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Review

The role of intramolecular interactions in the functional control of multiheme cytochromes *c*

Bruno M. Fonseca [a], Catarina M. Paquete [a], Carlos A. Salgueiro [b], Ricardo O. Louro [a,*]

[a] Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Av. da República, EAN, 2780-157 Oeiras, Portugal
[b] Requimte-CQFB, Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Campus Caparica, 2829-516 Caparica, Portugal

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ABSTRACT

Detailed thermodynamic and structural data measured in soluble monomeric multiheme cytochromes *c* provided the basis to investigate the functional significance of interactions between redox co-factors. The steep decay of intramolecular interactions with distance means that close proximity of the redox centers is necessary to modulate the intrinsic reduction potentials in a significant way. This ensures selection of specific populations during redox activity in addition to maintaining fast intramolecular electron transfer. Therefore, intramolecular interactions between redox co-factors play an important role in establishing the biological function of the protein by controlling how electrons flow through and are distributed among the co-factors.

1. Introduction

MacMunn presented the earliest report of cytochromes in the late 19th century and the interest in these cell components grew in the 1920's when David Keilin re-identified them as key players in respiratory chains [1]. Presently, cytochromes are known to be key players in fundamental cell functions such as electron transfer, gas transport, catalysis and also cellular signalling [2,3].

Cytochromes contain heme prosthetic groups, which are coordination complexes of a tetrapyrrole porphyrin ring with an iron. These co-factors confer characteristic absorption bands in the visible wavelength range that change with redox, spin and coordination state. Depending on the type of substituent at the periphery of the porphyrin ring, cytochromes can be classified as type *a, b, c* or *d*. The heme in *c*-type cytochromes is covalently bound through thioether bonds to cysteine residues of the polypeptide chain arranged in a typical CXXCH motif, where the histidine provides the proximal ligand to the iron [2].

The biogenesis of *c*-type cytochromes relies on complex molecular assemblies. The best characterized system is the cytochrome *c* maturation (Ccm) system that can be found in many species of Bacteria and Archaea. It is composed of eight proteins encoded by the *ccmABCDEFGH* gene cluster, which are located in the periplasm and/or cytoplasmic membrane. These proteins are responsible for the correct ligation of the heme to the apocytochrome *c*, while it is translocated to the periplasmic space. Thus, no cytoplasmic cytochrome *c* is known. These proteins are either periplasmic, associated to the cytoplasmic or outer-membrane or to extracellular electrically conductive appendages [4–7].

Although the first *c*-type cytochromes were found in aerobic or facultative anaerobic organisms [1], anaerobes contain a considerable variety of these proteins. The first cytochromes identified contained only a small number of heme co-factors [1,8,9] but a screen of the currently available genomic sequences revealed that the number of heme binding motifs per multiheme cytochrome *c* varies considerably. The most common numbers are two, three and four. Sequences containing five, eight or ten binding motifs are also relatively common. Although the protein structure with the largest number of hemes per polypeptide is a hexadecaheme cytochrome [10], genes coding for polypeptide chains that contain 43 and 45 heme binding motifs were identified in *Geobacter uraniumreducens* and *Aeromyxobacter* sp., respectively [11]. Almost all major groups of Bacteria and Archaea have multiheme cytochromes *c*. In some of these organisms, such as representatives of *Geobacter, Shewanella*, *Anaeromyxobacter* or *Desulfovibrio* genera, the number of multiheme cytochromes is so elevated that it corresponds to a high percentage of their proteome, having elicited the creation of the term "cytochromome" [12,13].

The fact that so many organisms contain a large number of genes coding for multiheme cytochromes *c* and that these cytochromes can bind so many hemes begs two questions: what are the selective advantages of containing so many redox co-factors in a single polypeptide, and why did the spatial distribution of the co-factors evolve to be the way it is?

* Corresponding author. Fax: +351 214411277.
E-mail address: louro@itqb.unl.pt (R.O. Louro).

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The detailed structural, thermodynamic and kinetic characterization reported in the literature for several multiheme cytochromes, following the pioneering efforts of Xavier [14], is reviewed here in order to formulate a proposal to answer these questions.

2. Structural and functional advantages

There are structural advantages in binding several heme co-factors to a single polypeptide. Besides contributing to the correct fold of the polypeptide chain, the hemes also confer stability to the protein structure. Structures are known of multiheme cytochromes *c* with as little as 23 amino acids per heme. The cross linkage arising from the heme *c* coordinating residues, with three bonds provided by the CXXCH motif, or four bonds in the case of hexacoordinated hemes, provides multiheme cytochromes *c* with greater stability compared with proteins of similar size [15]. The covalent binding of the *c*-type hemes prevents the statistical thermodynamic decay in affinity constants for equilibrium binding of multiple identical co-factors [16]. This means that the hemes can be stably positioned close to each other and in larger quantity per polypeptide chain. Indeed, all known multiheme cytochromes containing more than two hemes per polypeptide are of type *c* [17].

Having multiple redox centers in a single protein also provides functional advantages for electron transfer. When electrons have to be transferred across large distances, intramolecular electron transfer within a multicenter redox protein proceeds without the need for transient recognition and binding between successive physiological partners in the crowded cellular medium. The two main parameters that define the intraprotein electron transfer are the tunneling distance and the driving force [18,19]. For a tunneling distance between pairs of redox centres shorter than approximately 14 Å, the tunneling rates remain compatible with the physiological turnover of the protein [18]. For electron transfer over larger distances the introduction of several redox centers is the only way to ensure that the electron tunneling rate remains sufficiently fast [19]. This mode of long distance electron transfer is called hopping, and it allows electrons to flow at a rate compatible with the physiological turnover even when there are endergonic steps in the overall exergonic electron transfer chain [19].

Multiheme cytochromes *c* can access a larger range of redox activity because each redox center can be tuned to be active at a different reduction potential. This contrasts with proteins containing a single redox center, which have a range of redox activity limited by the Nernst curve. There are various factors that influence the reduction potentials of hemes and allow them to be tuned over a range of 1 V (from −550 to +450 mV) [17]. The two most relevant are: the heme environment, namely amino acid charges, dipoles and exposure to solvent, which can shift the potential by up to 500 mV [20]; and the nature of the axial ligands, which can shift the potential by as much as 650 mV [21]. Smaller effects arise from factors such as: non-planar distortions of the heme, orientation of the axial ligands, thermodynamic proton coupling (redox-Bohr effect), and heme–heme interactions [22].

3. Cooperative effects

Heme–heme interactions modify the reduction potentials of the hemes involved. These changes may be cooperative or anti-cooperative, with both having functional consequences. Cooperativity between co-factors is an exclusive characteristic of multicenter redox proteins. The uptake of an electron by one heme affects the affinity for electrons (reduction potentials) of the remaining oxidized hemes. When the uptake of an electron lowers the affinity for the uptake of another, the protein exhibits negative cooperativity (anti-cooperativity) and this increases the range of electrode potentials where it is redox active. Conversely, if the uptake of one electron increases the affinity for an electron at another site, the cytochrome exhibits positive cooperativity and a sharper transition in redox state of the protein. Indeed, the maximum cooperativity that can be attained by a multicenter protein is ultimately limited by the number of sites. Therefore, the increase in the number of hemes facilitates the emergence of cooperative processes [23].

The detailed characterization of the reduction potentials and interactions in multiheme cytochromes is a laborious task and has been reported for several examples. Of these, the following are soluble and monomeric in physiological conditions, the structure is known, and heme–heme interactions were determined experimentally: the triheme cytochromes c_7 (or Ppc) from the *Geobacteraceae* family [24,25]; the tetraheme cytochromes c_3 from the *Desulfovibrio* (*D*) and *Desulfomicrobium* genera [26–29]; the small tetraheme cytchrome *c* (STC) [30,31] and the tetraheme flavocytochrome *c* [32] from the *Shewanella* genus; and the diheme cytochrome c_4 from *Pseudomonas stutzeri* [33]. Fig. 1 shows the heme architecture of the target cytochrome structural families.

4. Distance dependence of electrostatic interactions

The experimental heme–heme interactions are composite parameters that can contain contributions from other processes. The most common is the redox-Bohr effect that arises from thermodynamic coupling between redox and acid–base groups that titrate in the physiological pH range. The heme, with a redox active iron and acid–base active propionates, is a well suited co-factor to establish such a heterotropic linkage. Whereas the electrostatic interaction between the electrons in hemes is repulsive and therefore anti-cooperative, the electrostatic interaction between electrons and protons is attractive and therefore cooperative. Consequently, the parsing of the redox-Bohr contribution is essential for correctly interpreting the values of the heme–heme interactions [30,34]. For those cases where this was done it is possible to observe that the redox interactions between pairs of hemes show a

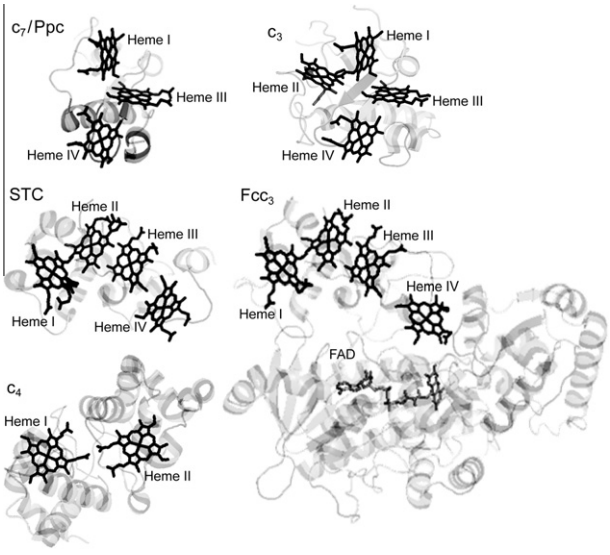

Fig. 1. Spatial arrangement of the hemes in the various cytochrome structural families analyzed in this work. The image was prepared using the program Pymol v0.99 to display the structures with the following PDB codes, corresponding to a representative of each protein family: 3H4N (PpcD from *Geobacter sulfurreducens*); 1UPD (cytochrome c_3 from *D. desulfuricans* ATCC 27774); 1M1Q (STC from *Shewanella oneidensis* MR-1); 1QJD (flavocytochrome c_3 from *Shewanella frigidimarina*); 1M70 (cytochrome c_4 from *Pseudomonas stutzeri*).

dependence with the iron-to-iron distance that follows a simple model of shielded electrostatic interactions (Fig. 2). This model considers a Coulombic decay enhanced by a Debye–Hückel shielding factor [35]. This is an indication that the overall dielectric environment within these multiheme cytochromes is very similar, despite the differences between number of hemes, heme architecture and amino acid composition. Considering only the heme domain of the flavocytochrome c_3, the overall ratio of protein length to heme for these proteins ranges from 23 to 27 amino acids for STC and cytochrome c_3, respectively. The diheme cytochrome c_4, which has a ratio of 95 amino acids per heme, displays a less effective electrostatic shielding of the hemes–heme interactions in agreement with a less hydrophilic environment.

The values of the redox interactions between pairs of hemes are typically positive as expected for the repulsive interaction between electrons. However, this simple correlation breaks down in the presence of redox-linked conformational changes that can modify the relative coordinates of the interacting centers or the electrostatic environment surrounding the interacting charges. These additional terms in the observed interaction are denominated non-Coulombic effects or mechano-chemical effects, which can enhance or diminish the electrostatic component of the interaction [28,36].

Non-Coulombic effects are evident in Fig. 2 for data measured for the cytochromes c_3, which are the most disperse [35]. Some of the heme-heme interactions measured for these proteins even display negative values when cooperative non-Coulombic effects dominate over the electrostatic effects. These redox-linked conformational changes were identified by comparative structural studies of the oxidized and reduced forms in several cytochromes c_3. They do not involve major structural rearrangements and were mainly associated with localized conformational changes in the side chains of amino acid residues located in the neighborhood of the heme groups and with conformational changes in the heme propionates [37–41].

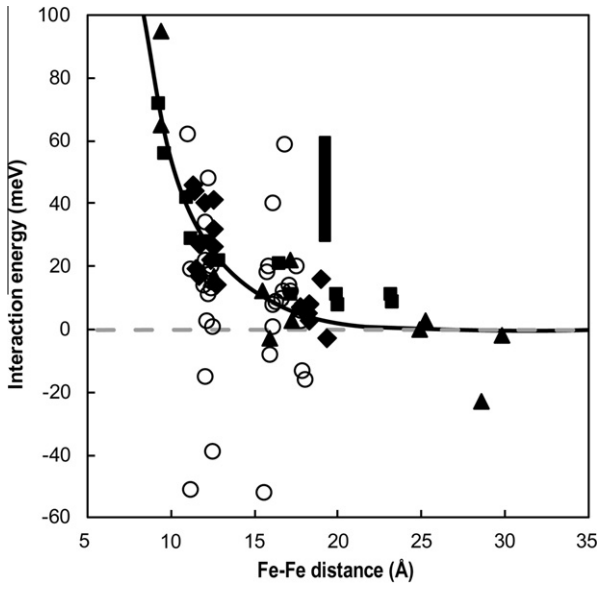

Fig. 2. Distance dependence of the pairwise interactions between the hemes. Squares illustrate data for the STC from the *Shewanella* genus [30,31]; Triangles illustrate data for the flavocytochromes c_3 from the *Shewanella* genus [32]; Diamonds illustrate data for the cytochrome c_7/Ppc from *Desulfuromonas* and *Geobacter* genera [24,25]; the rectangle illustrates the range of the interactions reported for the cytochrome c_4 [33]; open circles illustrate data for the cytochromes c_3 from the *Desulfovibrio* and *Desulfomicrobium* genera [27–29]. Distances were measured between iron atoms from the protein structures with the following PDB codes: 1M1Q; 2K3V; 1QJD; 1D4D; 1HH5; 2LDO; 3BXU; 3H4N; 3H34; 1RWJ; 1WAD; 2CTH; 1UPD; 2BQ4; 2CY3; 1W7O; 1M70, using the program Pymol v0.99. The solid line was obtained with a Debye–Hückel model of shielded electrostatic interactions considering and effective dielectric constant of 8.6 and Debye length of 7.7 Å [42].

5. A functional role for intramolecular interactions

Fig. 2, shows that heme-heme interactions affect the reduction potentials of the hemes, and that this effect of up to 100 mV decays rapidly with distance.

Fig. 1 shows that multiheme cytochromes with different physiological functions may present similar spatial arrangement of the hemes. These data suggest that the interplay of the heme reduction potentials with the redox, redox-Bohr and non-Coulombic effects, plays a role in establishing the functional specificity of each protein.

This hypothesis attributes a biological function to the heme–heme interactions in modulating the microscopic redox properties of multicenter redox proteins. Thermodynamically, this means that the population of the multiple *microstates* that mediate the progression between fully reduced and fully oxidized cytochrome is not fortuitous and has been selected to favor the physiological function.

To explore this hypothesis the redox speciation diagrams for these complex proteins need to be calculated on the basis of the experimentally determined heme reduction potentials and their interactions, pK_as of acid-base groups affected by the redox state of the protein and redox-Bohr interactions. Experimental data that allow this detailed analysis was collected for several multiheme cytochromes and are plotted in Fig. 3. Due to the close proximity of the hemes in all these multiheme cytochromes, intramolecular electron transfer is fast on the experimental time-scale, which ensures equilibrium among all *microstates* with the same number of oxidized hemes, in what are called macroscopic redox *stages*. The *stages* are linked by *steps* that involve the uptake or release of one electron by the cytochrome. In the case of a multicenter protein containing n redox centers, $n + 1$ macroscopic oxidation *stages* can co-exist in solution, each containing the *microstates* with the same number of oxidized hemes. The number of oxidized hemes provides the nomenclature for the macroscopic *stages* from 0 for the fully reduced protein to n for the fully oxidized state. For $n + 1$ macroscopic oxidation *stages* there are 2^{n+m} *microstates*, being m the number of redox-Bohr centers within the protein (see accompanying essay by Catarino and Turner).

The populations of these *microstates* for 15 soluble monomeric multiheme cytochromes are reported in Fig. 3, where the dominant *microstates* of partially oxidized *stages* are labeled to identify the hemes that are oxidized in each step.

Fig. 3 shows two aspects of the interplay between intrinsic reduction potentials of the individual hemes and the intramolecular interactions. The first is that in structurally identical proteins the tuning of the reduction potentials and interactions leads to dramatic differences in the dominant *microstates* as the proteins proceed from fully reduced to fully oxidized at physiological pH. This is observed for the triheme c_7/Ppc cytochromes. Proteomics and knock-out mutant studies on the five triheme cytochromes c_7 (PpcA-E) from *Geobacter sulfurreducens* suggested that this family of cytochromes plays a crucial role in driving electron transfer from the cytoplasm to the cell exterior, and assisting the reduction of extracellular acceptors [25]. In the case of PpcB and PpcE, several *microstates* are significantly populated in oxidation *stages* 1 and 2 and therefore, no preferential pathway for electron transfer can be established. In contrast, PpcA and PpcD appear to be optimized to interact with specific redox partners involving e^-/H^+ transfer via different mechanisms, where hemes I and IV are directly involved in the electron–proton coupling. In the case of PpcA, electron–proton coupling occurs between oxidation *stages* 1 and 2, whereas in PpcD oxidation *stages* 0 and 2 are involved in a two-electron transfer step coupled to

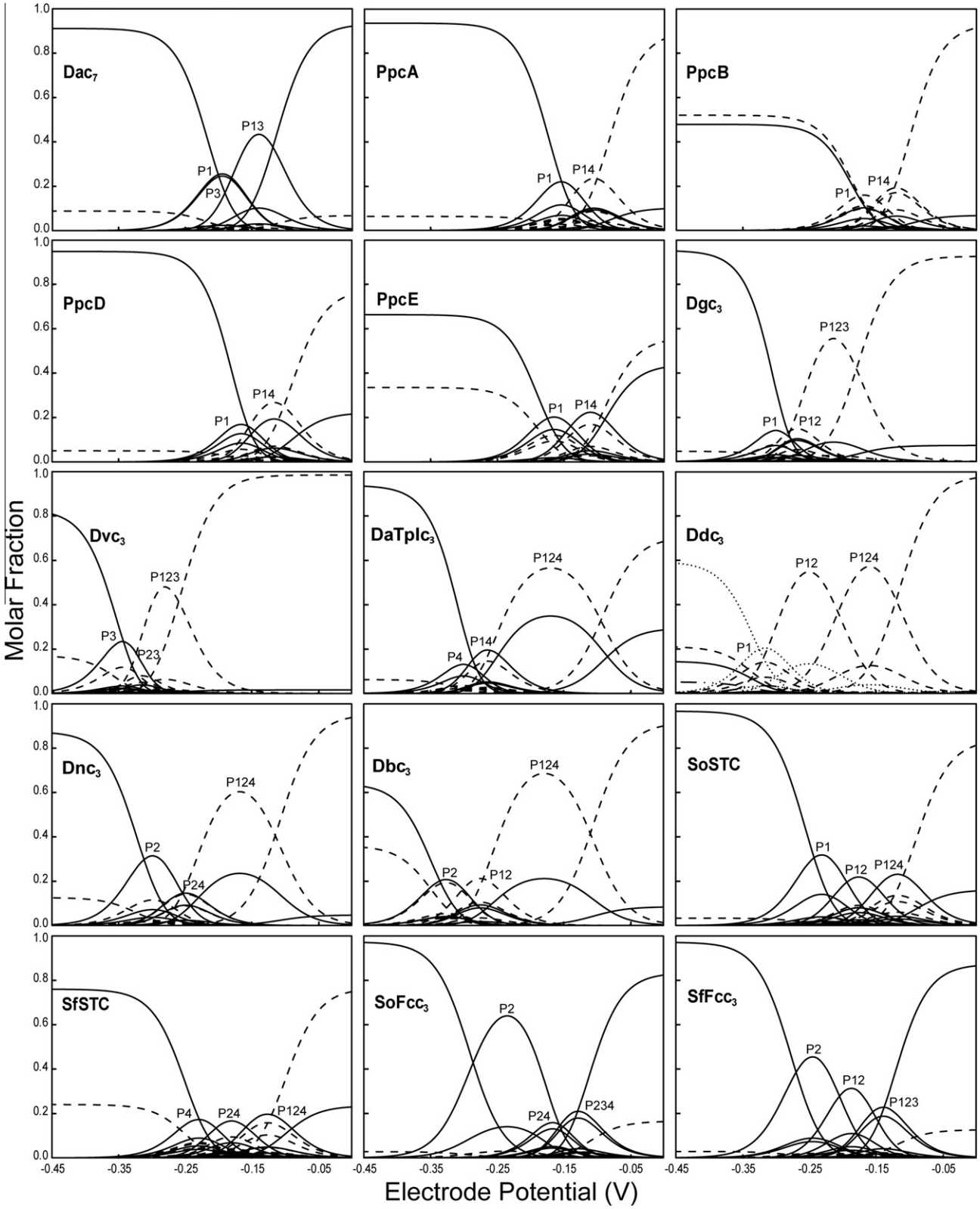

Fig. 3. Speciation diagrams of the *microstates* of the multiheme cytochromes *c*. The curves were calculated as a function of the electrode potential (SHE) using the thermodynamic properties determined experimentally [24–32]. The solid lines indicate the protonated *microstates* while the dashed lines indicate the deprotonated *microstates*. Dotted and dash-dot lines in the panel Ddc_3 indicate a second set of redox-linked protonations. The numerals above the lines indicate the oxidized hemes in the dominant *microstates*. Panels Dac_7 and PpcA-E correspond to data from the triheme cytochrome c_7 from *Desulfuromonas acetoxidans* and *Geobacter sulfurreducens*, respectively, at physiological pH 7.5 [24,25]. Panels Dgc_3, Dvc_3, $DaTplc_3$ Ddc_3 correspond to data from the type I tetraheme cytochrome c_3 from *D. gigas*, *D. vulgaris* Hildenbourgh, *D. africanus*, *D. desulfuricans* ATCC 27774, respectively, at physiological pH 7.0 [26–28]. Panels Dnc_3 and Dbc_3 correspond to data from the tetraheme cytochrome c_3 from *Desulfomicrobium norvegicum* and *Desulfomicrobium baculatum*, respectively, at physiological pH 6.15 [29]. Panels SoSTC and SfSTC correspond to data from the tetraheme cytochrome STC from *Shewanella oneidensis* MR-1 and *Shewanella frigidimarina*, respectively, at physiological pH 7.0 [30,31]. Panels $SoFcc_3$ and $SfFcc_3$ correspond to data from the tetraheme flavocytochrome c_3 from *Shewanella oneidensis* MR-1 and *Shewanella frigidimarina*, respectively, at physiological pH 7.0 [32].

the deprotonation of the redox-Bohr center [25]. The triheme cytochrome c_7 from *Desulfuromonas acetoxidans* was also implicated in the respiration of metals and sulfur [43,44]. In this cytochrome, no electron–proton coupling is observed in the physiological pH range but a clear selection of the intermediate stage populations is evident [24].

The second aspect is that structurally identical proteins can achieve the same functional properties via dramatically different detailed mechanisms. The tetraheme cytochromes c_3 from *Desulfovibrio* and *Desulfomicrobium* share a common architecture of the heme core, which is identical to that of cytochromes c_7 with the addition of the polypeptide section that binds heme II. The physiological function of these cytochromes is to accept electrons and protons from periplasmic hydrogenases, and deliver them to membrane-linked protein complexes, with a net energy transfer from the redox to the protonic centers [28,45]. In line with this common function and in contrast to what is observed for the triheme cytochromes, all cytochromes c_3 show a bias for some of the intermediate *stage* populations to be greatly diminished, an indication of positive cooperativity. Fig. 3 shows that a variety of strategies can be used to achieve this objective. For the cytochromes c_3 from *D. gigas*, *D. vulgaris* Hildenborough, *D. africanus* and *D. desulfuricans* ATCC 27774, a clear-cut positive cooperativity between a pair of hemes occurs [46]. In all cases the positive redox cooperativity is associated with an acid-base transition giving rise to a proton-coupled two-electron step. The speciation diagram for *D. desulfuricans* is slightly more complex since two redox-linked protonation events are observed in the physiological range, leading to a greater dispersion of the populations. For the cytochromes c_3 from the *Desulfomicrobium* genus the coupling with protonation is so important, in the context of a weaker heme–heme positive cooperativity, that it has been named proton-assisted two-electron step [29].

The STC cytochrome from *Shewanella* genus, a soluble 12 kDa protein isolated from the periplasmic space, presents a distinct spatial arrangement of the hemes which forms a bent chain. Knock-out mutations of the gene implicate this protein in iron reduction [47,48] and more recently in the reduction of DMSO [49]. Analyzing the panels corresponding to STC from *S. oneidensis* (SoSTC) and from *S. frigidimarina* (SfSTC), it is possible to verify that in both cases, at physiological pH, reduction of heme III which is the least exposed heme and therefore the less likely to make direct electron transfer to a redox partner, is strongly coupled to protonation. This suggests a role as gate-keeper by modulating the electrostatic environment via redox and redox-Bohr interactions with the other hemes. The consequence is that when the protein is fully oxidized, the deprotonated *microstate* is dominant, whereas in all the other redox *stages*, the protonated *microstates* are dominant, for both examples. In addition to this protonation step coupled with electron transfer, there is always a dominant *microstate* in every redox *stage*, indicating that there is an ordered distribution of the electrons within the protein [30,31]. The similarities at the structural level and in metabolic role are also translated in kinetic studies performed in vitro [50]. These studies show that for both SoSTC and SfSTC the heme with the more negative reduction potential is the major player in the uptake or release of electrons.

Finally, flavocytochrome c_3 (Fcc_3) is a 64 kDa soluble fumarate reductase from the *Shewanella* genus. This enzyme contains a tetraheme domain that is structurally homologous to the STC cytochrome and a flavin domain responsible for the unidirectional reduction of fumarate to succinate. This is a coupled two-electron, two-proton reaction. It is clear from the dominant *microstates* in both examples that the first pair of electrons is transferred to the FAD catalytic site while ensuring that heme IV, located near the catalytic site, remains reduced. This reduction is achieved by intramolecular re-equilibration among the hemes which is approximately three orders of magnitude faster than the electron transfer to the FAD, ensuring the transfer of a pair of electrons necessary for the catalysis [32,51]. Interestingly, the data for these two enzymes do not show significant e^-/H^+ coupling, which reveals that proton delivery to the catalytic site is not thermodynamically linked to the redox state of the hemes [32].

The data reported in Fig. 3 reveals clearly that for a similar heme core arrangement the combined effect of the reduction potentials and interactions brings about a detailed selection of the specific states of the protein that are active during the redox cycling. These allow for a versatile tuning of the detailed redox properties of multiheme cytochromes. Therefore, a common architecture can accommodate different functions, or alternatively a common architecture can be used to achieve a similar function by different detailed mechanisms.

6. Conclusion

The data compiled in this review argue in favor of an important role for intramolecular interactions in establishing a defined speciation during the redox cycling of multicenter cytochromes, a phenomenon called thermodynamic gating [40]. However, the electrostatic component of these interactions decays strongly with distance. As a consequence, hemes need to be located very close together to ensure an interaction magnitude that can significantly modulate their intrinsic reduction potentials. This in turn places constrains on the spatial organization of the hemes within the protein.

In conclusion, the architecture of multiheme cytochromes ensures fast intramolecular electron transfer and control over the *microstates* that are populated during the redox activity. These properties are essential to prevent the thermodynamic dissipation of the redox energy channeled through these proteins, diminishing the probability of unspecific interactions within the crowded cellular medium and guaranteeing a defined biological role.

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