lines are the result of the simultaneous fitting of the NMR and UV–visible data. The three upper panels show the pH dependence of haem methyl chemical shifts at oxidation stages 1 (Δ), 2 (○), and 3 (●). The broken lines in each panel represent the best fit for the wild-type protein. The chemical shift of the haem methyls in the fully reduced stage (stage 0) are not plotted since they are unaffected by the pH. The lower panel corresponds to the reduced fractions determined by UV–visible spectroscopy at pH 7 (●) and pH 8 (Δ). The open symbols and the filled symbols represent the data points in the reductive and oxidative titrations, respectively.

(nuclear Overhauser effect) connectivities between the haem groups were also analysed and showed the same set of connectivities for both the native and mutant proteins. Overall, these results demonstrated that the haem core arrangement is similar for all the proteins.

Redox characterization of haem groups and redox–Bohr centre
In order to probe the functional role of residue Met58 in the control of the redox properties and, concomitantly, in the global network of co-operativities, the detailed thermodynamic characterization of the four PpcAM58 mutants was carried out. The thermodynamic parameters of the wild-type protein were previously determined by fitting the pH-dependence of the chemical shifts of haem methyls 12¹CH₃¹, 7¹CH₃³, and 12¹CH₃⁴, measured in different stages of oxidation together with data from visible redox titrations obtained at pH 7 and 8, within the framework of an electrostatic model that considers four interacting centres: three haems and one protonatable centre [20]. In the present work, the same set of haem methyl groups were used to characterize the redox properties of PpcAM58 mutants. The NMR redox titrations followed by 2D-EXSY NMR spectroscopy showed that, as in the native cytochrome, the mutated proteins exhibit fast intramolecular and slow intermolecular electron exchange. To illustrate the stepwise oxidation of PpcAM58 mutants, 2D-EXSY NMR spectra obtained at pH 8 are shown in Supplementary Figure S3 (at http://www.bioscirep.org/bsr/033/bsr033e002add.htm). The same type of NMR spectra were collected at different pH values and the chemical shifts of haem methyls 12¹CH₃¹, 7¹CH₃³, and 12¹CH₃⁴ were measured for different oxidation stages (Figure 3). As for the fully reduced form (Supplementary Table S1), in the fully oxidized state (oxidation stage 3) the chemical shifts of the haem methyls of PpcA and PpcAM58 mutants are little affected (cf. open circles in each mutant with the correspondent dashed line in Figure 3). However, in the intermediate oxidation stages (stages 1 and 2) different scenarios were observed within the family of mutants. With exception of PpcAM58S, the chemical shifts of the haem methyls are quite different, in particular for those of haem III (Figure 3). By having essentially unaffected chemical shifts in the fully reduced and fully oxidized forms, the variations on
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Figure 4 Oxidation fraction of PpcAM58 mutants (solid symbols and continuous lines) and PpcA (open symbols and broken lines) at pH 6 and pH 8

Haem I, III and IV data are coloured in green, orange and blue, respectively. The haem oxidation fractions were calculated according to equation \( x_i = (\delta_i - \delta_0)/(\delta_3 - \delta_0) \), where \( \delta_i, \delta_0 \) and \( \delta_3 \) are the observed chemical shifts of each methyl in stage i, 0 and 3, respectively.

the chemical shifts at intermediate oxidation stages reflect important changes in the haem oxidation fractions during the oxidation of the proteins, i.e., changes in their redox parameters. As an example, the oxidation pattern of the haem groups of the mutant and wild-type cytochromes at pH 6 and 8 are indicated in Figure 4. The comparison between the haem oxidation fractions in the mutant and in the wild-type cytochromes shows that, with exception of PpcAM58S, the haem oxidation profiles at both pH values were significantly altered with haem III being the most affected one (cf. broken and continuous lines in Figure 4). The haem III oxidation fractions are clearly higher in PpcAM58D when compared with the wild-type protein, whereas those of PpcAM58K and PpcAM58N are significantly smaller.

In order to quantify the effect of the mutations on the redox properties of the haem groups, the thermodynamic model was fitted to the pH-dependence of the observed chemical shift of haem methyls, together with data from visible redox titrations (see the Materials and methods section). The thermodynamic parameters are indicated in Table 1. The quality of the fittings obtained for the pH-dependence of the paramagnetic chemical shifts and for the visible redox titrations clearly shows that the experimental data is well described by the model (Figure 3). In the four mutants, the redox and redox–Bohr interactions are weaker compared with those of PpcA (Table 1). However, as observed for PpcA, the smallest redox interactions are established between the haems that are structurally further apart (haems I and IV), and the strongest redox–Bohr interactions with haem IV. The lower values of the redox interactions, in the context of a conserved haem core, might have their origin on structural rearrangements of charged groups that cause variations in local dielectric constants [32]. The positive values of the redox interactions indicates that the oxidation of a particular haem renders the oxidation of its neighbours more difficult, which is expected on an electrostatic basis due to the repulsion between the electron negative charges. Similarly, the negative redox–Bohr interactions (between the haems and the redox–Bohr centre) indicate that the oxidation of the haems facilitates the deprotonation of the redox–Bohr centre and vice versa. The higher redox–Bohr interaction observed with haem IV in Met58 mutants, indicates that the redox–Bohr centre remains associated with haem IV, as observed in the native protein [13,14,20].
With the exception of PpcAM58S, the most affected haem reduction potential was that of haem III in the other mutants (Table 1). However, the reduction potential of haem III in these three mutants was differently affected. In PpcAM58D it decreased by 18 mV, whereas in PpcAM58K and PpcAM58N it is increased by 29 and 35 mV, respectively, in the fully reduced and protonated proteins. The variations observed suggest an important role for residue Met58 in the regulation of haem III properties.

Impact of the mutations on the haem oxidation order at physiological pH

The reduction potential of each haem group is affected by the oxidation state of neighbouring haems (redox interactions) and by the solution pH (redox–Bohr interactions). To evaluate the effect of the mutations on the haem midpoint reduction potentials ($E_{\text{app}}$), i.e. the point at which the oxidized and reduced fractions of each haem are equally populated) at physiological pH, the oxidation curves of the individual haems were computed from the thermodynamic parameters listed in Table 1. As for the wild-type protein, the redox interactions modulate the electron affinity of each haem so that the curves are non-Nernstian, showing some cross over during the redox titration (Figure 5). The mutant PpcAM58S shows similar haem oxidation profiles compared with the wild-type protein, which reflects their comparable redox parameters. Both proteins displayed the same haem oxidation order (I–IV–III) with the separation of the curves only slightly affected: the difference between the $E_{\text{app}}$ values of haems I and IV decreased by 6 mV and that between haems IV and III increased by 11 mV in the mutant (Table 2). By contrast, the haem oxidation profiles of PpcAM58D show a general lowering of the $E_{\text{app}}$ values of the haem groups compared with those of the wild-type protein. A significant decrease in the reduction potential is observed for haem III. This haem is more oxidized at low reduction potentials, i.e., in earlier stages of oxidation compared with PpcA (cf. Figures 4 and 5). Consequently, its $E_{\text{app}}$ is nearly identical with that of haem IV and the haem oxidation order is I–(IV, III). On the other hand, in the mutant PpcAM58K, the positively charged lysine residue caused a significant alteration of $E_{\text{app}}$ of haem III but in an opposite direction to that observed in PpcAM58D. Indeed, for PpcAM58K, the oxidation curve of haem III is well separated from those of the other haems at low reduction potentials.
Figure 5 Oxidized fractions of the individual haems for PpcAM58 mutants (continuous lines) and wild-type (broken lines) at pH 7.5
The curves were calculated as a function of the solution reduction potential using the parameters listed in Table 1. The midpoint reduction potentials ($E_{\text{app}}$) of the haems are also indicated. The $E_{\text{app}}$ values of PpcA were previously reported [20] and are $-152$ mV (haem I); $-108$ mV (haem III); and $-126$ mV (haem IV).

(Figure 5). Increasing the solution potential leads to the progressive oxidation of haems I and IV, which have negligible redox interaction, while keeping haem III essentially reduced. As a result, the oxidation curve of haem IV is basically unaffected by the oxidation of haem III, and hence, unaltered by the stronger interaction between these haems (Table 1), as it was in the wild-type protein. The actual order of the midpoint reduction potentials in PpcAM58K is I–IV–III, as observed for PpcA. However, despite showing the same order of oxidation, the difference between the $E_{\text{app}}$ values of haems III and IV, in the case of PpcAM58K, is 37 mV higher compared with the wild-type (Table 2). The haem oxidation profiles of PpcAM58N (Figure 5) are similar to those of PpcAM58K as a result of the similarity of their thermodynamic parameters (Table 1). However, in PpcAM58N the difference between the $E_{\text{app}}$ values of haems III and IV is 44 mV higher compared with the wild-type (Table 2). The results obtained for PpcAM58D and PpcAM58K can be rationalized on a pure electrostatic basis. Indeed, the replacement of a neutral methionine residue with negatively charged aspartic acid is expected to stabilize the oxidized form of the nearest haem (haem III) by lowering its reduction potential, whereas the opposite effect is expected by the introduction of a positive charge, as observed in PpcAM58K. On the other hand, it is rather striking that the neutral asparagine side chain at position 58 has a similar effect as the positively charged lysine residue, whereas the neutral serine residue has similar reduction potentials as the native. A structural model generated in silico (not shown) by mutating Met$^{58}$ to Asn$^{58}$ starting with solution structure of PpcA (PDB code 2LDO [14]) has revealed a possible hydrogen bond between main chain nitrogen atom of Lys$^{60}$ and OD1 of Asn$^{58}$. The partial positive charge on the amide nitrogen of the side chain can interact with the $\pi$-electron cloud of haem III. The asparagine side chain will be within van der Waals contact of haem III. This observation is also corroborated by the chemical shift values of Asn$^{58}$ and NH$_2$ side chain protons. The two protons are bonded to the same nitrogen atom and their identification is straightforward in the $^1$H–$^{15}$N HSQC spectrum (see red straight line connecting these signals in PpcAM58N inset in Figure 2). The chemical shifts of Asn$^{58}$ amide protons are 8.04 and 7.46 p.p.m., which are shifted to downfield from their average positions at 7.35 and 7.14 p.p.m. calculated from approximately 10$^4$ shifts as reported in the Biological Magnetic Resonance Data Bank (http://www.bmrb.wisc.edu/). Since in PpcA the non-haem aromatic rings, (Phe$^{15}$ and Phe$^{41}$) are in distant regions in relation to Asn$^{58}$ amide protons, the observed

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chemical shift of these protons can only be explained by the ring-current chemical shift contribution from haem III.

**Role of Met^58 on the functional mechanism of PpcA**

As shown above, the oxidation profile of the redox centres is highly dependent on the nature of the side chain at position 58. In order to evaluate the effect of the mutation on the electron transfer mechanism of PpcA, the relative contribution of each of the 16 possible microstates (see Supplementary Figure S1) was determined as function of the solution potential for each mutant (Figure 6). Such study was previously done for the wild-type protein and a coherent electron transfer pathway coupled to proton transfer was established [20]. The relative variation of the microstates in PpcA is also depicted in Figure 6 together with those obtained in the present work for PpcAM58 mutants. While in the wild-type protein a concerted $e^-/H^+$ transfer mechanism typical of PpcA, the relative contribution of each of the 16 possible microstates to the overall population in each stage is significantly altered in PpcAM58D, PpcAM58K and PpcAM58N (Figure 6). In fact, in the wild-type cytochrome the oxidation stage 0 is dominated by the fully protonated and protonated form $P_{0H}$, but the microstates of oxidation stage 1 are overcome by the $P_{14}$ curve, which intercepts earlier the curve of microstate $P_{0H}$. The microstate $P_{14}$ dominates the oxidation stage 2, whereas $P_{134}$ dominates stage 3. Thus, in these two mutants, a different preferential route for electrons is established, favouring a proton-coupled $2e^-$ transfer step between oxidation stages 0 and 2: 2 $P_{0H} \rightarrow P_{14} \rightarrow P_{134}$.

Taken together, these observations and the relative $E_{app}$ values of the haem groups, it is clear that haem III plays a key role in the functional mechanism of each cytochrome. Protein surroundings near haem III control its reduction potential relative to the other two haems, which in turn controls the microscopic redox states that can be accessed during the redox cycle, establishing preferential pathways for electron transfer. It is striking to note that one residue (Met^58) can fine tune the redox states that can be accessed by the protein enough to alter the functional pathways. Indeed, the replacement of the neutral side chain by the negatively charged one in PpcAM58D mutant altered the reduction potential of haem III in a way that this haem is no longer the last one to oxidize. In this case, the preferential pathway for $e^-/H^+$ transfer is altered. In mutant PpcAM58K, the introduction of a positively charged side chain at position 58 leads to a higher stabilization of the oxidized form of haem III, compared with the other two haems, and a different preferred electron transfer pathway was established. In wild-type, PpcAM58N and PpcAM58K, the order of oxidation of the haem groups is maintained, but in the mutants a different functional mechanism emerges in which a $2e^-$ step coupled with proton transfer is observed.

In summary, the reduction potential of haem III relative to the other two haems seems to be crucial in enabling these proteins to couple electron transfer with deprotonation of the redox-Bohr centre (Table 2). In fact, only preferential $e^-/H^+$ pathways are established if haem III shows higher reduction potential. However, the pathway varies with the separation between the $E_{app}$ values of haem III and its predecessor (haem IV) in the order of

| Protein     | Order of haem oxidation | $\Delta E_{app}$ (2nd/1st) (mV) | $\Delta E_{app}$ (3rd/2nd) (mV) | Electron transfer pathway |
|-------------|-------------------------|---------------------------------|---------------------------------|--------------------------|
| PpcA        | I-IV-III                | 26                              | 18                              | $P_{0H} \rightarrow P_{14} \rightarrow P_{134}$ |
| PpcAM58S    | I-IV-III                | 20                              | 29                              | $P_{0H} \rightarrow P_{14} \rightarrow P_{134}$ |
| PpcAM58D    | I-IV-III                | 20                              | 1                               | No preferential pathway  |
| PpcAM58K    | I-IV-III                | 13                              | 55                              | $P_{0H} \rightarrow P_{14} \rightarrow P_{134}$ |
| PpcAM58N    | I-IV-III                | 11                              | 62                              | $P_{0H} \rightarrow P_{14} \rightarrow P_{134}$ |
| PpcD        | IV-III                  | 6                               | 54                              | $P_{0H} \rightarrow P_{14} \rightarrow P_{134}$ |

For comparison the values previously obtained for PpcA and PpcD [20] were also included. $\Delta E_{app}$ (2nd/1st) is the difference between the $E_{app}$ values of the second and the first haem to oxidized; $\Delta E_{app}$ (3rd/2nd) is the difference between the $E_{app}$ values of the third and second haem to oxidized.
oxidation (Table 2). In the case of PpcA such separation was 18 mV and the route for electron transfer was: \( P_0 \rightarrow P_1 \rightarrow P_{14} \rightarrow P_{134} \). In the case of PpcAM58S the same route was observed but the slightly higher separation of the \( E_{\text{app}} \) values (29 mV against 18 mV in the wild-type) increased the contribution of microstate \( P_{14} \). Finally, in PpcAM58K the higher separation between \( E_{\text{app}} \) values of haem III and haem IV (55 mV against 18 mV in the wild-type) lead to a significant contribution of the microstate \( P_{14} \) so that a different preferred route for electrons was observed: \( P_0 \rightarrow P_{14} \rightarrow P_{134} \). This route was further reinforced in PpcAM58N, which shows a separation of 62 mV in \( E_{\text{app}} \) values of haems III and IV.

Interestingly, the results obtained from the thermodynamic characterization of the four Gs PpcA family homologues (PpcA, PpcB, PpcD and PpcE) have shown that only PpcA and PpcD, for which haem III has the highest reduction potential showed preferential pathways for electron/proton coupling [20]. Remarkably, in PpcD, for which oxidation order of the haem groups was I–IV–III and the difference between the \( E_{\text{app}} \) values of haems III and IV is comparable with that of PpcAM58K (54 mV in PpcD against 55 mV in the PpcAM58K), a similar electron transfer mechanism \( (P_0 \rightarrow P_{14} \rightarrow P_{134}) \) was observed.

Conclusions

In the present work, the role of residue Met58 on the redox properties of the trihaem cytochrome PpcA was studied by replacing it with serine, aspartic acid, asparagine or lysine. The results described show that Met58 is crucial in the regulation of the functional mechanism of PpcA, which is strongly dependent on the nature of the side chain at position 58. Indeed, the two mutants containing charged side chains, PpcAM58D and PpcAM58K affected significantly, albeit differently, the redox properties of the haems and altered the balance of the global network of cooperativities and the functional mechanism of the proteins. The data obtained suggests that the preferred \( e^-/H^+ \) transfer pathway observed for PpcA is strongly dependent on the reduction potential of haem III relative to the other haems and is favoured by a larger difference between the reduction potential of haem III and its predecessor. Overall, this study shows that the functional mechanism of PpcA relies on a fine-tuned balance of redox and redox–Bohr interactions that assure a coherent electron transfer pathway coupled to proton transfer, which allows the protein to perform \( e^-/H^+ \) energy transduction. The proper tuning of the reduction potentials is fundamental to achieve concerted \( e^-/H^+ \) transfer, which contributes to cellular energy transduction, which is intimately linked to the nature of the residue at position 58. The functional characterization of PpcA mutants as described in the present study opens up the possible development of strains carrying mutant cytochromes rationally designed to increase respiratory skills coupled to the cellular growth enhancement of Gs.

AUTHOR CONTRIBUTION

Carlos Salgueiro designed the study. Carlos Salgueiro and Leonor Morgado conducted and analysed the NMR experiments, calculated the thermodynamic parameters and wrote the paper. Leonor Morgado conducted the redox titrations followed by visible spectroscopy. Leonor Morgado, Joana Dantas and Telma Simões performed the bacterial growth and purification of PpcA M58 mutants.
Yuri Londer and P. Raj Pokkuluri provided the PpcA M58 mutant plasmids and key revisions of the paper.

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Role of Met\textsuperscript{58} in the regulation of electron/proton transfer in trihaem cytochrome PpcA from Geobacter sulfurreducens

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Figure S1  Electronic distribution scheme for a trihaem cytochrome with a proton-linked equilibrium showing the 16 possible microstates

The dark grey and light grey circles correspond to the protonated and deprotonated microstates, respectively. Hexagons represent haem groups, which can be either reduced (black) or oxidized (white). The microstates are grouped, according to the number of oxidized haems, in four oxidation stages connected by three one-electron redox steps. $P_0H$ and $P_0$ represent the reduced protonated and deprotonated microstates, respectively. $P_{ijk}H$ and $P_{ijk}$ indicate, respectively, the protonated and deprotonated microstates, where $i$, $j$ and $k$ represent the haem(s) that are oxidized in that particular microstate.
Figure S2 Diagram of a haem c numbered according to the IUPAC–IUB nomenclature [1]

Figure S3 Expansions of 2D-EXSY NMR spectra obtained for PpcA and PpcAM58D and PpcAM58K at different levels of oxidation (288 K and pH 8)

2D-EXSY NMR spectra of PpcAM58S and PpcAM58N are similar to those of PpcA and PpcAM58K and are not represented. Cross-peaks resulting from intermolecular electron transfer between the three oxidation stages 1–3 are indicated for the haem methyls 12CH3 (green broken lines), 7CH3 (orange broken lines) and 12CH3 (blue broken lines). Roman and Arabic numbers indicate the haems and the oxidation stages, respectively. In order not to overcrowd the figure, the 2D-EXSY NMR spectra with cross-peaks to oxidation stage 0 are not shown. The chemical shifts corresponding to the oxidation stage 0 for each haem methyl are listed in Supplementary Table 1.
Table S1 Chemical shifts (p.p.m.) of the haem protons of PpcAM58 mutants in the reduced state at pH 8.0 and 288 K
For comparison chemical shift values obtained for PpcA [2] are indicated in parentheses.

| Haem substituent | Mutant | Haem I  | Haem II | Haem III | Haem IV |
|------------------|--------|---------|---------|----------|---------|
| 5H               | M58D   | 9.61 (9.65) | 10.57 (10.58) | 9.00 (9.02) |
|                  | M58K   | 9.64     | 10.58   | 9.01     |
|                  | M58S   | 9.63     | 10.58   | 9.01     |
|                  | M58N   | 9.63     | 10.61   | 9.01     |
| 10H              | M58D   | 9.08 (9.12) | 9.84 (9.86) | 9.31 (9.33) |
|                  | M58K   | 9.12     | 9.68    | 9.32     |
|                  | M58S   | 9.12     | 9.82    | 9.31     |
|                  | M58N   | 9.11     | 9.76    | 9.33     |
| 15H              | M58D   | 9.25 (9.26) | 9.47 (9.45) | 9.50 (9.51) |
|                  | M58K   | 9.27     | 9.49    | 9.54     |
|                  | M58S   | 9.28     | 9.49    | 9.54     |
|                  | M58N   | 9.27     | 9.52    | 9.54     |
| 20H              | M58D   | 9.49 (9.50) | 10.14 (10.14) | 9.38 (9.39) |
|                  | M58K   | 9.51     | 10.15   | 9.40     |
|                  | M58S   | 9.51     | 10.14   | 9.39     |
|                  | M58N   | 9.51     | 10.17   | 9.40     |
| 21CH₃            | M58D   | 3.54 (3.56) | 4.33 (4.35) | 3.60 (3.61) |
|                  | M58K   | 3.56     | 4.32    | 3.62     |
|                  | M58S   | 3.56     | 4.33    | 3.62     |
|                  | M58N   | 3.56     | 4.33    | 3.62     |
| 71CH₃            | M58D   | 3.56 (3.58) | 4.11 (4.14) | 3.00 (3.02) |
|                  | M58K   | 3.58     | 4.13    | 3.01     |
|                  | M58S   | 3.58     | 4.13    | 3.02     |
|                  | M58N   | 3.58     | 4.13    | 3.03     |
| 121CH₃           | M58D   | 2.51 (2.55) | 3.48 (3.50) | 3.93 (3.95) |
|                  | M58K   | 2.55     | 3.44    | 3.94     |
|                  | M58S   | 2.55     | 3.48    | 3.94     |
|                  | M58N   | 2.54     | 3.45    | 3.95     |
| 181CH₃           | M58D   | 3.33 (3.34) | 3.87 (3.86) | 3.33 (3.34) |
|                  | M58K   | 3.34     | 3.86    | 3.34     |
|                  | M58S   | 3.35     | 3.86    | 3.35     |
|                  | M58N   | 3.35     | 3.87    | 3.35     |
| 31H              | M58D   | 6.28 (6.30) | 6.89 (6.91) | 6.00 (6.04) |
|                  | M58K   | 6.28     | 6.91    | 6.02     |
|                  | M58S   | 6.28     | 6.91    | 6.03     |
|                  | M58N   | 6.29     | 6.91    | 6.02     |
| 81H              | M58D   | 6.26 (6.29) | 6.68 (6.60) | 6.26 (6.28) |
|                  | M58K   | 6.31     | 6.47    | 6.27     |
|                  | M58S   | 6.31     | 6.66    | 6.26     |
|                  | M58N   | 6.28     | 6.66    | 6.28     |
| 32CH₃            | M58D   | 2.13 (2.14) | 1.77 (1.73) | 2.06 (2.06) |
|                  | M58K   | 2.13     | 1.77    | 2.05     |
|                  | M58S   | 2.13     | 1.78    | 2.06     |
|                  | M58N   | 2.13     | 1.77    | 2.06     |
| 82CH₃            | M58D   | 1.79 (1.79) | 2.97 (2.98) | 1.49 (1.55) |
|                  | M58K   | 1.85     | 2.97    | 1.55     |
|                  | M58S   | 1.83     | 2.95    | 1.54     |
|                  | M58N   | 1.79     | 2.98    | 1.54     |

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