Redox-Bohr and Other Cooperativity Effects in the Nine-heme Cytochrome c from *Desulfovibrio desulfuricans* ATCC 27774

CRYSTALLOGRAPHIC AND MODELING STUDIES

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The nine-heme cytochrome *c* is a monomeric multiheme cytochrome found in *Desulfovibrio desulfuricans* ATCC 27774. The polypeptide chain comprises 296 residues and wraps around nine hemes of type *c*. It is believed to take part in the periplasmic assembly of proteins involved in the mechanism of hydrogen cycling, receiving electrons from the tetraheme cytochrome *c*3. With the purpose of understanding the molecular basis of electron transfer processes in this cytochrome, we have determined the crystal structures of its oxidized and reduced forms at pH 7.5 and performed theoretical calculations of the binding equilibrium of protons and electrons in these structures. This integrated study allowed us to observe that the reduction process induced relevant conformational changes in several residues, as well as protonation changes in some protonatable residues. In particular, the surroundings of hemes I and IV constitute two areas of special interest. In addition, we were able to ascertain the groups involved in the redox-Bohr effect present in this cytochrome and the conformational changes that may underlie the redox-cooperativity effects on different hemes. Furthermore, the thermodynamic simulations provide evidence that the N- and C-terminal domains function in an independent manner, with the hemes belonging to the N-terminal domain showing, in general, a lower redox potential than those found in the C-terminal domain. In this way, electrons captured by the N-terminal domain could easily flow to the C-terminal domain, allowing the former to capture more electrons. A notable exception is heme IX, which has low redox potential and could serve as the exit path for electrons toward other proteins in the electron transfer pathway.

Protein electron transfer is a key process in metabolic chains. Usually, proteins involved in these chains, bearing one or more redox centers, show a high degree of complexity, which makes the understanding of the molecular basis of such processes a difficult task. Nevertheless, the knowledge of an increasing number of three-dimensional structures of such proteins in different redox states has helped, to a certain extent, to overcome this limitation. For instance, in the large multicenter redox enzyme cytochrome *c* oxidase, the knowledge of the crystal structure from different sources and in different redox states, as well as with different bound ligands (1–6), has allowed the suggestion of molecular mechanisms of electron and proton transfer. However, this example constitutes a very large and complex system, and it is still far from being completely understood. There are other examples, such as tetraheme cytochromes *c*3 (7–12), ferredoxins (13–16), rubredoxins (17, 18), and flavodoxins (19–22), that because of lower complexity, constitute good working systems to understand these types of processes. In fact, experimental evidence for the existence of cooperativity effects underlying electron transfer has already been reported in the literature for tetraheme cytochrome *c*3 of certain species of the *Desulfovibrio* genus (Refs. 10, 23, and 24, and references therein). These cooperativity effects include positive cooperativity in reduction as well as the redox-Bohr effect (25), which is a thermodynamic interdependence between reduction and protonation, and is clearly present in these types of cytochromes at physiological pH (Refs. 10, 23, and 24, and references therein). The understanding of these phenomena at the molecular level and the identification of the groups involved were possible only by the determination of the three-dimensional structure, in different oxidation states, of these cytochromes, either by x-ray crystallography (8, 10, 12), or by NMR spectroscopy (7, 9, 11).

When analyzing the effects of reduction using structures in different oxidation states, it is important to obtain these structures under controlled conditions, where the effects we want to study are evident, so that clear, significant, and interpretable differences can be observed. We have done this for the tetraheme cytochrome *c*3 from *D. desulfuricans* ATCC 27774 (DdA)† (10, 12). The structures that we determined at pH 7.6, together with the results from theoretical calculations, allowed us to identify the groups related to the redox-Bohr and positive cooperativity effects and understand, in this way, which are the key features that underlie the mechanism of electron transfer in this cytochrome. In conclusion, the study of simpler systems still constitutes an important and valuable contribution for the understanding of the mechanisms underlying the electron transfer process or even other types of processes associated with electron transfer.

One of these systems is the nine-heme cytochrome *c* (9HcA)

† The abbreviations used are: DdA, *Desulfovibrio desulfuricans* ATCC 27774; CEs, continuum electrostatics; MR, molecular replacement; MC, Monte Carlo; Tpi-c3 and TpiII-c2, types I and II tetraheme cytochrome *c*3, respectively; rmsd, root mean square deviation.
from DdA. It is a c-type cytochrome, with a molecular mass around 37 kDa, 296 residues, and nine heme groups covalently bound to the polypeptide chain. Eight of these nine hemes are arranged in two tetraheme c$_{5}$-like domains, with the extra, isolated heme located in between (26, 27). The heme-binding motif for all nine hemes is of type Cys-X-X-Cys-His and, until now, 9HcA has been isolated only from two strains of the Desulfovibrio genus, DdA (26, 27) and D. desulfuricans strain Essex (DdE) (28, 29). 9HcA is encoded by the first gene of an operon, which also encodes for three other proteins (9HcB, 9HcC, and 9HcD) and is mainly involved in sulfate respiration (30). The four proteins that compose this putative redox complex (9Hc) are similar in sequence to four of the proteins identified in another related redox complex, the Hmc complex (HmcB, HmcC, HmcD, HmcE, and HmcF), isolated from D. vulgaris Hildenborough (DvH) (31, 32). 9HcA has 39% sequence identity with the C-terminal region of the 16-heme high molecular mass cytochrome c (HmcA), 9HcB has 51% sequence identity with HmcB, 9HcC has 64% sequence identity with HmcC, and 9HcD has 30% sequence identity with HmcD (30). In fact, the high degree of similarity observed between the 9Hc and the Hmc complexes, together with the fact that the 9Hc complex is absent from those strains where the Hmc complex has been isolated (26, 27, 30) to include the 9Hc complex in the Hmc family. Moreover, it has been shown that 9HcA from DdA, similar to HmcA from DvH, accepts electrons from hydrogenase via the tetraheme cytochrome c$_{5}$ (27). Recently, experimental evidence for the presence of the redox-Bohr effect in 9HcA has been reported (33).

Theoretical methods applied to structural data have proven invaluable in understanding electron and proton transfer. Studies were performed in several cases to estimate and understand redox potentials in proteins, using microscopic (34–37) or continuum electrostatics (CE) (38–45) methods. Because of their computational efficiency, the CE methods were used for the more extensive simulations of the actual binding equilibrium of electrons and protons (10, 42, 44, 45). The most correct way to model coupling between reduction and protonation in proteins is to use methods introduced by us (42) that model the joint binding equilibrium of electrons and protons in proteins, using Monte Carlo techniques. Other similar approaches were later reported in the literature (43, 44), aiming to model the same type of effects. Multiheme cytochromes c$_{5}$ were extensively characterized using these theoretical methodologies aiming to understand coupling phenomena (10, 12, 42, 43, 45), with significant success. However, one limitation of the majority of these studies is the fact that only one conformation (x-ray structure) is used to characterize all possible states of the system, which is a significant simplification that leaves out the possibility that specific reorganization effects of reduction and protonation may occur. We reported recently (10, 12) studies that considered two x-ray structures (in the oxidized and reduced states) to understand protein reorganization effects and the way these influence the reduction and protonation phenomena in cytochrome c$_{5}$. This procedure was clearly better than previous approaches and allowed us to characterize very important effects in this redox protein, such as the phenomenon of positive cooperativity. This is the approach that we propose to follow here with 9HcA.

In the present work, we report the crystal structure of the oxidized and reduced forms of 9HcA from DdA at pH 7.5 and results obtained from theoretical studies based on these structures. This integrated study allowed the identification of the groups directly involved in the redox-Bohr effect. Similarly to what happens in the previously mentioned case of the tetra-heme cytochrome c$_{5}$, it was possible to provide evidence of the presence of positive cooperativity in reduction in this cytochrome and to identify the groups involved.

MATERIALS AND METHODS

Crystallization and Data Collection—Crystals of the oxidized form of 9HcA were obtained at pH 7.5 under conditions similar to those described previously (46). These crystals were then reduced with an excess of sodium dithionite that was added to the crystallization drop; the reduction process was followed by the change in the color of the crystal, from an initial red to a final pink-red. Color homogeneity was reached after about 5 min, an indication that the process was complete. Nevertheless, the crystals were still allowed to stay under these conditions for another 10 min, after which they were immediately harvested and flash-frozen, using a cryoprotecting solution containing 20% glycerol, 80% of the crystallization solution, and an excess of sodium dithionite.

Two data sets were collected at the European Synchrotron Radiation Facility in Grenoble, at 110 K: one from a crystal of the oxidized form, and another from a crystal of the reduced form. Both data sets were processed with the DENZO/SCALEPACK program (47), and the statistics obtained are listed in Table I. The crystals belong to the monoclinic space group, P2$_{1}$, with two molecules in the asymmetric unit. The data set obtained from the oxidized crystal had a maximum resolution of 1.5 Å, whereas that obtained from the reduced crystal had a maximum resolution of 2.0 Å.

It has been shown that a prolonged exposure to a high-flux Synchrotron radiation beam may induce reduction in some crystal structures, such as horseradish peroxidase (48). Because our high resolution (1.5 Å) diffraction data of the oxidized form of 9HcA at pH 7.5 were obtained from low-symmetry crystals (space group P2$_{1}$), such an x-ray-induced reduction effect might be possible and, as such, partially mask the structural differences described below between the oxidized and re-

| Oxidation state | Reduced | Oxidized |
|----------------|---------|----------|
| X-ray source   | BM14 (ESRF$^*$-Grenoble) | ID14-EH2 (ESRF$^*$-Grenoble) |
| Wavelength, Å | 0.799   | 0.993    |

*ESRF, European Synchrotron Radiation Facility.
$^*$ a.u., arbitrary units.
Values obtained using program R merge (76).

Values in parentheses refer to the last resolution shell, 2.0–2.0 Å for the reduced form and 1.55–1.50 Å for the oxidized form.
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TABLE II

|                        | Reduced             | Oxidized            |
|------------------------|---------------------|---------------------|
| Oxidation state        | 36457               | 36457               |
| R-factor R_{merge} (%)  | 18.5(2.7)           | 16.3(19.3)          |
| No. of nonhydrogen protein atoms$^a$ | 2294                | 5583                |
| No. of solvent molecules | 521                 | 675                 |
| Other ligands          | 1 acetate; 2 glycerols | 1 acetate; 3 glycerols |
| Model rmsd from ideality | 0.011               | 0.010               |
| Bond lengths (Å)       | 0.020               | 0.027               |
| Average B-factors (Å$^2$) | 37.24              | 32.25               |
| Protein main-chain 9HcA A (9HcA B) | 26.41 (28.36)    | 19.33 (21.48)       |
| Protein side-chain 9HcA A (9HcA B) | 26.99 (29.51)    | 22.27 (24.40)       |
| Solvent molecules      |                     |                     |
| Ramachandran plot statistics |                   |                     |
| % residues in most favored regions | 87.1               | 88.1                |
| % residues in allowed regions | 12.5               | 11.5                |
| % residues in generously allowed regions | 0.4                | 0.4                 |

$^a$ Including heme groups.
dielectric constant must be regarded in an average sense (and hence as an empirical parameter), given that the reorganization of a protein molecule is largely inhomogeneous (64, 66, 67). The optimal value for this dielectric constant was found in different works to be around 20 (58, 62, 68) for prediction of \( pK_a \) values in proteins.

The MC sampling of binding states was performed with the PETIT (Proton and Electron TITration) program (42, 45, 58). The ranges of \( pK_a \) and electrostatic potential were sampled at intervals of 0.25 \( pK_a \) units and 20 mV, respectively. The temperature was 300 K, and site pairs were selected for double moves when at least one pairwise term was >2 \( pK_a \) units. Averages were computed using \( 10^6 \) MC steps, and correlations were computed using \( 10^6 \) MC steps.

**Preparation of Structures for Calculations**—Because of the underlying molecular rigidity imposed, electrostatic calculations using CE methods are very sensitive to the exact conformations of groups, in particular of polar and charged groups. Therefore, special care must be taken to analyze the effect of particular conformational changes because non-relevant conformational differences can mask their effect. X-ray refinement may, in some cases, place side chains in more or less random positions, because of the lack of electron density to model them unambiguously. The way we chose to treat this problem was to compare all three x-ray structures analyzed here and their corresponding electron densities. If a side chain was found to be undefined in any of the structures, its overall conformation was set to one of the other structures. This may not represent the conformation of the side chain in solution, but in the absence of other information, this procedure does not introduce a differential effect on the calculations. Therefore, only conformational changes that are clearly visible in the electron density are analyzed.

Besides the three x-ray structures available (oxidized at pH 5.5, oxidized at pH 7.5, and reduced at pH 7.5), we must consider that several residues have alternate conformations. Some of these alternate conformations are potentially of considerable importance; therefore, an independent analysis must be carried out for the most important cases. If a structure has a residue with alternate conformations in an important region, two structures will be built. Thus, in the oxidized structure at pH 5.5, we have considered two structures for Ser-58, a “normal” one (Ox5.5N) and an “alternative” one (Ox5.5S). In the same way, for the oxidized structure at pH 7.5 two structures were built, corresponding to the two alternate conformations for the main chain of the loop comprising the residues from Val-118 to Lys-121, Ox7.5N and Ox7.5L. In the reduced structure at pH 7.5, Lys-212 showed alternate conformations, both of which interacted with the propionate side chains of heme III, and two more structures were considered, Red7.5N and Red7.5K. From the above considerations, we arrived at a total of six different structures to simulate. Because of computational limitations, we chose to consider only molecule A in all calculations. However, the most important structural differences (discussed below) are present in both independent molecules in the asymmetric unit.

As done previously, crystallographic water molecules with <50% relative accessibility were considered in the calculations (45, 58).

**RESULTS AND DISCUSSION**

**Structure Analysis**—The three-dimensional structure of nine-heme cytochrome c comprises 296 residues, 292 of which are visible in the electron density maps, and nine heme groups covalently bound to the polypeptide chain. All nine hemes have bis-histidinyl coordination, and all heme binding motifs are of the type CXXCH. The three-dimensional structure is divided into two domains, the N-terminal domain and the C-terminal domain, connected by a short polypeptide segment. Each domain comprises a \( c_3 \)-like region and an insertion, which is involved in the coordination of the isolated heme (heme IV) (Fig. 1) (26, 27). The structure of the two tetraheme domains resembles some specific features found in the type II tetraheme cytochrome \( c_3 \) (TpII-\( c_3 \)). In the N-terminal domain, heme I (the corresponding heme I in tetraheme cytochromes) is highly exposed to the solvent, such as in TpII-\( c_3 \) and in contrast to the Tpl-\( c_3 \). Also, more similar with some of the TpII-\( c_3 \), in the C-terminal domain, heme IX (the corresponding heme IV in tetraheme cytochromes) is not surrounded by a positively charged patch. In this same domain, a loop region, which characteristically interrupts the long \( \alpha \)-helix in the Tpl-\( c_3 \), is absent (as in TpII-\( c_3 \)).

![Fig. 1](attachment:image.jpg)

To compare and to assess the stereochemical quality of both molecules in the asymmetric unit, the PROCHECK and PROCHECK (69) programs were used (Table II). The results revealed that in the oxidized and reduced forms, the secondary structure elements are maintained, Asp188 being the only residue to lie in a generously allowed region of the Ramachandran plot in the two independent molecules in the asymmetric unit. In the oxidized form, the average value of the root mean square deviation (rmsd) between the Ca atoms of the two independent molecules in the asymmetric unit is 0.407. This value was calculated with LSQKAB (55) and ranges between 0.019 and 1.634 (not considering the first residue of the N-terminal do-
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main, which was very ill-defined in the electron density maps. High values of rmad (>1.0) are found in regions with different crystalline contacts. Likewise, those regions also show high values of rmad between the side-chain atoms (variation range between 0.045 and 6.17 Å). A similar behavior was observed for the reduced form. The average value of the calculated rmad between the Cα atoms of both molecules in the asymmetric unit is 0.459. In this case, these rmad values vary between 0.029 and 1.663 (again, not considering the first residue of the N-terminal domain, which were very ill-defined in the electron density maps). For the side-chain atoms the calculated rmad values ranged between 0.067 and 6.242. The key conclusion from this analysis is that, in both the oxidized and reduced forms, the major differences between the two independent molecules in the asymmetric unit are located at the molecular surface and are mainly attributable to different crystalline contacts. Also, with the exception of Ser-58 in the reduced form, none of these differences concern residues that experience redux-linked or pH-linked conformational changes.

In the reduced form of 9HeA, the side chain of Ser-58 and PrA-I show different conformations for both independent molecules in the asymmetric unit. In molecule A, there is a symmetry-related molecule interacting with this region; in particular, there is an electrostatic interaction between PrA-I and Tyr-179 from the symmetry-related molecule. On the other hand, in molecule B this region is completely exposed to the solvent. The observed differences also appear to be connected with the reduction process (see “Discussion”).

The final atomic coordinates have been deposited in the Protein Data Bank, with accession code numbers 1ovf and 1ofy.

Global Analysis of Titration Curves—From the analysis of Fig. 2, it is clear that the oxidized structure at pH 5.5 (Fig. 2a) shows less negative simulated redux potentials than the oxidized structure at pH 7.5 (Fig. 2b). This effect can result from two factors. First, the change to lower pH will protonate groups, leading to a larger population of protons, as indicated in Fig. 3 (there is a difference of 6.3 protons between the oxidized structures at pH 5.5 and 7.5), which will stabilize the reduced state (protons stabilize electrons). Second, the protein structure can experience conformational changes because of pH that can stabilize or destabilize each of the different redux states. To test the first factor, we simulated the two structures obtained at different pH values (5.5 and 7.5) at the pH value of the other structure (5.5 for the structure obtained at 7.5 and vice versa). Overall, a very similar distribution of titration curves was obtained for the different structures simulated at each pH value (5.5 and 7.5) (data not shown), despite the fact that differences do exist between the two x-ray structures. Therefore, the difference in pH seems to be the main factor in the shifting of redux titrations in the oxidized structures.

In the oxidized structure at pH 5.5, the midpoint redux potential differences between individual hemes are lower (i.e. the hemes reduce at closer redux potentials) than in the oxidized structure at pH 7.5 (Fig. 2). This also results from the different pH of the simulations, because the simulations done with each structure at the pH of the other structure show a reversion of this behavior, suggesting that it is probably an indirect effect of protonation. Indeed, at pH 5.5 there are a multitude of acidic groups that are closer to their titration midpoints, meaning that they are more prone to exchange protons with the medium. Upon reduction, these higher exchange propensities will result in concomitant proton captures that will tend to stabilize the electron captures, making the reduction process easier. This will counterbalance the effects of heme repulsion upon reduction and will facilitate the simultaneous electron capture by the heme groups, which will result in the proximity of heme redux potentials. The same behavior seems to occur when we consider the reduced structure at pH 7.5. This structure shows lower differences between individual heme mid point redux potentials than in the case of the oxidized structure at the same pH. The same explanation can be used here also, because one of the effects of the reduction is to favor the appearance of groups that display proton fluctuations.

Table III contains the midpoint reduction potentials for each individual heme, as calculated for each different structure analyzed, as well as the reduction order observed in each case. The results obtained show that there is a maximum change in midpoint redux potentials of 0.3, 2.1, and 1.4 mV, when the alternative Ox5.5B, Ox7.5L, and Red7.5K structures are considered relative to their normal counterparts, respectively. Nevertheless, the reduction order of the hemes for these alternative structures, in comparison with that of the corresponding normal ones, is unchanged. In contrast, there are significant differences between the results corresponding to structures obtained at different pH values and redux conditions. The reduction order changes more significantly between the reduced and oxidized structures at pH 7.5. However, the actual reduction order of this cytochrome should be something between these two extreme situations. Nevertheless, the C-terminal hemes VI, VII, and VIII are always the first to be reduced, whereas the N-terminal hemes (I, II, III, and V) are the last to be reduced, in all structures analyzed at pH 7.5.

Fig. 3 shows that reduction is accompanied by proton binding, which constitutes a clear indication that the redux-Bohr effect is present in this cytochrome. If only the simulation data for the experimental situations (i.e. the fully reduced state for the fully oxidized state and the fully oxidized state for the fully oxidized structure, both at pH 7.5) is taken into account, it is possible to verify that upon reduction the molecular capture 2.4 protons for a total capture of nine electrons. The groups responsible for this effect will be discussed below.

Comparison between the Oxidized Structures at pH 5.5 and pH 7.5—In this section we will focus our attention on those groups that exhibit conformational changes induced by the change of pH and that are relevant to the analysis of the differences observed between the oxidized and reduced forms at pH 7.5, discussed below. Also, of particular interest will be those groups that show a large protonation difference between the two oxidized forms (Table IV).

The Cα atoms from both oxidized forms at different pH values (pH 5.5 and pH 7.5) were compared using error-scaled difference distance matrices (program ESCET) (70, 71). The results showed that for both independent molecules in the asymmetric unit, the conformation of the protein backbone is generally invariant, within the error margin of both structures, the major conformational changes being located in the side chains of certain residues. Indeed, these conformational changes can be induced by changes in the protonation state because of the increase in pH. Therefore, theoretical calculations were carried out to identify which groups showed a significant difference in the protonation state between pH 5.5 and pH 7.5. Table IV lists all of the residues that showed protonation differences above 0.1 protons. Despite the fact that pH 5.5 is above the normal pKₐ of free acids, the particular architecture of this structure causes a multitude of acidic groups to show clear protonation, even at this pH.

Ser-58 shows a significant conformational change between pH 5.5 and 7.5. As described above, this residue is positioned close to heme I, and its side chain shows two alternate conformations at pH 5.5 (Fig. 4a). Rotamer A points away from the heme, and its Oy is within H-bonding distance of two water molecules (2.4 and 3.0 Å), whereas rotamer B is facing heme I.
and establishes an H-bond with the O$_{1A}$ of PrA-I (2.98 Å). At pH 7.5, this residue shows only one conformation, similar to rotamer A at pH 5.5 (Fig. 4b). Independent simulations were done with Ox5.5N and Ox5.5S structures, corresponding to both rotamers at pH 5.5, and no significant differences were found in the protonation of nearby groups. Therefore, the reason for these two conformations at pH 5.5 may lie in the considerably mixed population of protonation states for the two propionate side chains of heme I (see Table IV). Rotamer A may be stabilized by the protonated form of PrA-I, whereas rotamer

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**Fig. 2.** Redox titrations for each individual heme of 9HcA using the different structures, at the pH of the corresponding crystal. The conformations considered were the ones termed normal in the text. a, oxidized structure at pH 5.5. b, oxidized structure at pH 7.5. c, reduced structure at pH 7.5.
B may be favored by the deprotonated one. On the other hand, at pH 7.5 both propionates are deprotonated (Table IV), and yet the conformation found for Ser-58 is similar to conformer A found at pH 5.5, which seemed to be stabilized by the protonated form of PrA-I. This residue will be further discussed when comparing the oxidized and reduced forms.

Another relevant conformational change attributable to the change in pH is found in Glu-102. At pH 5.5 the glutamate is facing PrA-IV, and its O²⁻ atom makes an H-bond with the O²⁻A atom from PrA-IV (2.51 Å) (Fig. 5a), suggesting that a proton is shared between these two atoms. From Table IV it is clear that this proton comes from the propionate, which captures 0.858 protons at pH 5.5. In fact, this PrA-IV is the group in this structure that shows the clearest protonation at pH 5.5. At pH 7.5, Glu-102 adopts a different conformation, facing the solvent and away from PrA-IV (Fig. 5b), and in addition, it captures 0.105 protons while PrA-IV becomes deprotonated (Table IV). Nevertheless, the deprotonation effect that occurs in PrA-IV is more relevant, and the glutamate side chain is driven away. These two residues are strongly coupled and will be further discussed when comparing the oxidized and reduced forms at pH 7.5.

The change in pH also affects the backbone conformation of the loop that comprises residues Val-118 to Lys-121. This segment is located in the neighborhood of heme IV and shows only one conformation at pH 5.5 (Fig. 5a). However, at pH 7.5 it becomes disordered, and two alternate conformations were modeled (Fig. 5b). One conformer (B) is similar to that found at pH 5.5, whereas the other conformer (A) is placed closer to heme IV and is similar to this segment's conformation in the reduced structure (discussed below). The analysis of the theoretical calculations does not provide an explanation for this change. It does not correspond to any significant proton capture (results not shown), nor to any relevant change in the midpoint reduction potentials of the hemes (Table III) (the maximum change is 2.0 mV). Nevertheless, this effect may be related to a significant conformational change that is observed nearby. At pH 5.5 PrD-IV is facing PrA-IV, and its oxygen atoms are within H-bonding distance of two water molecules and of a main-chain nitrogen atom (Lys-121) (Fig. 5a). Both propionates show some degree of protonation at this pH, which is even more pronounced in PrA-IV (Table IV), and this may account for their close position. At pH 7.5 both propionates become deprotonated (Table IV), the repulsive forces increase between them,

### Table III

**Midpoint reduction potentials of individual hemes in the different structures used in the calculations**

| Reduction orders | O5.5N and O5.5S | O5.5N and O5.5L | Ox5.5N | Ox7.5N | Ox7.5L | Red7.5N | Red7.5K |
|------------------|-----------------|-----------------|--------|--------|--------|---------|---------|
| I                | −298.9          | −299.3          | −365.5 | −365.6 | −344.8 | −347.9  |         |
| II               | −273.3          | −272.9          | −305.7 | −305.8 | −288.1 | −288.2  |         |
| III              | −279.2          | −278.9          | −305.2 | −305.8 | −331.9 | −330.5  |         |
| IV               | −291.6          | −291.4          | −316.5 | −314.4 | −321.7 | −320.9  |         |
| V                | −278.1          | −278.5          | −340.1 | −340.3 | −374.7 | −373.4  |         |
| VI               | −255.2          | −255.2          | −284.3 | −284.0 | −284.4 | −284.5  |         |
| VII              | −267.8          | −268.4          | −284.6 | −286.1 | −268.6 | −268.1  |         |
| VIII             | −254.1          | −254.7          | −253.9 | −253.3 | −271.1 | −270.5  |         |
| IX               | −303.4          | −303.5          | −343.2 | −343.8 | −340.3 | −340.5  |         |

The potentials are calculated at the pH of the crystals. Ox5.5N, Ox7.5N, and Red7.5N correspond to the calculations made with the conformations with higher occupancy. Ox5.5S corresponds to the alternate conformation of Ser-58. Ox7.5L corresponds to the alternate conformation of Lys-212. Hemes belonging to the N-terminal domain are marked in bold, whereas hemes belonging to the C-terminal domain are underlined. Heme IV is in plain text.
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and PrD-IV moves away, changing its conformation and, consequently, its H-bond network. These changes lead to an increase of free space in the neighborhood of heme IV, thereby allowing the segment (Val-118 to Lys-121) to move to its new position (conformer A) (Fig. 5b). Overall, it seems that the disorder found at pH 7.5 for this loop is an indirect result of the change in PrA-IV protonation that occurred at this pH. Besides PrA-IV, the other two groups that show the highest difference in protonation attributable to pH are PrD-V and PrD-IX (Table IV). However, neither group shows conformational changes. Both propionates are buried inside the protein, and this could be the reason why PrD-V still shows some protonation (0.329) at pH 7.5. On the whole, the analysis made in this section provides evidence that the propionates are, in general, the groups that capture more protons.

Comparison between the Oxidized and Reduced Structures at pH 7.5—The structural comparison between the oxidized and the reduced structural models was done using program ESCET (70, 71) and taking the electron density maps into consideration. According to the difference distance matrix, the majority of the differences found between the Ca atoms of both structural models lie within their respective error margins. The rmsd between the Ca atoms of both structural models is 0.316 and 0.264 for molecules A and B, respectively (calculated with LSQKAB (55)). Except for the region that comprises residues 118 to 121 (discussed below), where the backbone shows a different conformation in the two redox forms (maximum rmsd value between Ca atoms is 1.733), the rest of the backbone seems to be insensitive to the reduction process. On the other hand, when the side-chain conformations are analyzed, important differences are observed between the two structural models. As mentioned before, some of these differences may be correlated with the protonation of certain groups because of the reduction process, i.e. the redox-Bohr effect. Therefore, all the relevant protonation differences at this pH, between the oxidized and the reduced structures, were also identified. Table V lists all the groups that experienced larger protonation changes (>0.1 protons) upon reduction. It is clear from Table V that only some of the propionate groups are capturing protons because of reduction, clearly showing their important role in the protonation process. In this section, we will discuss in detail all the significant conformational changes induced by the reduction process, as well as those groups that show significant protonation differences (Table V). It is important to note that, with the exception of Ser-58 in the reduced form, the same conformational changes found between the oxidized and the reduced form are observed for both independent molecules in the asymmetric unit.

PrA-I, PrD-I, and Ser-58 are three groups that are close to each other and experience a conformational change upon reduction (Fig. 4, b and c). As described above, in the oxidized structure at pH 7.5, Ser-58 has its side chain away from the heme and establishes a H-bond to a water molecule. PrA-I is facing the polypeptide chain, and its oxygen atoms (O1A and O2A) are within H-bond distance of three water molecules and of Val-57-N. PrD-I is also facing the polypeptide chain within H-bond distance of three water molecules. When the structure becomes reduced, in molecule A, PrA-I and PrD-I move away from the polypeptide chain while the Ser-58 side chain rotates and adopts a conformation similar to that found in rotamer B at pH 5.5. These changes induce a reorganization of the H-bond network, and two new H-bonds are established between Ser-58 Oγ and two carboxyl oxygen atoms from the propionate groups, PrA-I O1A and PrD-I O2D (2.44 and 3.23 Å, respectively). Both propionates of heme I (PrA-I and PrD-I) show a significant protonation change upon reduction by capturing 0.368 and 0.216 protons, respectively (Table V), which influences the conformational changes adopted by these groups. Nevertheless, even if one is able to explain the conformational change observed in PrA-I and PrD-I by considering this effect, the same explanation is not valid for the new conformation adopted by Ser-58 because, in this case, protonation of the propionates has a destabilizing effect, as discussed above. Therefore, a direct effect from heme reduction on the conformational changes cannot be excluded. As mentioned before, these conformational changes do not occur in molecule B, which shows the same conformations for these residues observed in the oxidized form. In this case, what we are seeing is probably a conjugated effect between reduction and the presence of different crystalline contacts. In molecule A, the conformational changes that occurred in PrA-I, PrD-I, and Ser-58, by themselves, do not change the redox potential of heme I significantly, but the observed redox-Bohr effect can contribute to the stabilization of the reduced state of heme I. For instance, if PrD-I was totally protonated, this could have a maximum effect of 43 mV on the potential of heme I. A note should be made to clarify what the maximum effect of a given protonatable group is on the reduction potential of a given heme. This corresponds to an approximate estimation based on electrostatic interaction energies between the heme and the protonatable group, using their relevant redox and protonation states, respectively. If there were no other interactions present in the system, the redox potential change attributable to the full protonation/deprotonation of the group could be predicted accurately from this interaction energy. In practice, given that many interactions exist in the system and given that a group may be only partially protonated/deprotonated, this measure can only be regarded as an estimate of the maximum possible effect of the protonatable group on the heme redox potential. To accurately model the effect, two simulations would be required, one with the normal system and another with a system where the influence of the group is eliminated, by an in silico mutation for instance (10).

The partial protonation of these two propionates, PrA-I and PrD-I, may have implications in the interaction with the TpI-cα. Both propionates are located in a large pocket, within the region where, according to previous studies (26), TpI-cα has the highest probability of docking to transfer electrons to SHcA. If one bears in mind that the docking of TpI-cα may be made through a positively charged region that characteristically surrounds heme IV of TpI-cα, the protonation of these two propi-
onates (and therefore reduction of negative charge) may directly contribute to the destabilization of the electron transfer complex and to the consequent release of TpI-c upon reduction of 9HcA.

Important conformational changes were also observed upon reduction on heme IV and its surrounding region. Glu-102 shows one of the most significant conformational changes attributable to reduction. In the oxidized structure, this acidic group is pointing toward the solvent (Fig. 5b), whereas in the reduced structure it is interacting with PrA-IV (Fig. 5b) in a similar fashion to what is observed in the oxidized structure at pH 5.5 (Fig. 5a). This constitutes clear evidence that this group...
is sharing a proton with the propionate. In fact, it is the propionate, and not the glutamate, that captures almost a complete proton upon reduction (see Table V). This conformational change can have a maximum interaction of \(-23.5\) mV with heme IV, which is a very significant value. On the other hand, the protonation of the propionate can have an opposite effect (a maximum of \(-58\) mV is possible because of protonation).

Concomitant with the conformational change observed in Glu-102, Arg-122 and Lys-106 and the segment Val-118–Lys-121 also experience a conformational change upon reduction. Arg-122 becomes disordered and moves away from the heme (Fig. 5, b and c), having a maximum effect of \(-14.7\) mV on the heme. However, this conformational change is counterintuitive in terms of reduction, because the reduction of the heme results

**Fig. 5.** Detailed view of heme IV and its surroundings. a, oxidized form at pH 5.5. b, oxidized form at pH 7.5. c, reduced form at pH 7.5. Carbon atoms are shown in yellow, oxygen atoms in red, nitrogen atoms in blue, and the iron atom in magenta; the H-bond distances are represented as dashed red lines. Prepared with PyMOL (77). Haem, heme; Prop, propionate.
in an attractive force. Nevertheless, the reason for this motion may be the protonation of PrA-122, which is the group that mostly interacts with Arg-122 in the oxidized state. The side chain of Lys-106, which in the oxidized form was not defined in the electron density (Fig. 5b), moves closer to the heme and establishes an interaction with Glu-102 (Fig. 5c) with a maximum contribution of 11.5 mV to the reduction of heme IV. On the other hand, the Val-118–Lys-121 segment experiences a conformational change that does not significantly influence the reduction of this heme and is probably attributable to electrostatic effects caused by the overall reduction process. This segment change from two alternate conformations modeled in the oxidized form at pH 7.5, (Fig. 5b) to a single one in the reduced structure (Fig. 5c), similar to that with higher occupancy found in the oxidized structure at pH 7.5. Altogether, the conformational changes found in the region surrounding heme IV are rather complex, and even if they may be attributable to reduction, their physical basis may reside mainly on protonation effects.

Significant protonation differences were found in Pr-DV and in Pr-DIX (Table V), although none of them shows any conformational changes. Both propionates are buried, and the effect of the protonation on the redox potential of their own heme can be as significant as 62 and 41 mV, respectively (upon full protonation).

Similarly to what was observed in the previous section, the propionates are the groups that experience either the most significant conformational changes and/or the most significant protonation effects upon reduction. Therefore, these groups seem to play a fundamental role in the stabilization of the reduced state of the hemes.

Analyzing Stabilization and Cooperativity Effects—As discussed previously (10, 12), the stabilization of electrons by the reduced structure may be the basis for positive cooperativity. Therefore, if the conformational changes observed between the oxidized and reduced states are occurring in the zone where a particular heme is titrating, it should be expected that its actual titration curve shifts from a typical oxidized structure curve toward a typical reduced structure curve. This could lead to an increase in the steepness of the actual redox titration curve, as seen in Fig. 6 for hemes I, II, III, V, VII, and IX, because the curves simulated with the reduced structure are above those simulated with the oxidized structure.

The stabilization effect afforded by the reduced structure can stem from different sources. It may come from conformational changes of specific residues, induced by reduction. For instance, a basic group, such as a lysine, can approach the heme upon overall reduction, and its positive charge will help to stabilize the electron. Alternatively, it may come from the protonation of some residues upon reduction, i.e. the redox-Bohr effect.

The results from the theoretical calculations shown in Table V allow us to predict that proton capture is clearly present in this cytochrome. Table V contains the total proton capture, from the oxidized to the reduced state, considering a change from the oxidized to the reduced structure (as it should be in a real situation). However, to analyze the actual relationship between proton capture in an individual group and reduction of an individual heme, the former must be occurring in the redox potential zone of the latter. This is what is contained in Table VI, where the effective protonation difference, i.e. whether the reduced structure can capture more protons than the oxidized one, in corresponding conditions, is present. This effect can be measured from the simulations if the protonation fraction is calculated for all groups at the midpoint potential (Table III) for both the oxidized and reduced structures. The difference between these two proton fractions (calculated at different redox potentials) gives the amount of protons that a specific group can capture in the reduced structure, in excess of what was captured in the oxidized one (Table VI). Considering that extra protons will stabilize the electrons and consequently the reduced state, the effect of these groups can be used to partly explain the stabilization of the reduced structure in comparison with the oxidized one. In fact, only two groups show effective protonation differences at the midpoints redox potentials of the hemes (Table VI) (PrA-I and PrA-IV), in contrast with the five groups present in Table V. PrA-IV is the major source of the difference in proton capture between the two structures (Table VI). This is attributable to the complex conformational changes that take place around it, mostly because of Glu-102 and Arg-122, as discussed above. Its effect can be different for different hemes, as shown in Table VI, given that proton capture depends on the redox potential at which the heme titrates. Potentially, the most affected hemes would be I, III, IV, V, and IX. However, to exert a noticeable effect, the titrating group should also have a significant interaction with the heme, which is certainly not the case for all hemes (see below).

Despite all that was said about stabilization of the reduced state, there are cases where a destabilization is observed, such as for hemes VI and VIII, where the redox titration curve of the reduced structure precedes the corresponding one for the oxidized structure (Fig. 6). This destabilization effect is more difficult to explain. An overall explanation would be to consider that the reduction of a certain heme can lead to conformational changes that stabilize its own reduced state but simultaneously will destabilize the reduced state of another heme, i.e. one heme pays the price of stabilization on another, the overall compensation being favorable. This is an indirect effect and, therefore, quite difficult to elucidate.

To identify the groups most important to the stabilization/destabilization effects present in the curves of Fig. 6, we have determined which were the ones that experienced conformational changes upon reduction in the vicinity of each heme. In addition, for each group, its effect on the individual heme reduction potentials was approximately estimated using group-heme interactions (data not shown), along with the effect of PrA-I and PrA-IV protonation on each heme reduction. In the absence of other factors, protonation will always stabilize the reduced state, whereas conformational changes can have either a stabilizing or a destabilizing effect. In hemes I, III, and VII, the overall stabilization of the reduced state is achieved by the contribution of both conformational changes and proton capture effects. In this case, the group that contributes the most, with both effects, is PrA-IV. Upon reduction this propionate experiences a small conformational change and a large proton capture that tend to stabilize the reduced state of all three hemes. Nevertheless, the contribution given by PrA-I, PrA-III, and PrA-VIII to the reduced state of their own heme should not be neglected. In hemes II and IX an overall stabilization of the reduced state also occurs. However, in this case, this effect is

| Residue | Red7.5 | Ox7.5 | Red 7.5–Ox7.5 |
|---------|--------|-------|---------------|
| PrA-I   | 0.235  | 0.019 | 0.216         |
| PrD-I   | 0.408  | 0.040 | 0.368         |
| PrA-IV  | 0.885  | 0.000 | 0.885         |
| PrD-V   | 0.812  | 0.329 | 0.483         |
| PrD-IX  | 0.250  | 0.026 | 0.232         |

Cooperativity Effects in Nine-heme Cytochrome c

TABLE V

Groups that show large protonation changes (>0.1 protons) from the simulation in the oxidized state using the oxidized structure at pH 7.5 to the simulation in the reduced state using the reduced structure at pH 7.5.

Highest occupancy conformers are considered.
small and mainly attributable to conformational changes, particularly those of the Lys-87 and Lys-189 side chains, which contribute to stabilize hemes II and IX, respectively. In hemes VI and VIII, the conformational changes clearly contribute to the destabilization of the reduced state in both hemes. Those that contribute the most to this effect are observed in Glu-222 and Arg-109, respectively. In the case of hemes IV and V, neither stabilization nor destabilization effects of the reduced state were observed. Indeed, heme IV constitutes an interesting case where large opposite effects are observed, but in the end, no net stabilization or destabilization is present. In this case, conformational changes contribute mostly to the destabilization of the heme reduced state, i.e. residues Glu-102 (−24 mV) and Arg-122 (−15 mV). On the contrary, the protonation of PrA-IV (which may influence its own heme by +50 mV) and the conformational change of Lys-106 (+12 mV) counteract the destabilizing effects. On the other hand, the major contribution to the destabilizing effect in heme V comes from entropic sources, i.e. occupational and tautomeric entropies (42, 58), that overcome the contribution given by the protonation of PrA-IV to the stabilization effect. We have calculated these effects on the reduced and oxidized structure simulations at the corresponding midpoint redox potentials of this heme and found that this entropic effect can destabilize the reduced state by −14.7 mV, which is quite substantial. Therefore, entropy can counteract the stabilization provided by proton capture, showing the importance of taking its influence into account (42, 58).

Stabilization effects of the reduced state are clearly present in this cytochrome (hemes I, II, III, VII, and IX), reinforcing the possibility of positive cooperativity in different hemes. Likewise, destabilization effects of the reduced state were also
observed (heme VI and VIII). This diversity of effects has been observed in large electron transfer complexes such as, for instance, in the case of the cytochrome c oxidases (Ref. 72 and references therein), where it was suggested to have a relevant contribution to the electron transfer mechanism.

**Studying the Redox-Bohr Effect**—From the above description, it is now clear that the redox-Bohr effect (25, 73) should be present in this cytochrome, playing a considerable role in regulating the heme redox potentials. Indeed, recent work by Reis et al. (33) has provided experimental evidence for the presence of the redox-Bohr effect in 9HeA in the physiological pH range. According to our calculations, the redox-Bohr effect allows 9HeA to capture a maximum of 2.4 protons upon full reduction. Interestingly, Reis et al. (33) proposed two acid-base centers in their thermodynamic interpretation of the experimental data.

The redox-Bohr effect can be functionally important in 9HeA, similar to what was found for tetraheme cytochromes c (74) and more generally for redox proteins involved in metabolic pathways. In this section we will carry out a detailed analysis of the redox-Bohr effect in this cytochrome and discuss its possible determinants.

One way to analyze the molecular causes of redox-Bohr effects on proteins, using theoretical methods, is to look at direct (electrostatic) interactions between protonatable and redox groups or sites. However, as we discussed in other works (42, 45), the consideration of direct interactions between sites does not fully capture their coupling; a more appropriate measure is the statistical correlation between their occupancies by protons or electrons. If two binding sites are always simultaneously empty or occupied, their correlation is 1; if they always have opposite occupancies (empty-occupied or occupied-empty), their correlation is 0. In practice, all-or-none effects do not seem to take place. In previously analyzed systems, the binding of electrons and/or protons with relevant couplings showed correlations with absolute values in the range 0.1 to 0.4 (42, 45). Because these phenomena require that the groups have the capacity to exchange ligands (protons and electrons), one should bear in mind that the occupancy of a group can influence correlation effects. In the case of 9HeA, the correlation values can be very high, as high as 0.45 (Fig. 7), and indicate that there are strongly interacting groups. In fact, the majority of the correlations involve hemes and their own propionates, as well as between the propionates themselves (negative correlations).

When analyzing the simulations done with the oxidized structure and those performed with the reduced structure, we see that, in general, the magnitude of the correlation values between hemes and protonatable groups and among protonatable groups increases in the reduced structure. This can be explained by the increased number of protons that can be exchanged in the reduced structure, which will lead to higher correlations. The same does not seem to happen with the correlation between the hemes (Fig. 7, a and d). Indeed, reduction makes the interaction between hemes III and IV decrease and disappear from the plot (correlations below 0.1 are not shown). Another effect of reduction is to narrow the zone where correlations are present; this may simply be the effect of narrowing redox titration ranges (Fig. 2, b and c). The correlation analysis revealed that the most strongly interacting hemes are in the same tetraheme domain. The N-terminal hemes (I, II, III, and V) do not interact with the C-terminal hemes (VI, VII, VIII, and IX). Heme IV, in the intermediate region, correlates significantly with the N-terminal hemes but not with the C-terminal hemes (at least within the imposed cut-off of Fig. 7). It is also worth mentioning that the most significant correlations occur in the N-terminal domain of 9HeA (including the intermediate heme IV region), providing evidence that this zone is the most active zone of this cytochrome in terms of binding of electrons and protons.

The correlations between hemes and propionate groups almost match the information present in Table V, i.e. the propionates with the higher correlations are those that capture protons upon reduction. Exceptions to this are the correlations between PrA-III and PrD-IV, two groups that do not show very significant proton capture, and their own hemes. On the other hand, not all proton-capturing groups show high correlations with hemes in both structures; this is the case of PrA-I and PrA-IV, where the correlations are not significant in the oxidized structure. The case of PrA-IV is possibly the result of the conformational change involving this propionate, Glu-102, Arg-122 and Lys-106. In fact, the correlation between PrA-IV and Glu-102, which is absent in the oxidized structure, in the reduced structure becomes the strongest correlation observed in this cytochrome. This effect can be explained by the fact that in the oxidized structure this propionate cannot capture as many protons as it can in the reduced structure. Another important correlation between protonatable groups occurs between the two propionates of heme I.

Their correlation in the oxidized structure is significant (see Fig. 7c), and becomes even higher in the reduced structure (see Fig. 7f). This is attributable to the conformational changes described above, which lead to a closer approach between the two groups. Simultaneously, the conformational change observed in Ser-58 can also influence them.

Overall, the groups that are more directly relevant for the redox-Bohr effect are the propionates, similar to what we found in previous work with other multi-heme cytochromes (40–42,45). The propionates that contribute more strongly to this effect are PrA-I, PrD-I, PrA-III, PrA-IV, PrD-IV, PrD-V, and PrD-IX. Despite the fact that all of these groups influence and are in turn influenced by heme reduction (the essence of the redox-Bohr effect), proton capture is not large in PrA-III and PrD-IV. It should be noted that, despite not being involved in proton capture, Glu-102 is also influencing this process substantially, as evidenced in the reduced structure where it is establishes a hydrogen bond with the protonated PrA-IV.

**A Functional Picture of Electron Transfer in 9HeA**—The present results indicate that the N-terminal and C-terminal domains of 9HeA seem to function in an independent manner. Higher correlation values between hemes are observed within these domains, but inter-domain heme-heme correlations are quite low. Heme IV, although being located between the two domains, clearly interacts more strongly with the N-terminal region and therefore may be functionally associated with it.

Reorganization effects are also more important in the N-terminal domain, resulting in stabilization of the reduced state of three (I, II, and III) of its four hemes. These stabilization effects have a component of proton capture, but the possibility for positive cooperativity effects resulting from conformational changes is also present. The C-terminal region presents less stabilization of the reduced states, and in two cases (VI and VIII) destabilization effects are present. Similarly to what happens with regard to reorganization effects, the N-terminal domain is also more active regarding the redox-Bohr effect and proton capture. Proton capture is almost exclusively associated with the propionate groups of the N-terminal domain, including (and very importantly) heme IV. Heme IV and its surroundings constitute a region where important redox-linked conformational changes occur and where substantial protonation effects are observed.

When analyzing the redox potentials of this cytochrome, we realized that the N-terminal domain (including heme IV) showed
higher redox potentials on average than the C-terminal domain (a notable exception being heme IX). Therefore, in thermodynamic terms, electron transfer should follow downhill, from the N-terminal domain to the C-terminal domain. This can also have kinetic consequences, because the free energy of reduction is an important component of the electron transfer kinetics. If we consider that electron transfer from the redox partner cytochrome c takes place near hemes I and II of the N-terminal domain, as suggested by our previous modeling studies, we see that this arrangement of redox potentials (in the absence of other controlling factors) can have a functional significance, allowing the electrons to quickly flow from the N-terminal to the C-terminal hemes, thus allowing the former to receive more electrons and the latter to pass them to whatever acceptor follows in the chain. The exception of heme IX in this redox potential arrangement is noteworthy and possibly with some physiological relevance. The fact that it shows a lower redox potential than the other hemes in the C-terminal domain (and consequently lower affinity for electrons) may be advantageous for passing electrons from the 9HcA to the next partner in the respiratory chain. If all hemes in the C-terminal domain had very high affinity for electrons, it would become difficult for another partner to capture them. Therefore, heme IX may play a key role in the electron transfer process downstream.

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Redox-Bohr and Other Cooperativity Effects in the Nine-heme Cytochrome c from Desulfovibrio desulfuricans ATCC 27774: CRYSTALLOGRAPHIC AND MODELING STUDIES

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