Conformational Component in the Coupled Transfer of Multiple Electrons and Protons in a Monomeric Tetraheme Cytochrome*

Ricardo O. Louro§, Isabel Bento‡§, Pedro M. Matias‡, Teresa Catarino‡, António M. Baptista‡, Cláudio M. Soares‡, Maria Arménia Carrondo‡, David L. Turner†, and António V. Xavier¶

From the ‡Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Rua da Quinta Grande, 6, Apt. 127, Oeiras 2780-156, Portugal, the ¶Departamento de Química da Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa, Monte da Caparica, 2825-114 Caparica, Portugal, and the †Department of Chemistry, University of Southampton, Southampton SO17 1BJ, United Kingdom

Cell metabolism relies on energy transduction usually performed by complex membrane-spanning proteins that couple different chemical processes, e.g. electron and proton transfer in proton-pumps. There is great interest in determining at the molecular level the structural details that control these energy transduction events, particularly those involving multiple electrons and protons, because tight control is required to avoid the production of dangerous reactive intermediates. Tetraheme cytochrome c₃ is a small soluble and monomeric protein that performs a central step in the bioenergetic metabolism of sulfate reducing bacteria, termed “proton-thrusting,” linking the oxidation of molecular hydrogen with the reduction of sulfate. The mechanochemical coupling involved in the transfer of multiple electrons and protons in cytochrome c₃ from Desulfovibrio desulfuricans ATCC 27774 is described using results derived from the microscopic thermodynamic characterization of the redox and acid-base centers involved, crystallographic studies in the oxidized and reduced states of the cytochrome, and theoretical studies of the redox and acid-base transitions. This proton-assisted two-electron step involves very small, localized structural changes that are sufficient to generate the complex network of functional cooperativities leading to energy transduction, while using molecular mechanisms distinct from those established for other Desulfovibrio sp. cytochromes from the same structural family.

Recent developments in techniques of structural biology have opened the way for probing the mechanisms used by biological macromolecules involved in energy transduction at the molecular level. The structural analysis of bacteriorhodopsin trapped in the M photointermediate state (1), the structures in the oxidized and reduced forms of cytochromes c₃ that perform a coupled two-electron step associated with proton transfer (2, 3), and the establishment of the coupled transfer of electrons and protons to the 3Fe-4S cluster of Azotobacter vinelandii ferredoxin (4) are just a few recent examples where results from different techniques are integrated in a description at the atomic level of the energy-transducing events. The phenomenon of energy transduction relies on coupled events (5), whether they involve only electrostatic interactions or structural rearrangements of the active sites or its surroundings (mechano-chemical coupling), which may be more important than the electrostatic component of the overall coupling (6). The pumping of proton(s) at the beginning of re-reduction of cytochrome c oxidase (7) can be described using a model in which the electrostatic attraction of electrons and protons is overcome (8), a situation that requires structural changes involving charged residues. Small proteins capable of performing energy transduction provide easier access to the structural bases for the underlying mechanisms, as the recent advances in the understanding of the proton pumping by the 26-kDa bacteriorhodopsin demonstrate (1,9).

This work reports the conformational changes underlying the positive cooperativity between redox centers (homotropic) and between redox and acid-base centers (heterotropic) observed in a 14-kDa tetraheme cytochrome c₃ isolated from Desulfovibrio desulfuricans ATCC 27774 (Ddc3). The analysis integrates data derived from the microscopic thermodynamic characterization of the hemes and acid-base centers (6), the structures obtained by x-ray crystallography in the oxidized and reduced states, and theoretical studies based on these structures. These data provide a coherent description of the mechanism that enables the efficient coupling of the transfer of two electrons with protons in the physiological pH range. The thermodynamically coupled transfer of electrons and protons observed in the soluble cytochromes c₃ has been termed “proton-thrusting” because it parallels the linkage between redox- and proton-transfer mechanisms of larger, membrane-embedded protein complexes involved in proton pumping (10).

MATERIALS AND METHODS

Crystallization—Crystals of the oxidized form of D. desulfuricans ATCC 27774 cytochrome c₃ were obtained as previously described (11). At pH 4.0, Ddc3 crystallizes in hexagonal space group P6₃,22, with cell parameters a = b = 62.71 Å, c = 111.09 Å at room temperature and one molecule in the asymmetric unit. These crystals were first transferred to another solution at higher pH containing polyethylene glycol 400
standard protocol. Fourier beginning with a simulated annealing refinement at 3,000 K using the working set containing the remaining 7963 reflections used containing data up to 3.5–
factors was carried out within the CCP4 (13) program package. The to extract diffraction intensities; conversion of intensities into structure The diffraction images were processed with the HKL program suite (12) using graphite-monochromatized Cu-K\(_\alpha\) radiation (\(\lambda = 1.5418 \text{ Å}\) from a Nonius FR-571 rotating anode generator operating at 45 kV and 99 mA. The oxidized and reduced forms of Ddc3 from the reduced protein solution were unsuccessful; and second, use of sodium dithionite as a reducing agent requires alkaline pH values to avoid degradation with formation of bisulfite.

**Data Collection**—Diffraction data from flash-frozen crystals of both the oxidized and reduced forms of Ddc3 at pH 7.6 were collected in-house at 110 K on an imaging plate detector (MAR 300-mm scanner) using graphite-monochromatized Cu-K\(_\alpha\) radiation (\(\lambda = 1.5418 \text{ Å}\) from a Nonius FR-571 rotating anode generator operating at 45 kV and 99 mA. The diffraction images were processed with the HKL program suite (12) to extract diffraction intensities; conversion of intensities into structure factors was carried out within the CCP4 (13) program package. The statistics of the data collection and processing are summarized in Table I.

**Structure Determination and Refinement**—The positioning of the previously obtained structural model for the oxidized form of Ddc3 at room temperature and pH 4.0 (14) in the crystal structures of the oxidized and reduced forms at 110 K and pH 7.6 was carried out by the molecular replacement method with the program AmoRe (15) using data up to 3.5-Å resolution. Clear solutions were obtained for both forms, with correlation coefficients of 69.8 and 68.8%, and R-factors of 33.0 and 34.2%, respectively. The reflection data set of the oxidized form was then split into a test set (504 reflections, i.e. a random sample containing ~6% of the 7877 measured independent reflections), used to calculate \(R_{\text{free}}\), and a working set, comprising the remaining 7373 reflections, used in the structure refinement. The reflection data set of the reduced form was likewise split into a (different) test set (544 reflections, i.e. a random sample containing ~6% of the 8507 measured independent reflections), and a (different) working set containing the remaining 7963 reflections used in the structure refinement.

The refinement of both redox forms was carried out with X-PLOR beginning with a simulated annealing refinement at 3,000 K using the standard protocol. Fourier 2|\(F_o\) – |\(F_c\)| and |\(F_o\)| – |\(F_c\)| maps were calculated at the end of each refinement run and inspected on a workstation using TURBO (16). Some side-chain positions were corrected and, at a later stage, individual thermal motion parameters were refined and water molecules and SO\(_4^-\) ions included in the model. In the final stages of refinement it was realized that the SO\(_4^-\) ion sites were partially occupied, because all atoms had a B-factor much higher than the refined overall B for the whole model. Also, side-chain 2-fold disorder models were included for a few protein residues as well as the propionate A of heme I (in both redox forms). The final R-values obtained were 0.189 and 0.179 for the oxidized and reduced forms, respectively, and the corresponding values of \(R_{\text{free}}\) were 0.240 and 0.205, respectively. The final refinement statistics are presented in Table II.

The models were analyzed with PROCHECK (17), and both molecules had sterically reasonable quality parameters within their respective confidence intervals. In the \(\varphi, \psi\) plots for the non-proline and non-glycine residues (not shown) only Cys-51 in both molecules lies outside the normally allowed regions, as previously discussed (14). Overall, 88.0% of the non-proline and non-glycine residues for the reduced form (82.6% for the oxidized form) lie within the most favored regions. The \(\varphi, \psi\) plots for proline and glycine residues (not shown) indicate that 60% (v/v) and 0.05 M Tris/HCl buffer at pH 7.6. An excess of sodium dithionite was then added to the drop, and a gradual color change in the crystals was observed. After approximately 3 h, the crystal color became uniform and similar to that observed for the reduced protein in solution, which was taken as an indication of a successful reduction. This procedure was necessary for two reasons: first, all attempts at crystallizing Ddc3 from the oxidized protein solution were unsuccessful; and second, use of sodium dithionite as a reducing agent requires alkaline pH values to avoid degradation with formation of bisulfite.

**Table I**

| Oxidation state | Reduced | Oxidized |
|-----------------|---------|----------|
| X-ray source    | Detector |
| Wavelength, Å   | Data processing |
| Space group     | Unit cell |
| No. molecules in atomic units (Z) | Resolution limits (Å) |
| No. of observations | No. of independent reflections |
| % completeness  | % I > 3\(\sigma\) (I) |
| Merging R-factor|         |

\(\alpha = 61.14, c = 107.48 \text{ Å}\)

**Table II**

| Oxidation state | Reduced | Oxidized |
|-----------------|---------|----------|
| Resolution limits (Å) | Final \(R_{\text{free}}\) (%) (test set) | Final \(R_{\text{free}}\) (%) (working set, \(F > 2\sigma(F)\)) |
| No. non-hydrogen protein atoms\(^a\) | No. solvent molecules | No. sulfate ions |
| Disordered residues\(^b\) | Model r.m.s. deviations from ideality\(^c\) |
| Bond lengths (Å) | Bond angles (°) |
| Estimated r.m.s. coordinate error (Å\(^d\)) | Numbering scheme |
| Protein chain | Heme groups |
| Sulfite ions | Solvent molecules |
| Solvent molecules |
| Average B-factors (Å\(^2\)) | Protein main chain (side chain) |
| Heme groups | Sulfite ions |
| Solvent molecules |

\(^a\) Values in parentheses refer to the last resolution shell, 2.07–2.00 Å for the reduced form, 2.12–2.05 Å for the oxidized form.

\(^b\) The occupation factor of the major component is in parentheses.

\(^c\) Calculations were made with X-PLOR.

\(^d\) Calculations were made with SIGMAA (46) using all reflections.

**Table III**

| Oxidation state | Reduced | Oxidized |
|-----------------|---------|----------|
| Protein chain   | Heme groups |
| Sulfite ions    | Solvent molecules |

Pro-21 in both molecules adopts a conformation slightly outside the most favored regions. There is some missing electron density in the surface loop containing Gly-72, particularly for Glu-73, possibly because of loop flexibility. As a consequence, this loop conformation is poorly defined. Overall, the refined model for the reduced form seems to be better than the refined model of the oxidized form. This may result in large measure from the better quality of the diffraction data obtained from the former (see Table I).

**Modeling Methods**—The modeling methodology used here has been previously described in detail (18). It consists of the calculation of free energy terms using finite-difference continuum electrostatics (19), followed by the sampling of redox and protonation states using a Monte Carlo method. The continuum electrostatic calculations were done with the program MEAD (20), using an ionic strength of 0.1 M, a temperature of 300 K, a molecular surface defined with a solvent probe radius of 1.4 Å, and a Stern (ion exclusion) layer of 2.0 Å. A two-step focusing procedure (21) was used, with consecutive grid spacings of 1.0 and 0.25 Å. The dielectric constants of the solvent and protein were respectively 80 and 15. The Monte Carlo calculations were done with the program...
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RESULTS

Thermodynamic Characterization—The redox and acid-base properties of Ddc3, reported in the literature (6), show that the reduction potentials of the four hemes are pH-dependent, a phenomenon termed the redox-Bohr effect (22, 23), and that this dependence involves two distinct pK_a values in the range 4.5–8.7. The two acid-base transitions were fit with pK_a = 4.6 and 5.0, and pK_a = 7.6 and 7.1, respectively.

Fig. 1 shows the oxidized fractions for each of the hemes versus solution potential at pH 6 derived from the thermodynamic data (6). Hemes I and II titrate together and more steeply than Nernst curves calculated using a value of n equal to 1. This is a consequence of the network of interactions between the centers and the similarity of the reduction potentials of these two hemes, which, assisted by a favorable redox-Bohr effect, results in an overall positive cooperativity favoring a coordinated two-electron step. This overall positive cooperativity will be referred to as a proton-assisted two-electron step.

Fig. 2 shows the dominant microscopic populations involved in the first two macroscopic steps of oxidation at pH 6, determined from the thermodynamic data and discriminated according to their protonation state. The macroscopic steps are defined as the transition between the different redox stages of the protein, each of which comprises all microscopic populations with the same number of oxidized hemes (24). The figure clearly shows that, when oxidation stage 2 is reached (two of the hemes are oxidized), the dominant microscopic population is that with the acid-base center located close to heme I deprotonated. This selectivity for particular microscopic populations is an important feature for the efficiency of energy transduction (25, 26), because it ensures that the majority of the molecules are in the same state, thus able to react in a consistent manner with the specific redox partners upstream and downstream in the redox chain. Selection for particular microstates can be considered directional in the sense that certain groups in the protein structure are specifically used, avoiding short-circuiting through nonspecific reactions. This may result from selective evolutionary pressure that favors the interactions of particular active groups with the physiological partners (27).

Comparison between the Oxidized and Reduced Structures—The crystals obtained for the oxidized and reduced states of the protein at pH 7.6 (see “Structure Determination and Refinement” under “Materials and Methods”) yielded broadly similar structures. It must be noted that at pH 7.6 the structures are expected to represent the fully deprotonated oxidized state (pK_a = 4.6 and 5.0), and a partially protonated condition in the reduced state (pK_a = 7.6 and 7.1). Thus, to assess the changes linked only to reduction, the structure models of both redox states were also compared with the structure of the oxidized state at pH 4.0 previously reported (14). The models of both redox states at pH 7.6 were superimposed with X-PLOR (28) using the main-chain and heme ring atoms. The overall r.m.s. values obtained for this fit are 0.39 Å for main-chain, 1.26 Å for side-chain, and 0.20 Å for heme ring atoms. However, for the analysis of the localized differences, the electron density was regarded as an important factor in deciding which of the observed changes were real and which were likely to be artifacts caused by poor definition of side chains due to positional disorder in some of the surface residues.

Three main areas in the structure show a clear difference between the oxidized and reduced states of Ddc3: the side chains of Glu-26 and Lys-27, Propionate A of heme I, and the neighborhood of heme II. Because these were all regions where the structural models of the oxidized states (at pH 4.0 and 7.6) were in agreement, it seems reasonable to consider that the differences observed in the reduced state do indeed result from the reduction of Ddc3 rather than deprotonation.

Side Chains of Glu-26 and Lys-27—In both structures of the oxidized state the Glu-26 Oe1 forms a hydrogen bond with Lys-27 NZ, whereas its Oe2 is hydrogen-bonded to a water molecule. In the reduced state, Glu-26 has a different side-chain rotamer, there is no hydrogen bond to Lys-27, and Oe1 is now hydrogen-bonded to a water molecule.

Propionate A of Heme I—At pH 7.6, propionate A of heme I in the oxidized state is 2-fold disordered, with one of the conform-
ers identical to the only one observed in the oxidized state at pH 4.0, which is protonated. In the reduced state at pH 7.6, this group is also 2-fold disordered, but both conformers are different from any of those obtained in the structures of the oxidized state. Interestingly, one of the oxygen atoms (O1A) in conformer 2 of the reduced state establishes a hydrogen bond with the carbonyl oxygen of Tyr-43 (2.94 Å), indicating that a proton is located between these two atoms. Fig. 3 represents the region around Heme I in the reduced and oxidized states at pH 7.6.

The Neighborhood of Heme II—Propionate A of heme II, Glu-61, Lys-75, and His-76 are compared in Fig. 4 for the oxidized and reduced states at pH 7.6. In the structure of the reduced state, the side chain of Glu-61 is nearly perpendicular to the heme plane, although not interacting with it, and establishes a hydrogen bond with Lys-75 Nε. By contrast, the Glu-61 side chain in the oxidized states is nearly parallel to the heme plane and the hydrogen bond observed in the reduced state is absent. Furthermore, the associated electron densities are poorly defined in both structures of the oxidized state, suggesting that the side chain of the Lys-75 is flexible.

In the oxidized and reduced states at pH 7.6, the Nε2 atom of His-76 is oriented toward both oxygen atoms of the carboxylate group in Propionate D of heme II. However, some differences are observed, with the Nε2 atom of His-76 in the oxidized state closer to O1D (2.73 Å) than O2D (3.56 Å), although the presumed hydrogen bond has an atypical geometry (angle N–H...O close to 90°). In contrast, at pH 4.0 its distance is too long to form a significant hydrogen bond with either O1D or O2D (3.38 and 3.23 Å, respectively). This structural rearrangement may be explained considering that both groups are protonated, with rapid exchange of the propionate proton between O1D and O2D. In the reduced state, a clear hydrogen bond is observed between His-76 Nε2 and O2D (2.72 Å).

In the structure of the oxidized state at pH 4.0, the Nδ1 atom of His-76 is hydrogen-bonded to a water molecule (OW 264, 2.77 Å). However, this water molecule is absent in the oxidized state at pH 7.6, and is again clearly observed in the reduced state at pH 7.6 (OW 384, 2.68 Å). This result suggests that, at pH 7.6, His-76 Nδ1 has lost its proton in the oxidized state but regained it upon reduction of the cytochrome, possibly as a consequence of the redox-Bohr effect.

Theoretical Calculations—To identify the specific residues that may participate in the redox-Bohr effect, theoretical calculations based on the x-ray structures in the oxidized and reduced states were performed to determine the proton and electron populations at different values of pH and reduction potential for different groups. These calculations give titration profiles that deviate from a Henderson-Hasselbalch curve and the midpoint titration of all protonatable groups (pKα1/2) in the fully oxidized and fully reduced states can be estimated from these data (Fig. 5). The calculations show that the groups with substantial modifications in their predicted pKα values between the two structures, and with either pKα1ox, pKα1red, or both, in the range from pH 6 to 8, are as follows: the propionates of heme I, propionate A of heme II, propionate D of heme IV, and His-76. The N terminus is the only other residue predicted to titrate in this pH range, but the change of its pKα values is negligible.

The roles of propionate D of heme I and the free His (when it is present) seem to be a consistent feature over several Desulfovibrio species (18, 29, 30). In the oxidized structure there are no indications that either of the propionates of heme I is protonated at pH 7 in any of the possible conformers. In contrast, there is clear evidence in the reduced structure that at least one proton is bound to these groups, both from the crystallographic as well as from the theoretical point of view. If conformer 1 of propionate A is considered, the calculations show that propionate D contains a proton at pH 7, given that its pKα1D is around this value (black square in Fig. 5). If conformer 2 of propionate A is considered, the calculations show that this same propionate has a proton, given that its pKα1D is above 7 (black diamond in Fig. 5). The crystallographic evidence referred to in Fig. 4 also points to the presence of such a proton, which is shared with the carbonyl group of Tyr-43.

DISCUSSION

Redox Coupling—Fig. 4 reveals that Glu-61 has a very large redox-linked conformational modification. This residue is located close to hemes I and II and is expected to be charged in the physiological pH range as reported in Fig. 5. The observed movement of its negatively charged carboxylate side chain away from heme II is consistent with the observed enhancement of the electron affinity of heme II upon reduction. The continuum electrostatic calculations of the electron binding curves using the coordinates of the oxidized and reduced structures confirm this effect (Fig. 6).

The curves corresponding to the reduced and oxidized structures cannot hold simultaneously, because a transition between the two structures necessarily occurs as oxidation proceeds. Thus, if this transition takes place within the region where heme II titrates, the actual titration curve will follow the dotted curve in the fully reduced region and gradually shift to the solid curve as oxidation proceeds (Fig. 6). This shift leads to a steeper function, characteristic of positive cooperativity. To show that the conformational change occurring in Glu-61 (whose potential effect has previously been calculated (29)) is that responsible for this positive cooperativity, three mutants were modeled. The plot identified as “E61A” in Fig. 6 contains the calculated data of the mutation of Glu-61 and shows that the change into Ala destroys this positive cooperativity, resulting in a flatter curve, indicating a slight negative cooperativity. To investigate the origin of this negative cooperativity two other mutants were modeled. The mutation of Lys-62 to Ala...
(plot identified as “K62A” in Fig. 6) retains the positive cooperativity, but the double mutant of Glu-61 and Lys-62 into Ala (plot identified as “E61A-K62A” in Fig. 6) generates a situation where no significant cooperativity is observed (almost superimposable curves for the oxidized and reduced structures), indicating that Lys-62 was the main cause of the negative cooperativity.

Thus, the redox-linked structural changes associated with the side chain of Glu-61 make a fundamental contribution to the positive cooperativity observed for the titrations of hemes I and II in the thermodynamic data, even though the structural bases for the enhanced affinity of heme I for electrons upon reduction are not clearly observed in the current set of structures. This may be a consequence of having to model the propionates of heme I in two different conformations, resulting in an ambiguous assignment of redox transitions from fully oxidized to fully reduced. Indeed, the average of all possible transitions between the structures in the oxidized and reduced states, used in this analysis, may be different from the actual situation in oxidation stage 2 if the transitions have different probabilities (see below).

The results reported in Fig. 6, together with the knowledge from experimental data that the titrations of hemes I and II are essentially complete in stage 2, have the consequence of showing that at least part of the redox-related conformational modifications observed between the structures in the oxidized and reduced forms must have already taken place when the two-electron-oxidized stage is reached. This is an important piece of information that would be very hard to obtain by other means, because a sample in an intermediate stage of oxidation is heterogeneous due to the large number of possible microscopic redox populations and thus not easily amenable to structural studies by either NMR or x-ray crystallography.

Redox-Bohr Coupling—From the detailed thermodynamic characterization of the cytochrome, it is possible to determine the redox-linked difference in protonation of the protein within the experimental pH range. To analyze this coupling, the thermodynamic, structural, and theoretical data have to be correlated.

According to the structures and theoretical calculations, the propionates of heme I are clearly involved in the redox-Bohr effect. The modifications between the structures of the reduced and oxidized states show that the coupling between electrons and protons includes a conformational contribution in addition to the electrostatic interaction (Figs. 3 and 5). Moreover, the difference observed in the structures of the oxidized state obtained at pH 4.0 and 7.6 indicates an acid-base transition of the propionates of heme I compatible with the thermodynamic data. These observations are consistent with the participation of propionate D of heme I in the redox-Bohr effect, as observed in other cytochromes c$_4$ (24, 31, 32). From the thermodynamic characterization of the protein, it can be concluded that the effect of these propionates corresponds to the redox-Bohr effect of the acid-base center with pK$_{a}^{red}$ = 5.0 and pK$_{a}^{ox}$ = 7.1 (referred to as center V (6)), which has a very large influence on the reduction potentials of heme I (6).

In contrast with other cytochromes c$_3$, a second redox-Bohr effect was observed in Ddc3 in the physiological pH range (6). The remaining groups that titrate in this pH range with substantial differences in their pK values between the oxidized and reduced state are His-76, propionate A of heme II, and propionate D of heme IV (Fig. 5). The redox-linked structural differences observed for the His-76 (Fig. 4), as well as those between the low and high pH structures in the oxidized state, provide strong evidence that this residue plays a role in the overall redox-Bohr effect measured experimentally. Thus, it is only subject to the influence of the reduction of the heme and the conformational modification of the close-by Glu-61. The propionate D of heme IV also has predicted pK$_{a}$ values higher than the normal range for solvent-exposed propionates and should therefore also contribute to the redox-Bohr effect. This propionate is buried in the structure, but contrary to what is observed in the structures from Desulfotibrio vulgaris Hildenborough (33), D. vulgaris Miyazaki F (34), and Desulfovibrio gigas (35), it does not establish hydrogen bonds with any protein atom, which is evidence of its protonated form. Thus, the combined effect of these residues accounts for the redox-Bohr effect defined from the thermodynamic data with pK$_{a}^{red}$ = 4.6 and pK$_{a}^{ox}$ = 7.6 (referred to as center VI (6)).

The above assignments for the residues participating in the redox-Bohr effect are supported by theoretical calculations on the overall difference in protonation of the protein between the oxidized and reduced states. Fig. 7 plots this overall difference as derived from the thermodynamic characterization (6) and compares it with the combined effect calculated for these groups. The calculated curves are in reasonable agreement with the experimental data considering the approximations of the modeling strategy (18). In particular: (i) some of the residues involved in the redox-Bohr effect had to be modeled in two different conformations, which results in an ambiguous assignment of redox transitions from fully oxidized to fully reduced, namely for the propionates of heme I; (ii) the average of all possible transitions was taken here as the proton difference to use, but these transitions may actually have different probabilities, which would necessarily modify the maximum height and breadth of the theoretical curves.

Interestingly, the participation of several acid-base groups in the redox-Bohr effect of Ddc3 has a precedent in the Bohr effect of hemoglobin, in which the total Bohr effect is the result of
partial contributions of several amino acid side chains to modifying the oxygen affinity (36).

Despite the participation of several acid-base groups in the overall redox-Bohr effect, two clear redox-Bohr influences can be distinguished in the thermodynamic characterization, and specific microscopic populations are selected in the first two steps of oxidation (Fig. 2). These populations are involved in the proton-coupled two-electron step, and the thermodynamic selectivity for specific states in the “catalytic” cycle is a fundamental property of energy-transducing proteins, because it facilitates the directionality of the whole process due to the selection of particular centers in the protein structure.

Conclusions—The present work reveals that monomeric tetrahem Ddc3 has characteristics that are fundamental for energy transduction: (i) thermodynamic linkage of the various centers is observed (37) with both cooperative and anti-cooperative interactions between the centers giving rise to a selection for particular microscopic states (i.e. thermodynamic gating); (ii) proper tuning for binding multiple electrons and protons, which is achieved via small localized structural changes involving the hemes, their propionate substituents, and charged amino acid side chains in their close vicinity (mechano-chemical coupling). The preferential involvement of the heme propionates in the redox-Bohr effect of heme proteins had already been reported in the literature (38, 39) and is a consequence of the dual capacity of the heme prosthetic group as a redox- and acid-base center, which facilitates the coupling between the two ligands. The detailed thermodynamic properties that favor a coupled two-electron step modulated by a redox-Bohr effect in Ddc3, i.e. a proton-assisted two-electron step, are different from those reported for other structurally homologous cytochromes $c_i$ (2, 3, 24, 32). Thus, the study of these various proteins yields information on the variety of strategies that Nature can employ to achieve a common function using the same structural arrangement of the redox active sites. This situation is identical to that recently reported for transmembrane cytochrome $c$ ox-
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**FIG. 7. Differences in protonation between the oxidized and reduced states.** The thick line was determined from thermodynamic parameters reported in the literature for Ddc3 (6). The thin lines are taken from the result of theoretical studies, by choosing average protonation differences of selected groups and combinations of groups. When the group displays different conformations, the proton difference is taken as the average of the results obtained with these individual conformations in the oxidized and reduced structures. This is the case of propionate A from heme I. The curves correspond to successive additions of the protonation of different groups, starting from the heme I propionate A, corresponding to the curve with circles. Open squares, addition of heme I propionate D; triangles, addition of heme II propionate A; solid squares, addition of heme IV propionate D; diamonds, addition of His-76.

**FIG. 6. The role of redox-linked conformational changes of Glu-61 in positive cooperativity effects.** The plot identified as “normal” contains the redox titration curves of heme II for the oxidized (solid lines) and reduced (dotted lines) structures, at pH 7.6, computed from Monte Carlo simulations of the binding thermodynamics of electrons and protons. The curves labeled E61A, K62A, and E61A-K62A represent the results of replacement of Glu-61 and Lys-62 by alanine.

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...mechanism by which redox-linked conformational changes occur. The thin lines...
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Conformational Component in the Coupled Transfer of Multiple Electrons and Protons in a Monomeric Tetraheme Cytochrome

Ricardo O. Louro, Isabel Bento, Pedro M. Matias, Teresa Catarino, António M. Baptista, Cláudio M. Soares, Maria Arménia Carrondo, David L. Turner and António V. Xavier

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