Published in final edited form as:

Biochemistry. 2010 September 21; 49(37): 8043–8052. doi:10.1021/bi100663u.

Multiconformation Continuum Electrostatics Analysis of the Effects of a Buried Asp Introduced Near Heme a in *Rb. sphaeroides* Cytochrome c Oxidase

Jun Zhang and M.R. Gunner*

Physics Department J-419, City College of New York, 160 Convent Ave., New York, NY 10031

Abstract

Cytochrome c oxidase (CcO) reduces O₂ to water via a series of proton coupled electron transfers generating a transmembrane electrochemical gradient. Coupling electron and proton transfer requires changing buried residues pK_as at each stage in the reaction cycle. Heme a is a key cofactor in the CcO electron transfer chain. Mutation of Ser44 to Asp has been reported (Mills et al Biochemistry (2008) 47, 11499-11509), changing the hydrogen bond acceptor from His102, the Heme a axial ligand in *Rhodobacter sphaeroides* CcO. This adds an acidic residue to the CcO interior. The electrochemical behavior of Heme a in wild type and S44D CcO is compared using the continuum electrostatics program MCCE. The introduced, deeply buried Asp remains ionized at physiological pH only when the nearby heme is oxidized. Heme a reduction is now calculated to be strongly coupled to Asp proton binding, while with Ser44 it is weakly coupled to small protonation shifts at multiple sites, increasing the pH dependence in the mutant. At pH 7, the partially ionized Asp44 is calculated to lower the Heme redox potential by 50 mV as expected given the thermodynamics of coupled electron and proton transfers. This highlights an inconsistency in the experimental results where a low Asp pK_a is found together with a stabilized reduced Heme. The stabilization of a model complex heme oxidation by a hydrogen bond to the axial His ligand calculated with Continuum Electrostatics and with Density Functional Theory was in good agreement.

Keywords

cytochrome c oxidase; Heme a; MCCE; continuum electrostatics; E_m; pK_a; proton coupled electron transfer

Cytochrome c oxidase (CcO) is the terminal protein of the aerobic respiratory chain in plants, animals and many bacteria. There are four redox centers within the protein: Cu_A, Heme a, Cu_B and Heme a₃. Electrons are sequentially transferred from cytochrome c to Cu_A, on to Heme a and then to the binuclear center composed of Cu_B and Heme a₃, where an oxygen molecule is reduced to water. In common with other intrinsic membrane electron transfer proteins, CcO strongly influences the free energy of ionization of the deeply buried sites within the protein (1, 2). The electron transfers between the redox sites then lead to

*To whom correspondence should be addressed. Telephone: 212-650-5557. Fax: 212-650-6940. gunner@sci.ccny.cuny.edu.

Supporting Information **Available:** The energy breakdown that leads to the calculated Asp pK_a and pK₇ while Heme a is fixed in the oxidized state and reduced state are presented in Table S1 and Table S2. The atomic charges sets used in MCCE calculation are in Table S3. Figure S1 gives the total protein proton uptake as a function of the total charge of Asp44 (or Ser44) plus Heme a. The change in E_m of a Bis His heme model complex due to the presence of a hydrogen bond acceptor from one of the His ligands calculated with DFT or Continuum Electrostatics continuum electrostatics (Pages 9-12). These materials are available free of charge via the internet at <http://pubs.acs.org>.

changes in the pK_a s thus changing protonation states of appropriate residues at each stage of the reaction cycle (3, 4). It is these coupled reactions that allow the electron transfer reactions to drive transmembrane proton transfer (4, 5). In CcO, synchronized with the 4 electron transfers needed to form water, eight protons are removed from the cell interior. Four are transported to the binuclear center for addition to the substrate oxygen while the other four are pumped to the outer side of the membrane, adding to the electrochemical proton gradient across the membrane (6-8).

Heme a, the focus of the work reported here, is an a-type, low-spin heme with two axial histidine ligands (9). A non-redundant survey of 132 b and c type hemes (10), has shown that with His a an axial ligand, 75% of the distal His nitrogens (those not involved in ligation to the heme iron) make a hydrogen bond to the protein. Of these 40% are to a backbone carbonyl and 3.2% are to the side chain of Ser as found for Heme a in *Rb. sphaeroides* CcO. Asp, which will be introduced as a hydrogen bond acceptor in the work presented here, is found 9.5% of the time. In CcO Heme a_3 has a single His ligand leaving a site free to bind oxygen for reduction to water. His 419, the Heme a_3 ligand makes a hydrogen bond to Gly 294, the Heme a ligand His 421 to the Val 417 backbone carbonyl and His 102 to the side chain of Ser44 (*Rb. sphaeroides* residue numbering). An HSSP analysis of 2264 oxidase analogues with as little as 32% percent identity and 46% percent similarity (11) shows the backbone Gly and Val hydrogen bond associated with the Heme a_3 and a ligands are 100% conserved. In contrast, a Ser hydrogen bond is found in only 6% of the oxidases, while 93% of the sequences have a Gly in this position and 1% have Ala. In the bovine crystal structure Gly30 (bovine numbering), aligned with Ser44 in the sequence is the hydrogen bond acceptor from the His61 ligand to Heme a. However, sequence alignment does not always result in structural conservation. For example, the *Paracoccus denitrificans* structure (PDB ID: 3HB3) has Ser 46 in the aligned sequence, but the side chain of Thr50 is in a better position to hydrogen bond to the His ligand of Heme a.

Recent studies have reported on the changes in the electron transfer reactions when the Ser hydrogen bond donor to the Heme a axial ligand is replaced with Gly, Asn or Asp in *Rb. sphaeroides* CcO (12). Visible and EPR spectroscopy and x-ray crystallography show that the structure of the protein is not significantly perturbed by the mutations. The electron transfer rate from Cu_A to Heme a has been measured by a light activated Ruthenium donor (13, 14). At pH 7-8 in wild-type oxidase the electron transfer from Cu_A to Heme a occurs at rates from $2 \times 10^4 \text{ s}^{-1}$ in bovine (13) or *P. denitrificans* (15) to 9×10^4 in *Rb. sphaeroides* (14) CcO. A reaction free energy difference of 40-50 meV, is derived from the observation that 12-18% of the electrons remain on Cu_A rather than proceed to Heme a. The electron transfer from Cu_A to Heme a in the Asn and Gly mutant remains faster than the rate of electron transfer from Cytochrome c to Cu_A of $\approx 7 \times 10^4 \text{ s}^{-1}$. However, with Asp44, reduction of Heme a occurs at $\approx 125 \text{ s}^{-1}$ at pH 8. Now as the pH is lowered, a second, fast phase of electron transfer is found, which is indistinguishable from that of the wild-type protein in these experiments ($> 3 \times 10^4 \text{ s}^{-1}$). These results support a model where the fast, low pH rate occurs in the presence of a protonated Asp44 (12). As the pH is raised when Heme a is oxidized the Asp is deprotonated. However, Heme a reduction slows as it is now tightly coupled to proton binding by the Asp. The fast and slow phases have equal amplitudes near pH 5.5, the apparent pK_a of Asp44. In addition, at pH > 6.5 Heme a reduction by Cu_A is now favored by at least 60 meV, as no reduced Cu_A is seen at equilibrium (12).

The results suggest that an Asp placed in a deeply buried site in the transmembrane cytochrome c oxidase has a pK_a of ≈ 5.5 , only 1.8 pH units higher than an Asp in solution. The energy penalty for removing the ionized form of a residue from water is more significant than for the neutral form, shifting the equilibrium constant to favor charge neutralization (16-18). Thus, surveys show that most acidic and basic amino acids are on the

surface of the protein (19). However, a significant minority is in fact buried, and these often represent residues that are important for enzyme function (20). Buried, native amino acids exist in locations that are optimized by evolution. Calculations of the buried acidic and basic residues in 500 proteins suggest that they remain ionized at physiological pH due to interactions with the backbone dipoles and other amino acid side chains (19). The Asp44 that was introduced into CcO represents a charge introduced into a naive site. A recent study introduced Asp, Arg, Glu and Lys into 25 sites in the hydrophobic interior of *Staphylococcus nuclease* and 6 sites in Ribonuclease H (21). With few exceptions the introduced acid or base has a pK_a greatly shifted to favor the neutral form. In addition, when this new residue is charged the protein is often significantly destabilized. It is thus of interest to understand how CcO is able to accommodate this new charge at Asp44.

Cytochrome c oxidase has been the subject of extensive computational studies with a wide variety of methods. Recent studies have employed QM/MM to study the energetics of oxygen reduction (22). The role of key amino acids for controlling proton pumping have been studied by QM/MM (23), Empirical Valence Bond molecular dynamics (24), dynamic electrostatic analysis (25) and similar techniques (26). A previous MCCE (Multi-Conformer Continuum Electrostatics) study of cytochrome c oxidase (27) determined the E_m and pK_a of the redox centers and key residues in O, E and R states (4).

The work presented here aims to understand the behavior of CcO following the introduction of Asp44. The redox potential of Heme a and pK_a of Asp44 are calculated and compared with the results in the wild-type protein. The Asp represents a probe of the coupling between electron and proton transfer inside the protein. If the introduced Asp is deprotonated, the free energy of electron transfer from Cu_A to Heme a becomes coupled to Asp protonation changes introducing new pH dependence to the reaction. The responses of the protein to adding a negative charge by reducing the heme or deprotonating Asp44 are compared. The inconsistency of the Asp having a low pK_a , while heme oxidation is more favorable than in wild type CcO is explored.

Material and Methods

The wild type and derivative structures

Two structures of CcO from *Rhodobacter sphaeoides* are used as input to the calculations. The wild-type X-ray structure 2GSM (28) with a resolution of 2.0 Å (designated WT_S44) (Table 1). The S44D mutant X-ray crystal structure provided by the Ferguson-Miller group (Michigan State University) (12) (designated Mut_D44). Both wild-type and mutant structures were relaxed with GROMACS (29) as described below to yield WT*_D44 and Mut*_D44 (with * indicating it is a product of GROMACS optimization). Ser44 in the WT_S44 was replaced with an ionized Asp and the resultant structure relaxed with GROMACS to yield WT*_D44. In addition Asp44 in WT*_D44 and Mut*_D44 was changed to Ser within MCCE to yield WT*_S44 and Mut*_S44 without additional GROMACS backbone changes. In all cases MCCE further relaxes the structure by allowing selection of different side chain positions (30). In addition to the mutation site of residue 44 in chain A, there are 12 surface residues that are not seen in the mutant crystal structure and are labeled as Ala. MCCE rotamer packing was used to recover the 2GSM wild-type sequence, yielding structures Mut*_S44# and Mut*_D44# (# indicating recovery of the 12 surface residues). Overall, there are 7 structures used in this study, including 4 structures with Ser44 (WT_S44, WT*_S44, Mut*_S44 and Mut*_S44#) and 3 structures with Asp44 (WT*_D44, Mut*_D44 and Mut*_D44#) (Table 1).

In silico mutation of Ser44 to Asp

Ser is smaller than Asp. If added to the wild type 2GSM structure the Asp44 to His102 distance is 2.85 Å but the Asp side chain clashes with the backbone of Phe 47 and Met 106. GROMACS molecular dynamics (version 3.3.2) (29) is used to relax the structure. Relaxation uses the GROMACS default mode, in vacuum with a short, <10 ps, trajectory aimed at relaxing van der Waals clashes. Standard ionization states are used so Asp44 is ionized. No atoms are constrained and termination occurs when the energy shifts are less than 0.01 kJ/mol. The final backbone RMSD between WT*_D44 and the original 2GSM wild type structure is 0.14 Å. The backbone in the vicinity of Asp44 shifts by ≈ 0.3 Å to accommodate the larger side chain. The hydrogen bond distance between Asp44 and His102 is now 3.1 Å (Figure 1). In the X-ray crystal structure of S44D mutant the hydrogen bond distance is 2.7 Å. It moves to be 2.88 Å after GROMACS minimization.

MCCE simulation and analysis

MCCE combines continuum electrostatics and molecular mechanics to calculate the pK_a and electrochemical midpoint potentials, E_m s, for amino acids and cofactors, allowing full side chain rotamer sampling and a fixed backbone (30, 31). MCCE version 2.4 has updated non-electrostatic parameters, and entropy and protein surface corrections. Previous simulation results showed the method provides values that agree with experimental redox potentials for hemes and quinones in large membrane proteins (32-35), with hemes in proteins with experimental E_m s varying by 800 mV (36) and with amino acid pK_a s (30). In the benchmark calculations, 65% of the site values have errors less than 1 pH unit or 60 mV, and 92% less than 2 pH units or 120 mV (30, 36).

The four key cofactors, Cu_A, Heme a, Heme a₃ and Cu_B, and the amino acids that serve as their ligands combined into four complexes as described in earlier MCCE calculations (27). An atomic partial charge distribution for Cu_A, with 2 Cu, side chains of Cys 252 and 256 and His 217 and 260 of chain B was calculated using B3LYP method, LANL2DZ basis set and CHELPG algorithm in Gaussian 98 (37-39). Metal centered charge sets (36, 40) are used for Heme a and Heme a₃ (27). Cu_B is ligated to three His residues (284, 333, 334) with a water that can lose a proton to form the Cu_B-hydroxy complex (27). Atomic charge sets are provided in the supplementary material.

The energy of the hydrogen bond to the heme His ligand—The DFT package, Jaguar (41), is employed to determine how well the shift of the Heme E_m by the hydrogen bond to the His ligand is captured by a classical continuum electrostatics program such as MCCE. The difference in the total energy of the separated and combined hydrogen bond acceptor and heme-ligand complex gives the hydrogen bond energy (Supplementary material Figure S2). The single point energy of a Bis-His-Heme model complex (without propionic acids) is calculated with and without an Asp, Asn or Gly as hydrogen bond acceptor from the His. The classical electrostatic hydrogen bond energy is obtained with Coulomb's law with atomic partial charges derived from each Jaguar calculation. Both Coulomb's law and DFT calculations use a dielectric constant of 6.

Previous studies of the interaction between the di-cation Cu_B²⁺ and Heme a₃, 4.8 Å apart in the binuclear center, showed that very large interactions can be overestimated in the classical continuum electrostatics calculations (42). Cu_B is much closer to Heme a₃ in the binuclear complex than the hydrogen bond donor here is to Heme a. In addition, the Cu_B is doubly charged while Ser44 is neutral and Asp44 is singly charged. Good agreement is found between the classical and DFT calculations for the range of interaction energy encountered here. The calculated energy difference in the two methods is less than 0.7 kcal/mol when the H-bond interaction is less than 8 kcal/mol (480 meV). Both the DFT and

continuum electrostatic analysis shows the energy of the hydrogen bond to the His ligand is stronger in the oxidized Fe (III) than the neutral Fe (II) complex. A neutral hydrogen bond partner shifts the E_m by less than 90 mV while an optimal interaction with an ionized Asp can lower the E_m by as much as 500mV, with a dielectric constant of 6. Small differences in hydrogen bond strength are found with the simple metal centered charge set for the heme used here and the Jaguar derived NBO charges (Supplementary material, Table S4). The NBO charges, which are calculated for each heme-imidazole complex generally reproduce the DFT energies better than other charge sets. The results support the use of the continuum electrostatics energy to obtain the shift in Heme a E_m that occurs when the hydrogen bond to the axial heme ligand is changed.

MCCE analysis of the E_m of Heme a—A 35Å slab of low dielectric constant material is added with IPECE (34) to simulate the membrane low dielectric environment. DelPhi (43-45) is used within MCCE to solve the Poisson-Boltzmann equation with an internal dielectric constant for the protein and membrane of 4 and 80 for the solvent. The external salt concentration is 150 mM. Reference redox potentials in water are 264mV for the Cu_A complex (46), -60mV for the Heme a bis-His complex (47) and -120 for Heme a₃ aquo-His (9). PARSE (48) charges and radii are used for the amino acids and the AMBER (49) force field for torsion and Lennard-Jones parameters. The interaction between Heme a₃ and Cu_B is corrected as described in reference (42). The pK_{sol} used for Asp in MCCE is 4.75, the value for a carboxylate side chain. The measured Asp $pK_{a,sol}$ is lowered in a tripeptide to 3.8 because the neighboring amide backbone dipoles must point their positive end towards the side chain with the larger carbonyl pointing away (50). The interaction of the Asp with its neighboring amides is counted explicitly in the MCCE calculations so using the higher carboxylic acid $pK_{a,sol}$ prevents double counting the interaction with the backbone. Benchmark MCCE studies show that the average error of calculated and measured Asp $pK_{a,s}$ is ≈ 0.4 pH units too low rather than too high as would be expected if 3.8 provided a better $pK_{a,sol}$ (30).

Given the Boltzmann distribution of side chain and ligand positions and charge generated by Monte Carlo sampling as a function of pH and E_h the energy terms that determine the E_m of a redox cofactor can be decomposed as (51):

$$nF E_m^{mfe} = nF E_{m,sol} + \Delta \Delta G_{rxn} + \Delta G_{bkn} + \Delta G_{res}^{mfe} \quad 1$$

where $E_{m,sol}$ is the E_m in aqueous solution (52). G_{rxn} is the desolvation energy penalty moving the cofactor from water into protein G_{bkn} is the electrostatic and non-electrostatic interaction of the cofactor with the protein backbone. ΔG_{res}^{mfe} is the mean field pair-wise interaction between the cofactor and residues in the protein in the distribution derived by Monte Carlo sampling. In each case the difference in interaction with the oxidized and the reduced group is given. ΔG_{res}^{mfe} provides the mean-field approximation for the conformer pair-wise interactions, calculated as described in (27), while the full micro-state energies are used in the Monte Carlo sampling. The reorganization energy due to conformational or protonation changes coupled to the redox reaction creates small differences between the calculated E_m and the E_m^{mfe} (31). A similar equation can be used to explore the factors leading to the shift in Asp pK_a in the protein.

The pK_7 is the effective pK_a for the residue when the ionization and conformation of the rest of the protein is fixed in their Boltzmann distribution found at pH 7 (27):

$$pK_7' = pK_{sol} + \Delta\Delta G_{rxn} + \Delta G_{pol} + \Delta G_{res}^{mfe} \quad 2$$

The pK_a and pK_7 will be different because of changes in the charge of the rest of the protein moving from pH 7 to the pH of the true pK_a .

Results and Discussion

The side chain of Ser44 makes a hydrogen bond to His102, an axial ligand to Heme a in CcO from *Rhodobacter sphaeroides*. A Gly at the aligned position in the sequence makes the hydrogen bond in bovine CcO (All residues are in subunit I with *Rb. sphaeroides* numbering unless noted). Replacing Ser44 with Asp allows exploration of what happens when Heme a reduction becomes strongly coupled to proton import to a buried residue (12). This mutation also tests the ability of this protein to accommodate a new charge (21). The calculations were carried out with structures derived from the wild-type protein 2GSM and from a structure of the S44D mutant (personal communication Shelagh Ferguson-Miller). Four structures with a Ser and three with an Asp at position 44 were prepared from these two structures (Table 1). All numbers in the text represent averages of the calculations of the proteins with Ser or with Asp unless otherwise stated. The results with individual structures are provided in Table 2. The change in outcome with different structures provides one estimate of the variability of the calculations. That reproducible values are found with the structure of the mutated protein and with the Asp imposed on the wild-type structure indicates that the changes seen here can be assigned to the Asp itself and not to other diffuse changes in the mutated protein structure.

E_m of Heme a at pH7

The measured Heme a E_m in *Rb. sphaeroides* CcO is ≈ 330 mV, similar to the bovine enzyme E_m of 340 mV (53, 54). Analysis of the *Rb. sphaeroides* structure 1M56 with an earlier version of MCCE yielded a value of 360 mV (27). The redox potential of Heme a here is calculated in the wild-type Ser44 and Asp44 mutant structures using MCCE2.4 with the other three redox centers (Cu_A , Cu_B and Heme a3) fixed in their oxidized state. This parallels the conditions of the electron transfer measurements, where electron transfer from Cu_A to Heme a is measured in the oxidized protein by flash activation of a Ruthenium labeled cytochrome c electron donor (12). The calculated E_m at pH 7 for Heme a in the Ser44 structures is 379 ± 7 mV, in good agreement with experimental measurements and previous MCCE studies. The values found with the initial WT_S44 structure, the structure derived from the mutant x-ray structure (Mut*_S44) and the wild type structure first relaxed around Asp44 in GROMACS and then mutated back to Ser44 (WT*_S44) differ by only 10 mV (Table 2, Figure 2).

The E_m of Heme a in the Asp44 structures is 50 ± 15 mV lower than with Ser44 at pH 7 (Table 2, Figure 2). The E_m was analyzed to determine what energy terms are modified in the mutant protein (Equation 1). Heme a in both wild-type and mutant structures have essentially the same desolvation energy and interactions with the backbone. It is the partially ionized Asp, which is calculated to have a pK_a near 7 that stabilizes the charge on the Heme, shifting the E_m down. If the Asp is fixed in the protonated state, the Heme a redox potential is 374 mV, negligibly different from 378 mV with the native Ser. The similarity of the E_m with a protonated Asp or with Ser is consistent with experimental findings that an oxidase with an Asn at position 44 behaves very much like the wild-type protein (12). The neutral Asp and the Asn have a similar shape and distribution of charges. However, when the Asp is fixed in its deprotonated state with charge -1, the Heme a redox potential drops to 116 (WT*_D44) to 61 (Mut*_D44) mV, providing a lower limit for the E_m in the mutant (Table 2, Figure 3).

pK_a of Asp44 in the mutated structures—The newly introduced Asp can be compared with the 17 Asp found in the native CcO. Ten are deeply buried, as defined as having lost at least 6.8 kcal/mol solvation energy, sufficient to shift the pK_a by 5 pH units in the absence of other interactions with the protein (19). Of these buried Asp five are calculated to be fully ionized at pH 5 and only three are not mostly ionized at pH 7. This indicates that the protein ‘pays back’ the stabilization of the ionized Asp by water lost within the protein through interactions with charged and dipolar groups in the protein.

The Asp44 pK_a is calculated to be ≈ 7 with Heme a oxidized in all structures. Thus, the newly introduced charge remains significantly ionized at neutral pH. The energy breakdown shows the expected, large desolvation penalty for this buried Asp, shifting the pK_a up, while the electrostatics pair-wise interaction between the Asp and positively charged Heme a shifts the pK_a down (equation 2, Table S1 in supplementary material). Interactions with the backbone stabilize the deprotonated Asp, while interactions with other side chains favor the protonated state. The Asp makes a hydrogen bond to the His102, the ligand to Heme a. The distance varies from 2.86 to 3.10 Å in the different structures (Table 1). At pH 7, the His stabilizes Asp ionization by -34 meV in the structures derived from 2GSM (WT*_D44) to -85 meV in the structures derived from the mutant x-ray structure where the Asp-His hydrogen bond is shorter (Mut*_D44). The more favorable interaction also leads to an increase in the fraction of Asp ionized and lower Heme a E_m when the Asp is forced to be ionized (Table S1). The oxidized heme stabilizes the deprotonated Asp by 240-280 meV (Table S1). When the Heme a is reduced, the Asp pK_a always shifts to be above 15 (Figure 3, Table S2).

Comparison of E_m and pK_a shifts in the protein—The E_m of a bis-His a-type heme is -60 mV in solution (52), while it is calculated to be 380 in the wild-type protein, a shift of 320 meV destabilizing the oxidized heme. In the presence of the ionized Asp the E_m shifts to 116 mV (WT*_D44 structure). With Heme a oxidized the side chain pK_a shifts from 3.8 for an Asp in solution to 7.2 in the protein. (Figure 3) This represents a 144 meV destabilization of the charge on the Asp. The mean field energy analysis of the MCCE Monte Carlo conformer population can compare the factors that shift the ionization free energy of Heme a and Asp44 (Table 2, equation 2). The Heme, with its bigger radius, has much smaller desolvation penalty than the Asp (55), while the backbone favors Asp ionization, due to the overall positive backbone potential within all proteins (50). The interactions with the other residues are more favorable for Heme a than for the Asp. The mutual interaction between the ionized Asp and Heme stabilize each charge by ≈ 250 meV. As the Heme E_m is dependent on the Asp charge, the Asp pK_a is dependent on the Heme ionization state. The pK₇ is 7.2 with the Heme oxidized and 11.8 with the Heme reduced (Supplementary table S1, Eqn 2). This value represents the cost of protonating the Asp at pH 7. It is lower than the true pK_a of 15-18 (Table 2) since the protein as a whole is more negative at the higher pH. Thus, in these calculations at pH 7 the Asp is 40% ionized when the Heme is oxidized and is fully protonated when the Heme is reduced. The resultant E_m of Heme a is 345 mV when the titration is free to be coupled to Asp44 protonation.

The experimental pK_a of Asp44 is estimated to be close to 5.5 (27) when the heme is oxidized while it is calculated to be ≈ 7.2 here. There are a variety of sources of error that could be responsible for the higher pK_a. The results rely on the ability of continuum electrostatics to calculate how a group making a hydrogen bond to the axial ligand interacts with the heme (27). The interaction of a number of hydrogen bond acceptors from an axial ligand in a bis-His Heme were calculated with DFT using the Jaguar program (41). There results were compared with those derived with Coulomb's law, the simplification of the Poisson–Boltzmann analysis used in MCCE, which is valid for a uniform dielectric constant. The classical electrostatics treatment models the heme E_m shifts quite well, so is unlikely to

be a source of the error in Asp pK_a (see Supplementary material). Thus, these calculations suggest that the hydrogen bond donor to the His changes the heme E_m via through space, electrostatic interactions rather than by modifying the interaction of the His ligand with the heme.

The GROMACS minimization is carried out with the Asp ionized, which will tend to pull the structure to stabilize this state lowering the pK_a . However, the crystal structure is made with Heme a reduced and will therefore have a neutral Asp44. Mutating the Ser in the wild-type structure to Asp also starts with a protein equilibrated around a neutral group. X-ray structures with the oxidized heme/ionized Asp show the Asp is very disordered (personal communication Shelagh Ferguson-Miller) so may be able to sample positions that are not available here. The optimized Asp-His hydrogen bond is slightly longer than found in the x-ray structure. The shorter bond does strengthen the Asp-His interaction. However, comparing the Mut*_D44# structure with a hydrogen bond of 2.86Å with the Wt*_D44 structure with a 3.10Å distance shows the pK_a actually increases only from 7.0 to 7.2 as the bond is lengthened. Thus, the pK_a is weakly dependent on the Asp to His distance. Lastly, preliminary calculations of the pK_a s of newly introduced acidic and basic residues into the interior of *Staphylococcal nuclease* (27) shows that MCCE can overestimate the desolvation penalty of a charge introduced into a site in the protein interior by mutation, raising the pK_a of introduced acidic residues. The problem appears to be that the analysis underestimates protein relaxation around the novel charge especially when the protein is given a low dielectric constant of 4 as done here. Calculation with a higher dielectric constant would be expected to lower the Asp pK_a .

pH dependence of the Heme a E_m —The calculated pH dependence of the Heme a E_m in the Ser44 structures is 34 mV/pH unit as derived from the slope of the linear regression line from pH 6 to 9. Earlier MCCE calculations also found a pH dependence of ≈ 30 mV/pH (27). A range of values from ≈ 10 (53) to 30 (56) mV/pH unit have been found in bovine CcO. When Ser44 is replaced with an Asp the pH dependence of Heme a increases to ≈ 50 mV/pH (Figure 2 a).

The E_m of Cu_A—The E_m of Cu_A was calculated as a function of pH. The redox potential of Cu_A and its pH dependence is independent of the residue at position 44. This is consistent with the electron transfer kinetics of Cu_A reduction by cytochrome c being unaffected by the mutation (12). However, the results are sensitive to which initial x-ray structure is the basis of the calculations. The E_m is 320mV in the 2GSM structures at pH 7, ≈ 30 mV more negative than in the structures derived from the mutant CcO structure. The difference arises from small changes in relatively distant surface residues of chain B. These residues are further away from the other three redox active centers and so do not affect their E_m s significantly.

The pH-dependence of the Cu_A E_m is ≈ 24 mV/pH unit in the 2GSM-derived structures and ≈ 45 mV/pH unit in the structures derived from the mutated protein x-ray structure (Figure 2 b). Earlier MCCE calculations, using the 1M56 structure of *Rb. sphaeroides* CcO also found a pH dependence of 30 mV/pH (27). In bovine CcO measurements show a smaller pH dependence of <10 mV/pH unit for the Cu_A E_m (pH 7.0 to 8.1) (53, 57). The G° for electron transfer from Cu_A to Heme a is obtained from $-nF(E_{m,CuA} - E_{m,Heme})$ (Figure 4). In the analysis of the electron transfer from Cu_A to Heme a the $E_{m,CuA}$ is always taken from the structure Mut*_S44 which has the smallest Cu_A pH dependence, while $E_{m,Heme}$ was compared for the 7 different structures (Figure 2 a).

The free energy of electron transfer from Cu_A to Heme a—At pH 7 the average G° in the Ser44 structures is -75 meV. This is in reasonable agreement with the experimental

value of -40 meV in the *Rb. sphaeroides* protein (14). The G° is -30 meV in the Asp44 mutant, while the experimental value is <-60 meV (12). While the calculated G° for the mutant is consistent with the measured value given the error range of the calculations, the change moving from wild-type to mutated protein is not.

A thermodynamic analysis (Fig. 5) shows that the driving force for electron transfer from Cu_A to Heme a should be less favorable when the starting state is Asp44·Hem^{ox} since Asp proton binding adds a cost to the Heme a reduction. This assumes the E_m of Cu_A is the same if the Asp is ionized or protonated and that the Heme a E_m is similar with Ser44 or a protonated Asp44 (Figure 4). These conditions are supported by these calculations and by earlier experiments (12). The shift in Heme a E_m is only 45 meV here because the calculated Asp pK_a in the mutant is ≈7 so the cost to protonate it at pH 7 is small. If the Asp pK_a were 5.5 as found experimentally the driving force for Heme a reduction would be 90 meV less favorable since Asp⁻ is more stable and so stabilizes the starting state. The electron transfer from Cu_A to Heme a would now be uphill. That experimentally the reduced Heme a is more stable than in the wild-type protein, while the Asp pK_a is so low, reveals that there must be additional factors stabilizing AspH:Heme a^{red} that are not captured here.

Response of the protein to deprotonating Asp44 or reducing Heme a—The proton uptake from the protein that accompanies Heme a reduction with the other cofactors held oxidized was determined (Figure 6). There are 0.5 protons taken up from solution at pH 7 when Heme a is reduced in the Ser44 structures (Fig. 6 Supplementary material S1). This increases to 0.6-0.7 at pH 9. As has been seen in earlier MCCE calculations (27) His 93 and Glu 182, whose protonation is coupled together, are the most important proton acceptors in the Ser44 structures. Increasing the pH to 9, increases the total proton uptake by 0.2 protons bound to the H93/E182 cluster.

With the addition of Asp44, when the ionization of Asp44 remains in equilibrium with the heme redox state, 0.75 protons are taken up when Heme a is reduced at pH 7. It increases to 1.15 at pH9 where Asp44 is fully ionized in the oxidized Heme initial state. The uptake is mostly to Asp44, while now none is to His 93 or Glu182 (Figure 6b). No other residue significantly changes proton uptake with pH. One goal of these calculations is to identify potential proton donors to the Asp. However, the nearest ionizable residues that contribute small amounts to proton uptake (<0.05 H⁺/e⁻) are Arg 52, 481 and 482, which are 12-13 Å away. Other residues >20 Å away, show protonation shifts at the limits of the MCCE sampling noise of ≈0.02. As the sites that respond to the change in charge at Asp44 and Heme a are relatively far away, the protonation shifts throughout the protein are the same when the Asp loses a proton when the heme remains reduced or when the oxidized heme is reduced with Asp fixed in its protonated form. In each case the charge on the Asp44:Heme changes from 0 to -1 and in either case the rest of the protein binds 0.3 protons to keep the net change in charge of -0.7 (Supplementary material S1). When the Asp is deprotonated the protein as a whole loses 0.7 protons. When the heme is reduced the protein binds 0.3 protons. Thus, the protein is behaving as a buffer, reducing the effect of the change in charge in and around Heme a by small, changes distributed throughout the protein (1).

It has been suggested that an internal site donates the proton required for Heme a reduction to Asp44 (12). This requires a residue or group of residues that is protonated in the Asp⁻:Heme^{ox} state and deprotonated in the AspH:Heme^{red} state. Deprotonating the Asp with the heme held oxidized at pH 7 only changes the net charge of the protein by -0.2. Thus, 0.8 protons are distributed to the ensemble average protonation of other residues throughout the protein. However all of these sites are quite far from the Asp and no individual residue is found whose equilibrium protonation is strongly coupled that of Asp44.

The low energy pathway for proton coupled Heme a reduction in the presence of Asp44

The experimental study of the Asp44 CcO and the calculations reported here support an Asp that is significantly deprotonated at neutral pH when Heme a is oxidized and fully protonated when the heme is reduced at pH 7. Long-range electron transfer from Cu_A is now coupled to proton uptake. The rate of proton coupled electron transfer is from 10-130 s⁻¹ while it is ≈90,000 s⁻¹ in the wild-type protein (12) a decrease of ≈10³-10⁴ fold. The reaction cannot be truly concerted (the diagonal path in Figure 5) as the protons and electrons come from different donors. Rather it will follow one of the two paths around Figure 5, either first protonating Asp44 (forming AspH:Heme^{ox}) or reducing heme (Asp⁻¹:Heme^{red}) (58). The calculations provide an estimate of the relative energy of the two possible intermediates. Assuming Cu_A is the electron donor, the reaction through Asp⁻¹:Heme^{red} state is 200 meV uphill. Thus, at room temperature the electron first pathway goes through an intermediate that will be present in a ratio with the ground state of 1:2,000. Electron transfer could populate thermally populate Asp⁻¹:Heme^{red} by a fast, but unfavorable electron transfer (59, 60). This would slow the rate 2,000 fold even if the electron transfer from Cu_A is unchanged and proton uptake not rate limiting. However, though this intermediate is certainly low enough in energy to be thermally accessible a proton must still be bound to the Asp in order to generate a stable product.

The proton first pathway via AspH:Heme^{ox} has an energy cost close to 0 meV given the Asp44 pK₇ of 7.2 or 100 meV uphill if the pK_a is 5.5 as found experimentally. Thus, this path is energetically favored. The Asp is deeply buried and not connected to the surface by any of the evolved proton pathways. Within 5 Å of the Asp there are Ala46, Gly41, Ile43, Leu42 and 105, Met106, Phe47, Thr48 and Val40 and 45. These provide no easy pathway for proton transfer to Asp44. In addition, in these structures there are no cavities that could hold a water near the Asp. Without some distortion of the structure delivery of a proton to the Asp could be quite slow.

Concluding remarks

These calculations highlight a number of aspects of the behavior of the S44D CcO mutant (12). Here an Asp is introduced into a region where there are no polar groups except one Thr and the His from which the Asp will accept a hydrogen bond. There are no cavities large enough to hold a water in the available x-ray crystal structures. Added buried acidic or basic residues are often found to have significant pK_a shifts to stabilize the neutral form of the residue and significant destabilization of the protein when the mutated site is ionized (21). However, here the Asp remarkably retains a fairly low pK_a if Heme a is oxidized and the wealth of experiments carried out on the mutant shows the protein remains functional in the Asp⁻:Heme^{ox} state. The interaction with the oxidized heme lowers the pK_a by 5.5 pH units (Fig. 5). The stability of the protein hosting the Asp can be explained given that the net charge on the protein with the ionized Asp (Asp⁻:Heme^{ox}) is the same as that of the native protein with a reduced Heme a (Ser:Heme^{red}) (Supplementary information Fig. S1). Thus, this anionic mutation is stabilized by the factors that support Heme a reduction.

The calculations are dependent on the interaction between the oxidized Heme a and Asp44, the hydrogen bond acceptor from the His ligand. A hydrogen bond of this kind is found in 75% of b and c type hemes collected in a non-redundant database (10). The similarity of the shift in the heme E_m calculated in a model complex with Density Functional Theory and with Coulomb's law shows the Asp interacts with the heme in a manner that can be treated as a through space interaction (Supplementary material page 9-12).

The calculated pK_a of the Asp is ≈1.5 pH units higher than measured. MCCE analysis of benchmark pK_as reported 84% of the errors are <1.5 pH units (30). The fact that all

structures used here were obtained with a neutral Asp will tend to raise the pK_a . The analysis is still able to account for much of the stability of the ionized Asp, which has lost ≈ 14 Kcal/mol of the energy that would stabilize an ionized Asp in water due to its position deep in the protein. This desolvation penalty would lift the pK_a by over 10 pH units in the absence of favorable interactions with the protein. The nearby oxidized Heme a is most important factor stabilizing the deprotonated Asp, while the backbone dipoles and the hydrogen bond to the His also lower the pK_a .

The analysis reveals an inconsistency in the model that has been derived from the experimental data. This combines a pK_a for Asp44 below 7, obligatory protonation of the Asp when the heme is reduced and a more favorable electron transfer from Cu_A to heme, while the Cu_A electrochemistry is unaffected by the residue at position 44 (12). The thermodynamic analysis of the coupled electron transfer shows that a low Asp44 pK_a stabilizes the Asp44⁻:Hem^{ox} state. This will make Asp44H:Hem^{red} less favorable lowering the heme E_m . It remains an open question what additional factors are stabilizing the reduced heme in the mutated protein.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by 1R01GM084028-01A2 with infrastructure support from NIH 5G12 RR03060.

We are grateful to Ling Qin and Shelagh Ferguson-Miller (University of Michigan, East Lansing, MI) for providing the coordinates for the S44D x-ray crystal structure prior to publication and Christian Fufezan for his work on the analysis of the hydrogen bonds to the heme ligands in his non-redundant heme protein database. We thank Dr. Yifan Song and Jianxun Lu for helpful discussion.

References

1. Rich PR. Towards an understanding of the chemistry of oxygen reduction and proton translocation in the iron-copper respiratory oxidases. *Aust J Plant Physiol.* 1995; 22:479–486.
2. Michel H. Cytochrome c oxidase: catalytic cycle and mechanisms of proton pumping—a discussion. *Biochemistry.* 1999; 38:15129–15140. [PubMed: 10563795]
3. Verkhovsky MI, Belevich I, Bloch DA, Wikstrom M. Elementary steps of proton translocation in the catalytic cycle of cytochrome oxidase. *Biochim Biophys Acta.* 2006; 1757:401–407. [PubMed: 16829227]
4. Brzezinski P, Larsson G. Redox-driven proton pumping by heme-copper oxidases. *Biochim Biophys Acta.* 2003; 1605:1–13. [PubMed: 12907296]
5. Mitchell R, Mitchell P, Rich PR. Protonation states of the catalytic intermediates of cytochrome c oxidase. *Biochim Biophys Acta.* 1992; 1101:188–191. [PubMed: 1321666]
6. Babcock GT, Wikström M. Oxygen activation and the conservation of energy in cell respirations. *Nature.* 1992; 356:301–308. [PubMed: 1312679]
7. Ferguson-Miller S, Babcock GT. Heme/copper terminal oxidases. *Chem Rev.* 1996; 96:2889–2907. [PubMed: 11848844]
8. Wikstrom M. Cytochrome c oxidase: 25 years of the elusive proton pump. *Biochim Biophys Acta.* 2004; 1655:241–247. [PubMed: 15100038]
9. Reedy CJ, Gibney BR. Heme protein assemblies. *Chem Rev.* 2004; 104:617–649. [PubMed: 14871137]
10. Fufezan C, Zhang J, Gunner MR. Ligand preference and orientation in b- and c-type heme-binding proteins. *Proteins.* 2008; 73:690–704. [PubMed: 18491383]

11. Schneider R, S C. The HSSP database of protein structure-sequence alignments. *Nucleic Acids Res.* 1996; 24:201–205. [PubMed: 8594579]
12. Mills DA, Xu S, Geren L, Hiser C, Qin L, Sharpe MA, McCracken J, Durham B, Millett F, Ferguson-Miller S. Proton-dependent electron transfer from CuA to Heme a and altered EPR spectra in mutants close to Heme a of cytochrome oxidase. *Biochemistry.* 2008; 47:11499–11509. [PubMed: 18847227]
13. Winkler JR, Malmstrom BG, Gray HB. Rapid electron injection into multisite metalloproteins: intramolecular electron transfer in cytochrome oxidase. *Biophysical Chemistry.* 1995; 54:199–209. [PubMed: 7749059]
14. Zaslavsky D, Sadoski R, Wang K, Durham B, Gennis R, Millett F. Single electron reduction of cytochrome c oxidase compound F: resolution of partial steps by transient spectroscopy. *Biochemistry.* 1998; 37:14910–14916. [PubMed: 9778367]
15. Farver O, Grell E, Ludwig B, Michel H, Pecht I. Rates and Equilibrium of CuA to Heme a Electron Transfer in *Paracoccus denitrificans* Cytochrome c Oxidase. *Biophys J.* 2006; 90:2131–2137. [PubMed: 16387770]
16. Grunwald E, Berkowitz BJ. The measurement and correlation of acid dissociation constants for carboxylic acids in the system ethanol-water activity coefficients and empirical activity functions. *J Am Chem Soc.* 1951; 73:4939–4944.
17. Warshel A, Russell ST. Calculations of electrostatic interactions in biological systems and in solutions. *Q Rev Biophys.* 1984; 17:283–422. [PubMed: 6098916]
18. Barrette WC, Johnson HW, Sawyer DT. Voltammetric evaluation of the effective acidities (pK_a) for Brønsted acids in aprotic solvents. *Anal Chem.* 1984; 56:1890–1898. [PubMed: 6093632]
19. Kim J, Mao J, Gunner MR. Are acidic and basic groups in buried proteins predicted to be ionized? *J Mol Biol.* 2005; 348:1283–1298. [PubMed: 15854661]
20. Bartlett GJ, Porter CT, Borkakoti N, Thornton JM. Analysis of catalytic residues in enzyme active sites. *J Mol Biol.* 2002; 324:105–211. [PubMed: 12421562]
21. Isom DG, Cannon BR, Castaneda CA, Robinson A, Garcia-Moreno B. High tolerance for ionizable residues in the hydrophobic interior of proteins. *Proc Natl Acad Sci USA.* 2008; 105:17784–17788. [PubMed: 19004768]
22. Siegbahn PEM, Blomberg MRA. Energy diagrams and mechanism for proton pumping in cytochrome c oxidase. *Biochim Biophys Acta.* 2007; 1767:1143–1156. [PubMed: 17692282]
23. Ghosh N, Prat-Resina X, Gunner MR, Cui Q. Microscopic pK_a analysis of Glu286 in cytochrome c oxidase (*Rhodobacter sphaeroides*): Toward a calibrated molecular model. *Biochemistry.* 2009; 48:2468–2485. [PubMed: 19243111]
24. Xu J, Voth G. Redox-coupled proton pumping in cytochrome c oxidase: Further insights from computer simulation. *Biochim Biophys Acta.* 2008; 1777:196–201. [PubMed: 18155154]
25. Olsson MHM, Warshel A. Monte Carlo simulations of proton pumps: On the working principles of the biological valve that controls proton pumping in cytochrome c oxidase. *Proc Natl Acad Sci USA.* 2006; 103:6500–6505. [PubMed: 16614069]
26. Quenneville J, Popovic DM, Stuchebrukhov AA. Combined DFT and electrostatics study of the proton pumping mechanism in cytochrome c oxidase. *Biochim Biophys Acta.* 2006; 1757:1035–1046. [PubMed: 16458251]
27. Song Y, Michonova-Alexova E, Gunner MR. Calculated proton uptake on anaerobic reduction of cytochrome c oxidase: Is the reaction electroneutral? *Biochemistry.* 2006; 45:7959–7975. [PubMed: 16800622]
28. Qin L, Hiser C, Mulichak A, Garavito RM, Ferguson-Miller S. Identification of conserved lipid/detergent-binding sites in a high-resolution structure of the membrane protein cytochrome c oxidase. *Proc Natl Acad Sci U S A.* 2006; 103:16117–16122. [PubMed: 17050688]
29. Van Der Spoel D, Lindahl E, Hess B, Groenhof G, Mark AE, Berendsen HJ. GROMACS: fast, flexible, and free. *J Comput Chem.* 2005; 26:1701–1718. [PubMed: 16211538]
30. Song Y, Mao J, Gunner MR. MCCE2: Improving protein pK_a calculations with extensive side chain rotamer sampling. *J Comp Chem.* 2009; 30:2231–2247. [PubMed: 19274707]
31. Mao J, Hauser K, Gunner MR. How cytochromes with different folds control heme redox potentials. *Biochemistry.* 2003; 42:9829–9840. [PubMed: 12924932]

32. Lancaster CRD, Michel H, Honig B, Gunner MR. Calculated coupling of electron and proton transfer in the photosynthetic reaction center of *Rhodospseudomonas viridis*. *Biophys J*. 1996; 70:2469–2492. [PubMed: 8744288]
33. Alexov EG, Gunner MR. Calculated protein and proton motions coupled to electron transfer: electron transfer from Q_A⁻ to Q_B in bacterial photosynthetic reaction centers. *Biochemistry*. 1999; 38:8253–8270. [PubMed: 10387071]
34. Song Y, Mao J, Gunner MR. Calculation of proton transfers in bacteriorhodopsin bR and M intermediates. *Biochemistry*. 2003; 42:9875–9888. [PubMed: 12924936]
35. Zhu Z, Gunner MR. Energetics of quinone-dependent electron and proton transfers in *Rhodobacter sphaeroides* photosynthetic reaction centers. *Biochemistry*. 2005; 44:82–96. [PubMed: 15628848]
36. Zheng Z, Gunner MR. Analysis of the electrochemistry of hemes with E_ms spanning 800 mV. *Proteins*. 2009; 75:719–734. [PubMed: 19003997]
37. Frisch, MJ.; Trucks, GW.; Schlegel, HB.; Scuseria, GE.; Robb, MA.; Cheeseman, JR.; Zakrzewski, VG.; Montgomery, JA.; Stratmann, RE.; Burant, JC.; Dapprich, S.; Millam, JM.; Daniels, AD.; Kudin, KN.; Strain, MC.; Farkas, O.; Tomasi, J.; Barone, V.; Cossi, M.; Cammi, R.; Mennucci, B.; Pomelli, C.; Adamo, C.; Clifford, S.; Ochterski, J.; Petersson, GA.; Ayala, PY.; Cui, Q.; Morokuma, K.; Malick, DK.; Rabuck, AD.; Raghavachari, K.; Foresman, JB.; Cioslowski, J.; Ortiz, JV.; Baboul, AG.; Stefanov, BB.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Gomperts, R.; Martin, RL.; Fox, DJ.; Keith, T.; Al-Laham, MA.; Peng, CY.; Nanayakkara, A.; Challacombe, M.; Gill, PMW.; Johnson, B.; Chen, W.; Wong, MW.; Andres, JL.; Gonzalez, C.; Head-Gordon, M.; Replogle, ES.; Pople, JA. Gaussian 98, Revision A.9. Gaussian, Inc; Pittsburgh PA: 1998.
38. Becke AD. Density-Functional Thermochemistry .3. The role of exact exchange. *J Chem Phys*. 1993; 98:5648–5652.
39. Hay PJ, Wadt WR. Ab initio effective core potentials for molecular calculations Potentials for the transition metal atoms Sc to Hg. *J Chem Phys*. 1985; 82:270–283.
40. Gunner, MR.; Honig, B. Calculations of proton uptake in *Rhodobacter sphaeroides* reaction centers. In: Breton, J.; Vermeglio, A., editors. *The Photosynthetic Bacterial Reaction Center: Structure, Spectroscopy and Dynamics II*. Plenum; New York: 1992. p. 403-410.
41. Jaguar 6.5. Schrodinger, LLC; Portland, OR: 2005.
42. Song Y, Mao J, Gunner MR. Electrostatic environment of hemes in proteins: pK_as of hydroxyl ligands. *Biochemistry*. 2006; 45:7949–7958. [PubMed: 16800621]
43. Nicholls A, Honig B. A rapid finite difference algorithm utilizing successive over-relaxation to solve the Poisson-Boltzmann equation. *J Comp Chem*. 1991; 12:435–445.
44. Bharadwaj R, Windemuth A, Sridharan S, Honig B, Nicholls A. The fast multipole boundary element method for molecular electrostatics: An optimal approach for large systems. *J Comp Chem*. 1995; 16:898–913.
45. Rocchia W, Alexov E, Honig B. Extending the applicability of the nonlinear Poisson-Boltzmann Equation: multiple dielectric constants and multivalent ions. *J Phys Chem B*. 2001; 105:6507–6514.
46. Ostermeier C, Iwata S, Michel H. Cytochrome *c* oxidase. *Current Opinion in Structural Biology*. 1996; 6:460–466. [PubMed: 8794157]
47. Vanderkooi G, Stotz E. Reductive alteration of Heme a hemochromes. *J Biol Chem*. 1965; 240:3418–3424. [PubMed: 14321382]
48. Sitkoff D, Sharp KA, Honig B. Accurate calculation of hydration free energies using macroscopic solvent models. *J Phys Chem*. 1994; 98:1978–1988.
49. Cornell WD, Cieplak P, Bayly CI, Gould IR, Merz KM J, Ferguson DM, Spellman DC, Fox T, Caldwell JW, Kollman PA. A second generation force field for the simulation of proteins, nucleic acids, and organic molecules. *J Am Chem Soc*. 1995; 117:5179–5197.
50. Gunner MR, Saleh MA, Cross E, ud-Doula A, Wise M. Backbone dipoles generate positive potentials in all proteins: origins and implications of the effect. *Biophys J*. 2000; 78:1126–1144. [PubMed: 10692303]

51. Gunner MR, Mao J, Song Y, Kim J. Factors influencing energetics of electron and proton transfers in proteins. What can be learned from calculations. *Biochim Biophys Acta*. 2006; 1757:942–968. [PubMed: 16905113]
52. Vanderkooi G, Stotz E. Oxidation-reduction potentials of Heme a hemochromes. *J Biol Chem*. 1966; 241:3316–3323. [PubMed: 5913120]
53. Moody AJ, Rich PR. The effect of pH on redox titrations of Heme a in cyanide liganded cytochrome c oxidase - Experimental and modeling studies. *Biochim Biophys Acta*. 1990; 1015:205–215. [PubMed: 2153404]
54. Wang K, Zhen Y, Sadoski R, Grinnell S, Geren L, Ferguson-Miller S, Durham B, Millett F. Definition of interaction domain for the reaction of cytochrome c with cytochrome c oxidase: II Rapid kinetics analysis of electron transfer from cytochrome c to *Rhodobacter sphaeroides* cytochrome oxidase surface mutants. *J Biol Chem*. 1999; 274:38042–38050. [PubMed: 10608873]
55. Bockris, JOM.; Reddy, AKN. *Modern Electrochemistry*. Vol. 1. Plenum; New York: 1973.
56. Artzbatanov VY, Konstantinov AA, Skulachev VP. Involvement of intramitochondrial protons in redox reactions of cytochrome alpha. *FEBS Lett*. 1978; 87:180–185. [PubMed: 24554]
57. Erecinska M, Chance B, Wilson DF. The oxidation-reduction potential of the copper signal in pigeon heart mitochondria. *FEBS Lett*. 1971; 16:284–286. [PubMed: 11945962]
58. Graige MS, Paddock ML, Bruce JM, Feher G, Okamura MY. Mechanism of proton-coupled electron transfer for quinone (Q_B) reduction in reaction centers of *Rb. sphaeroides*. *J Am Chem Soc*. 1996; 118:9005–9016.
59. Woodbury NW, Parson WW, Gunner MR, Prince RC, Dutton PL. Radical-pair energetics and decay mechanisms in reaction center containing anthraquinones or benzoquinones in place of ubiquinone. *Biochim Biophys Acta*. 1986; 851:6–22. [PubMed: 3524681]
60. Xu Q, Gunner MR. Temperature dependence of the free energy, enthalpy and entropy of P⁺Q_A⁻ charge recombination in photosynthetic reaction centers. *J Phys Chem B*. 2000; 104:8035–8043.

Abbreviations

CcO	Cytochrome c Oxidase
MCCE	Multi-Conformer Continuum Electrostatics

Figure 1.

Comparison of the region around Heme a in 2GSM (WT_S44) and WT*_D44. Heme a and its two distal ligands are shown. Ser44 and the original backbone are purple. Asp44 and nearby backbone after GROMACS minimization are yellow. Nearby residues Val40, Leu105 and Thr48 in green change position slightly. His 102, is 2.9Å from S44 in WT_S44. The Asp-His H-bond distance is 3.1Å in WT*_D44 after Gromacs minimization.

Figure 2. pH dependence of (a) Heme a and (b) Cu_a E_m s. Structures with Ser44: WT_S44 (□), WT*_S44 (△), Mut*_S44 (◇) and Mut*_S44# (○). Structures with Asp44: WT*_D44 (▲), Mut*_D44 (◆) and Mut*_D44# (●). The lines connect the average E_m s for all structures with S44 or with D44.

Figure 3.

- a). The Heme a E_m titration curve when D44 is fixed to be neutral (●), ionized (○) or allowed to remain at equilibrium the Heme at pH 7 (●). The redox potential of Heme a E_m in solution of -60mV is shown.
- b). The D44 pK_a titration when Heme a is fixed to be reduced (■) or oxidized (+1 charge) (□ Mut*_44D and △ WT*_44D). The Asp carboxylic acid side chain solution pK_a of 4.75pH is shown.

Figure 4.

The free energy difference between Heme a and Cu_A as a function of pH. The G° for electron transfer from Cu_A to Heme a is obtained from $-nF(E_{m,CuA} - E_{m,Heme})$. $E_{m,CuA}$ is that found in the structure Mut*_S44 (Fig. 2 b), while $E_{m,Heme}$ was compared for the 7 different structures (Fig. 2 a). Symbols are as found in Figure 2.

Figure 5.

Comparison of Heme a reduction with Ser44 and Asp44. E_m^0 : the Heme a redox potential in wild type CcO with Ser44 or in the WT*_D44 protein when Asp is fixed in its neutral, protonated state. E_m^{-1} : the redox potential when Asp44 is fixed in its ionized state. E_m : the calculated E_m in the Asp44 protein when Asp ionization is kept at equilibrium with the pH and heme redox state. In these calculations at pH 7 the Asp is partially ionized when the Heme is oxidized. Heme ionization is coupled to proton uptake so that the Asp is fully protonated when Heme a is reduced. Data from WT_S44 and WT*_D44 calculations.

Figure 6. pH dependent proton uptake when Heme a is reduced. (a) S44 (b) D44 structures. ■ total proton uptake, × uptake to His93 and Glu182 pair; △ to the rest of protein; ○ to Asp44.

Table 1
Structure Definitions

Designation	Comments	H-bond to H102 Å ^a	RMSD 2GSM Å ^b
WT_S44	Protein Databank structure 2GSM	2.85	0
WT*_D44	MCCE mutates D44 S44 in WT_S44 (2GSM) followed by GROMACS relaxation	3.10	0.14
WT*_S44	MCCE mutates D44 S44 in WT*_D44	3.58	0.14
MUT_D44	S44D X-ray structure; 12 surface residues labeled Ala	2.71	0.37
MUT*_D44	GROMACS relaxation of MUT_D44	2.88	0.44
MUT*_S44	MCCE mutates D44 S44 in MUT*_D44	3.60	0.44
MUT*_D44 [#]	MCCE replaces surface Ala with wild-type residues in MUT*_D44.	2.86	0.44
MUT*_S44 [#]	MCCE replaces surface Ala with wild-type residues in MUT*_S44	3.69	0.44

Designation: WT: structure derived from 2GSM; MUT: structure derived from x-ray crystal structure from S. Ferguson-Miller of the D44S mutant with 12 surface residues designated as Ala; GROMACS optimization carried out with ionized Asp at position 44 once for each parent structure. Relaxation for other structures carried out with MCCE optimization of side chain packing.

* Structure minimized using GROMACS.

[#] 12 unresolved side chains in the MUT_structure reverted to the 2GSM wild-type sequence.

^a Distance from the side chain oxygen of Ser or Asp44 to the N⁺ proton of His 102.

^b The RMSD of the backbone atoms.

Table 2

Energy terms contributing to the E_m shift of Heme a and pK_a shift of Asp 44.

Structures	E _m	G _{rxn}	G _{pol}	ΔG _{ele} ^{mf}		E _{m,sol}	Fraction charge of D44 @pH 7		
				Asp44	Others		Ox	Mid	
WT_S44	378	362	177		-101	-60			
WT*_S44	387	363	189		-105	-60			
Mut*_S44	375	374	177		-120	-60			
Mut*_S44#	372	373	180		-119	-60			
Avg_S44	378±6	368±6	181±6		-111±10				
WT*_D44	345	363	189	-34	-113	-60	0.45	0.18	
Mut*_D44	329	374	180	-85	-87	-60	0.85	0.33	
Mut*_D44#	318	373	182	-117	-94	-60	0.88	0.37	
Avg_D44	331±14	370±6	184±5	-78±41	-98±13				
Diff D44-S44	-47	2	3	-78	13				
ASP44	pK	G _{rxn}	G _{pol}	Heme a	others	pK _{sol}			
With heme oxidized									
WT*_D44	7.2	600	-97	-392	34	4.75			
Mut*_D44	7.3	630	-65	-482	58	4.75			
Mut*_D44#	7.0	628	-48	-539	53	4.75			
Avg	7.2±0.1	619±17	-70±25	-471±74	48±13	4.75			
With Heme reduced									
WT*_D44	15.2	600	-97	-127	26	4.75			
Mut*_D44	17.8	630	-65	-173	17	4.75			
Mut*_D44#	17.9	628	-49	-199	57	4.75			
Avg	17.0±1.5	619±17	-70±24	-166±36	33±21	4.75			
Diff D44-S44	9.8	0	0	-305	15				

Table 2: Analysis of: (Top) The redox potential (E_m) of Heme a at pH 7. (Bottom) The pK_a of D44 in the presence of the oxidized or reduced Heme a. Structures are defined in Table 1. E_{m,sol}: the reference E_m of Heme a in aqueous solution. pK_{a,sol}: pK_a of the Asp carboxylate side chain in solution. G_{rxn}: the difference in desolvation energy of reactant and product. G_{pol}: the difference of the electrostatic and non-electrostatic interaction of product and reactant states with the backbone. ΔG_{ele}^{mf}: the mean field energy difference of the electrostatic pair-wise and van der Waals interactions

with the residues in the protein in the reactant and product. The oxidized Heme a and deprotonated Asp are the product states. See Equation 1 and 2 for more complete description of terms. pK_{sol} and pK_a are in pH units, the E_M is in mV, all energies are in meV. 58 meV or 5.7 kJ/mol is the energy need to change a pK_a by 1 pH unit at 25°C.