Electrostatic Environment of Hemes in Proteins: pKₐs of Hydroxyl Ligands†

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ABSTRACT: The pKₐs of ferric aquo—heme and aquo—heme electrochemical midpoints (E_mₐₚₐₜ) at pH 7 in sperm whale myoglobin, Aplysia myoglobin, hemoglobin I, heme oxygenase 1, horseradish peroxidase and cytochrome c oxidase were calculated with Multi-Conformation Continuum Electrostatics (MCCE). The pKₐs span 3.3 pH units from 7.6 in heme oxygenase 1 to 10.9 in peroxidase, and the E_mₐₚₐₜ range from −250 mV in peroxidase to 125 mV in Aplysia myoglobin. Proteins with higher in situ ferric aquo—heme pKₐs tend to have lower E_mₐₚₐₜ. Both changes arise from the protein stabilizing a positively charged heme. However, compared with values in solution, the protein shifts the aquo—heme E_mₐₚₐₜs more than the pKₐs. Thus, the protein has a larger effective dielectric constant for the protonation reaction, showing that electron and proton transfers are coupled to different conformational changes that are captured in the MCCE analysis. The calculations reveal a breakdown in the classical continuum electrostatic analysis of pairwise interactions. Comparisons with DFT calculations show that Coulomb’s law overestimates the large unfavorable interactions between the ferric water—heme and positively charged groups facing the heme plane by as much as 60%. If interactions with Cu₈ in cytochrome c oxidase and Arg 38 in horseradish peroxidase are not corrected, the pKₐs calculations are in error by as much as 6 pH units. With DFT corrected interactions calculated pKₐs and E_mₐₚₐₜs differ from measured values by less than 1 pH unit or 35 mV, respectively. The in situ aquo—heme pKₐ is important for the function of cytochrome c oxidase since it helps to control the stoichiometry of proton uptake coupled to electron transfer [Song, Michonova-Alexova, and Gunner (2006) Biochemistry 45, 7959—7975].

Heme cofactors are found in many proteins. Cytochromes having two axial ligands contributed by the protein are six-coordinate and function as redox intermediates in electron transfer chains. However, five-coordinate hemes with one His ligand and one open position are the most common heme motif (1). The open site can bind different molecules for transport or catalysis. Hemoglobin and myoglobins are O₂ transporters (2). Heme oxygenase is an essential protein in heme metabolism, using three O₂s and seven electrons to degrade heme to biliverdin (3). Peroxidases reduce toxic hydrogen peroxide to water and then carry out one electron oxidation of a wide range of substrates (4, 5). Cytochrome c oxidase is the terminal electron acceptor in the respiratory chain (6—9). Here Heme a₁ forms a binuclear center with a copper complex (Cu₈) that reduces O₂ to water. The protein uses the released chemical energy to pump protons across the membrane. Other five-coordinate heme proteins carry out NO storage and transport (10), steroid synthesis (11, 12), and O₂, CO, and NO sensing (13, 14).

Hemes can carry out many biological functions because the protein, especially the active site residues, modulates the ligand binding specificity and resultant chemistry. The diversity of in situ heme functions highlights important interactions of proteins with their cofactors and substrates. Extensive studies have explored how ligand geometry (15—17) and the protein scaffolding (18) affect in situ heme properties (1). The range of redox free energy and pKₐs of protein-bound hemes and their ligands show the importance of electrostatic interactions (19, 20). For example, six-coordinate bis-His—hemes have E_mₐₚₐₜ ranging from −410 to +360 mV with the redox differences being predominately due to the intraprotein electrostatic environment (1, 20—22). In these proteins the loss of solvation energy (15, 23, 24), interactions with the protein backbone and with other residues (15, 20—22, 25), and local conformation changes on ionization changes (20, 26) determine the thermodynamic equilibrium measured by pKₐ and E_mₐₚₐₜ.

Theoretical studies have analyzed hemes in different systems using a number of techniques. The large span of E_mₐₚₐₜ in cytochromes has been subject to analysis by Protein Dipole Langevin Dipole (PDDL) (27, 28), continuum electrostatics (CE) (20, 21, 29, 30), and other techniques (31—37). E_mₐₚₐₜ of the two hemes in quinol:fumarate reductase have been determined within 30 mV of experimental values using MCCE, allowing the low- and high-potential heme to be assigned to the distal and proximal positions in the structure (38). The heme propionic acid pKₐ and their influence on the pH dependence of cytochrome E_mₐₚₐₜ have been considered by CE analysis (20, 38—40). The pKₐs and

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1.36 kcal/mol

58 meV.

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$E_{\text{ms}}$ of aquo–heme $a_3$ and $\text{Cu}_{\text{aq}}$ in cytochrome $c$ oxidase have been analyzed by CE ($41$–$43$) and density functional theory (DFT) ($44$). DFT and QM/MM calculations have been used to explain the unusual low-spin state of ferric aquo–heme ($45$–$47$) and to study the hydroxylation mechanism ($48$–$51$) of cytochrome P450.

In the work presented here, $pK_a$ and $E_{\text{ms}}$ of aquo–heme in myoglobins from sperm whale and *Aplysia*, hemoglobin I, heme oxygenase 1, and horseradish peroxidase are calculated with Multi-Conformation Continuum Electrostatics (MCCE) ($20$, $38$). The calculated $pK_a$ is compared with the pH of the acid–alkaline transition, which is attributed to the deprotonation of a water molecule as the sixth heme ligand ($52$). Calculations are also carried out on cytochrome $c$ oxidase. Here the $pK_a$ is compared with the pH dependence of the proton release on oxidation of heme $a_3$ ($53$). Several of these proteins have positively charged groups in the active site. The interaction of the heme with these charges is compared in CE and DFT methods, showing that CE overestimates the interaction. With the appropriate corrections, MCCE calculations reproduce the experimental $pK_a$ and $E_{\text{ms}}$, showing how the protein structures yield the observed in situ reaction energetics. The differences in the shifts of aquo–heme $pK_a$ and $E_{\text{ms}}$ in different binding sites show that electron and proton transfers can be coupled to different active site conformational changes. The aquo–heme $E_{\text{ms}}$ is not a factor in the function of myoglobin or hemoglobin, nor are the $pK_a$s important for the reaction mechanisms of most of the proteins studied here. However, the aquo–heme $pK_a$ in cytochrome $c$ oxidase appears to determine whether heme $a_3$ binds a proton during the reduction of the binuclear center ($43$). Thus, this $pK_a$ helps to control the electroneutrality of the oxidase reduction. The comparison of calculated and measured $pK_a$ and $E_{\text{ms}}$ in the smaller and simpler proteins provides a benchmark for the analysis of the more puzzling oxidase system.

**METHODS**

Structures of sperm whale myoglobin ($1A6G$, $1A6K$, $1A6M$, $1A6N$, $1HJT$, $1JP6$, and $1JP9$), *Aplysia* myoglobin ($1MBA$ and $5MBA$), monomeric clam hemoglobin I ($1B0B$, $1EBT$, $1FLP$, and $1MOH$), rat heme oxygenase 1 ($1DVE$, $1IVE$, $1IX4$, $1ULX$, and $1VGI$), horseradish peroxidase ($1ATJ$, $1H55$, $1H57$, $1H58$, $1H5A$, $1HSC$, $1HSD$, $1HSE$, $6ATJ$, and $7ATJ$), and *Rhodobacter sphaeroides* cytochrome $c$ oxidase ($1M56$) were obtained from the Protein Data Bank ($54$). All crystal waters are deleted and protein cavities filled with continuum water. Multi-Conformation Continuum Electrostatics (MCCE) samples both residue ionization state and rotamer position as a function of $pH$ and $E_h$ ($55$–$57$). The cytochrome $c$ oxidase calculations use the model generated for analysis of proton uptake coupled to anaerobic reduction in MCCE2.0 ($43$). In this larger protein a rotation angle of $120^\circ$ is used to make side chain rotamers, reducing the number of conformers. All other calculations use MCCE 2.2, which adds local optimization of side chain positions, pruning to delete energetically indistinguishable conformers and corrections of the CE energy terms for the errors in the dielectric boundary due to extra surface conformations (see www.sci.ccny.cuny.edu/~mcce). Preselected side chain rotamers are generated in $60^\circ$ increments around each rotatable bond for all amino acids. In each protein, a water or hydroxyl is added to the open heme coordinate position. The aquo–oxygen is placed on the heme iron with square bipyramidal geometry and a $1.95$ Å Fe–O bond length. Hydrogens are added in a tetrahedral geometry with a $0.96$ Å H–O bond length. Then additional hydroxyl and water conformations are generated in $30^\circ$ increments around the Fe–O bond, creating $12$ conformers for both water and hydroxyl.

Look-up tables are calculated for electrostatic and non-electrostatic conformer self and conformer–conformer pairwise interactions. The electrostatic pairwise interactions and reaction field energies are determined with a finite-difference technique to solve the Poisson–Boltzmann equation using the program DelPhi ($58$–$60$). Focusing ($61$) is used to ensure a final grid spacing of $> 2$ grids/Å. The protein residues are given PARSE charges and radii ($62$). The protein has a dielectric constant, $\epsilon$, of 4 while the surrounding water has an $\epsilon$ of 80 with a salt concentration of 150 mM. Cytochrome $c$ oxidase is surrounded by a 32 Å slab with $\epsilon = 4$ placed with IPECE to bury the fewest ionizable residues to simulate the membrane ($63$). The Lennard-Jones interactions are calculated with AMBER parameters ($64$), rescaled by $0.25$ ($65$). A metal-centered charge set is used for hemes with a $+2$ or $+3$ charge on the ferrous and ferric Fe and $-0.5$ on each N atom of the porphyrin as used previously in cytochrome benchmark calculations ($20$, $21$). The water–heme ligand uses TIP3 charges, and the hydroxyl charge, calculated with B3LYP ($66$) in Gaussian98 ($67$), has $+0.2$ on H and $-1.2$ on O. For the $a$-type heme in cytochrome $c$ oxidase, a $+0.3$ charge is placed on the formyl group C and $-0.3$ on O.

MCCE calculates the shift in $pK_a$ or $E_{\text{ms}}$ when a group is moved from aqueous solution to the protein. The solution $pK_a$ ($pK_{a,\text{sol}}$) and $E_{\text{ms}}$ ($E_{\text{ms,\text{sol}}}$) are obtained from measurements in water. $pK_{a,\text{sol}}$ for amino acids are taken from studies of peptides ($68$, $69$). The heme propionic acids are treated as described previously with a $pK_{a,\text{sol}}$ of 4.9 ($20$). The five-coordinate hemes are assumed to bind a water or hydroxide as the sixth ligand. Heme and its His and water or hydroxide ligands are treated as a complex. A $pK_{a,\text{sol}}$ of 9.6 for the hydroxyl in the ferric ($70$, $71$) and 10.9 in the ferrous ($71$) His–aquo–heme has been measured in microperoxidase 8 ($MP8$) which has a heme c. The water $pK_{a,\text{sol}}$ are assumed to be the same for hemes a and c. The $E_{\text{ms,\text{sol}}}$ for the His–water heme is $-120$ mV, and $-200$ mV ($72$) for the His–hydroxyl heme. The value of $-120$ mV is slightly higher than the measured value of $-140$ mV ($72$, $73$), giving an 80 mV shift from His–hydroxyl, consistent with the $1.3$ $pK_a$ shift between the $pK_a$s of ferric and ferrous aquo–heme ($70$, $71$). With the same ligands, the $E_{\text{ms}}$ of heme a is $100$ (I) to $160$ ($74$, $75$) mV more positive than that of heme c. An $E_{\text{ms,\text{sol}}}$ of $-20$ mV for His–water heme $a_3$ and $-100$ mV for His–hydroxyl heme $a_3$ is used here. No explicit pairwise interactions of water or hydroxide ligands with the His ligand or heme to which they are bound are considered since these interactions are included in $E_{\text{ms,\text{sol}}}$ and $pK_{a,\text{sol}}$. The reaction field energies in both solution and the protein were calculated for the aquo–heme complexes. A systematic shift of $-0.74$ $\Delta pK$ unit ($-1$ kcal/mol) is added to the reference reaction field energy of ferric hydroxyl–heme in the solution ($AG_{\text{RX-N,\text{sol}}}$) and 0.5 $\Delta pK$ unit (0.67 kcal/mol) to that of ferrous water–heme. This raises all ferric heme $pK_a$s by 0.74 pH unit and raises the aquo–heme $E_{\text{ms}}$ by 30 mV. Systematic
shifts are also applied to the ionized form of ionizable amino acids on the basis of pKₐ benchmark studies (43, 56, 65).

Possible microstates with different ligand and side chain position and protonation states are subjected to Monte Carlo sampling. A microstate is made up of one conformer for each residue, cofactor, and water. The energy of microstate n (\( \Delta G^n \)) is the sum of the electrostatic and nonelectrostatic energies (20):

\[
\Delta G^n = \sum_{i=1}^{M} \delta_i \left[ \{2.3m_i \kappa_i T(pH - pK_{sol}) + n_i F(E_n - E_{m,sol})\} + (\Delta \Delta G_{\text{rxn},i} + \Delta G_{\text{pol},i}) \right] + \sum_{i=1}^{M} \sum_{j=i+1}^{M} \delta_i \delta_j |\Delta G_{ij}| \tag{1}
\]

where the summation is over the total M conformers of all residues in the protein; for each conformer i, \( \delta_i = 1 \) if it is present in the current microstate n and 0 if it is not. Each residue has one conformer with \( \delta_i = 1 \) and the rest \( \delta_i = 0 \). In the double summation \( \delta_i \) and \( \delta_j \) are both 1 only when conformer i and j are from two different residues. \( \kappa_i T \) is 0.59 kcal/mol (25.8 meV), and \( m_i \) is 1 for bases, −1 for acids, and 0 for polar groups and waters. \( n_i \) is the number of electrons gained or lost compared to the ground state conformer. For example, if an oxidized conformer is defined as the ground state, it has \( n_i = 0 \) and the reduced conformer has \( n_i = 1 \); F is the Faraday constant. \( pK_{sol} \) is the pKa and \( E_{m,sol} \), the midpoint potential of the ith cofactor in solution. \( \Delta \Delta G_{\text{rxn},i} \) is the difference between the conformer reaction field energy in solution and in the protein (desolvation energy). \( \Delta G_{\text{pol},i} \) is the pairwise electrostatic and nonelectrostatic interaction of the conformer with the backbone and with side chains that have no conformational degrees of freedom. The torsion energy for each conformer is added to \( \Delta G_{\text{pol},i} \). \( \Delta G_{ij} \) is the electrostatic and Lennard-Jones pairwise interaction between each pair of conformers in the microstate. The limits on the summation of the interconformer terms ensure that each interaction is counted once. Monte Carlo sampling establishes the Boltzmann distribution of the different conformers of each residue at 25 °C. Residue pKₐs and \( E_{m,sol} \) are determined from the fraction group ionization in a series of Monte Carlo simulations at different pHs or \( E_{m,sol} \) (eq 1). Multiflip (76) between closely coupled residues is implemented (63). The SOFT function is not used (56). For pKₐ calculations the heme is fixed in its oxidized state, and the probability of residue protonation, including that of the aquo–heme, is determined as a function of pH (56). For \( E_{m} \) calculations the pH is fixed at 7 and the \( E_{m} \) varied. All groups are allowed to sample different protonation states throughout the heme titration.

**Analysis of the MCCE pKₐs and \( E_{m,sol} \).** The \( E_{m} \) or pKₐ changes on moving a group from solution to its position in the protein. Thus

\[
pK_{\text{a}} = pK_{\text{h,sol}} - m \Delta \Delta G_{\text{protein}} \tag{2}
\]

where pKₐ is the group pKₐ in the protein, m is −1 for an acid and +1 for a base, and

\[
E_{m} = E_{m,sol} - \Delta \Delta G_{\text{protein}} / nF \tag{3}
\]

where \( E_{m} \) is the heme in situ \( E_{m} \) and \( n \) is the number of electrons. The shift in energy of ionization (\( \Delta \Delta G_{\text{protein}} \)) is due to different changes of the reaction reactant and product energies when the reaction is moved into the protein. This term can be broken down in MCCE into the sum:

\[
\Delta \Delta G_{\text{protein}} = (\Delta \Delta G_{\text{rxn}} + \Delta G_{\text{pol}}) + \Delta G_{\text{res}} \tag{4}
\]

including the change in reaction field energy from solution (\( \Delta \Delta G_{\text{rxn}} \)), the added interaction with the rigid backbone (\( \Delta G_{\text{pol}} \)), and the side chains in their ionization and conformational states equilibrated at the given pH and \( E_{m} \) (\( \Delta \Delta G_{\text{res}} \)) (20, 43, 77).

MCCE uses continuum electrostatics to calculate the interaction between the aquo–heme and charges and dipoles in the protein. Selected interactions with the ferric heme porphyrin together with its imidazole and water or hydroxyl ligand were also calculated using Gaussian 98 (67). The heme and imidazole are taken from the crystal structure of sperm whale myoglobin (1A6K), with water or hydroxyl added in GaussView (67). Each structure is then optimized using the B3LYP method (66) with the LANL2DZ basis set (78). Hydroxyl–hemes are expected to be low spin (53, 79) while water–hemes are generally high spin (53, 80). The water–heme is given a net charge +1 and spin 1/2, while the hydroxyl–heme has a net charge 0 and spin 1/2. The change in system energy is then calculated with an external charge of +1 or −1 placed next to the aquo–heme complex in positions shown in Figure 1. All atoms are fixed in the structure optimized before the charge is added.

Nine external charges are placed on a plane parallel to the porphyrin plane located 4, 6, 8, 10, or 12 Å from the heme (Figure 1). Points that overlap the water or imidazole ligand are removed. The interactions are also calculated with Coulomb’s law with the metal-centered charges used in the MCCE calculations with \( \epsilon = 1 \). For the ferric hydroxyl–heme, Coulomb’s law reproduces the DFT interactions with an error of <10% and a slope of 0.94 with the exception of points within 4 Å of the heme plane (Figure 2). However, for the water–heme, the Coulomb’s law interactions with the points above the heme plane are significantly larger than those obtained with DFT. In contrast, for charges on the heme
of the interactions of the standard amino acids with external charges.

The ferric water–heme interaction of a simple external charge at the position of Arg 38 in peroxidase or CuB in oxidase calculated with DFT is only 60% of that found with Coulomb’s law. The interaction with a charge at the position of Arg 99 in hemoglobin I is 65% as large. The influence of the charge distribution for the external group on the correction was examined by using a PARSE Arg charge distribution, rather than a single +1 charge. The DFT interaction is now 63% of that found with Coulomb’s law, very similar to that found with a unitary charge model. The influence of the charge distribution of CuB is more complex and cannot be described by a simple scaling factor. The details are discussed in ref 43.

Corrections are applied to interactions of the water–heme with Arg 99 in hemoglobin, Arg 38 in peroxidase, and CuB in oxidase. However, pairwise electrostatic interactions in MCCE are calculated with the Poisson–Boltzmann equation (PB), a CE analysis which takes into account the different dielectric constants of the protein and water (30, 81). The scaling factor derived for particular heme–residue geometry from the comparison of Coulomb’s law and DFT interactions in a vacuum (Figure 2) is used to scale the individual PB interactions in MCCE. Corrections used for CuB can be found in ref 43.

Interactions with residues that are on the heme plane or far away from the heme are shown to need no correction. Residues that have significant interactions with the aquo–heme which are not corrected because of their position include the following: all of the heme propionates; Arg 45 which is hydrogen bonded to a propionic acid in myoglobin; Lys 179 which is hydrogen bonded to a propionate in hemoglobin I; Arg 136 and Asp 140 which form an ion pair on the porphyrin edge furthest from the propionates in hemoglobin I (Figure 4).

FIGURE 2: Comparison of interactions with a positive charge (blue ○) near the heme face, (red □) in the plane of the heme and near the heme edge, and (brown ▲) within 4 Å of the heme plane as shown in Figure 1 calculated with DFT or CE Coulomb’s law with \( \epsilon = 1 \). (A) Neutral, ferric (hydroxyl–heme). The ideal straight line through the origin with a slope of 1 is shown. (B) Cationic, ferric water–heme. The line through the points, \( y = 0.95x - (3.2 \times 10^{-3})x^2 \), is the best fit to a binomial function for the points near the heme face (blue). (C) (Hydroxyl–heme) – (water–heme). The line through the points is \( y = 0.89x + (1.1 \times 10^{-3})x^2 \).

RESULTS AND DISCUSSION

The ferric aquo–heme \( pK_a \)s were calculated with MCCE in myoglobins from sperm whale and \( Aplysia \), hemoglobin I, heme oxygenase 1, horseradish peroxidase, and cytochrome \( c \) oxidase and compared to measured values. These \( pK_a \)s span 3.3 pH units from 7.6 in heme oxygenase 1 to 10.9 in the peroxidase. Two sets of calculations are presented. In the first set, standard Poisson–Boltzmann continuum electrostatics (CE) calculations are used for all pairwise interactions between the aquo–heme and the surrounding protein residues. In the second, an additional correction derived from DFT calculations is applied. Calculated \( pK_a \)s are compared to the experimental values. Calculations of the heme \( E_m \)s are discussed below.

The \( pK_a \)s calculated using the standard CE-based pairwise interactions in MCCE (20) do not provide consistent agreement with the experimental data (Figure 3). Calculated \( pK_a \)s for the myoglobins and heme oxygenase are within 1 pH unit of the measured values. However, errors as large as 6 pH units are found in horseradish peroxidase and cytochrome \( c \) oxidase. In these proteins a nearby positive charge in the heme distal pocket lowers the calculated \( pK_a \)s, while the myoglobins have no charges in similar positions. As described in the Methods section CE and DFT calculations
provide very similar values for pairwise interactions between a charge and a neutral hydroxyl—heme, probably because the electronic polarization does not contribute much to this smaller, favorable interaction. For the cationic water—heme, both methods also yield similar results when the charge is on the heme edge. However, when the charge is above the heme plane near the iron, the interactions derived with a CE analysis are significantly larger than those obtained with DFT calculations. The closer the charge is to the heme plane, the larger the discrepancy (Figure 2). The large unfavorable interactions are relaxed when electronic polarization is considered in the DFT calculations. The DFT corrections are applied to the pairwise electrostatic interaction of the water—heme with Arg 99 in hemoglobin I, Arg 38 in horseradish peroxidase, and CuO in cytochrome c oxidase to recalculate the pKₐ,s. Now calculated pKₐ,s in the six proteins agree with the measured values within 1 pH unit. Analysis of the MCCE calculations can then show how interactions and conformational changes shift the pKₐ,s in the six proteins.

**Sperm Whale Myoglobin.** Sperm whale myoglobin has a folded six-helix core (Figure 4). The pKₐ of the ferric aquo—heme is 8.95 (52), little different from the pKₐ,sol of 9.6. MCCE calculations with seven different crystal structures give an average aquo—heme pKₐ of 9.1 ± 1.0, in good agreement with experiment. This heme is close to the protein surface. However, pKₐ ≈ pKₐ,sol not because the heme does not interact with the protein but rather because a number of effects cancel (Table 1). The water—heme, with a net +1 charge, has 7 ΔpK units (9.52 kcal/mol) less solvation energy in the protein than it does in water, but the hydroxyl—heme, which has a large dipole moment, has 5 ΔpK unit desolvation energy. Thus, the resultant desolvation penalty (ΔΔG_rsa) favors the water—heme complex raising the pKₐ by about 2 pH units. The backbone amide dipoles tend to raise the electrostatic potential within all proteins, shifting residue pKₐ,s (25, 82) and cytochrome Enₐs (20). Here they favor the hydroxyl—heme, lowering the pKₐ by 1 pH unit. The E helix, which contains His 64 in the distal pocket, is the largest contributor.

There are 4 Arg, 14 Glu, 7 Asp, and 19 Lys in the protein and all of them are >95% ionized between pH 7 and pH 9. However, most of these charges have little effect on the aquo—heme pKₐ, because they are solvent exposed. Arg 45, which is hydrogen-bonded to the D-ring propionic acid, does shift the pKₐ down by 0.6 pK unit (propionate A and D designated as assigned in the PDB structure files). The two heme propionic acids are both fully ionized and together shift the pKₐ up by 2.3 units. As these are all near the heme edge, the CE interaction agrees with the DFT values (Figure 2). His 64 in the distal heme pocket near the aquo—ligand undergoes a conformational change with the change in the aquo—heme protonation state (Figure 4). The His is always neutral, but the δ proton tautomer stabilizes the positive water—heme by 3–4 ΔpK units, while the protonated ε tautomer stabilizes the hydroxyl—heme by 1.6–2.5 ΔpK units. In different PDB structures the His tautomer is restricted by the rigid backbone to different extents, yielding the dependence of the calculated pKₐ on the starting structure. Arg 45 not only lowers the aquo—heme pKₐ by direct electrostatic interaction but it also lowers the pKₐ indirectly by favoring the ε His 64 conformer.

**Aplysia Myoglobin.** Myoglobin from the sea hare Aplysia limacine has a folded six-helix structure (Figure 4). It is calculated to have a ferric aquo—heme pKₐ of 7.7, 2 pK units lower than that of sperm whale myoglobin, in agreement...
with the experimental value of 7.6 (52). The Aplysia and sperm whale proteins have the same protein folds and are 27% identical. The analogue of myoglobin His 64 is Val 64 in the Aplysia distal pocket, providing a bigger cavity, so the heme is slightly better solvated, favoring the oxidized heme (52). However, the biggest difference between the two proteins is in interactions with other residues are quite long range. However, the biggest difference between the two proteins is in the heme distal pocket, providing a bigger cavity, so the heme is deeply buried in the transmembrane 12-helix subunit I of Heme Oxygenase I (4) and Arg 99, on the proximal side of the heme, shifts the pKₐ down by 1.5 pH units, while the two propionic acids, both of which are ionized, shift the pKₐ up by 2.2 pH units.

Heme Oxygenase 1. Rat heme oxygenase 1 is a multihelical bundle with two three-helix motifs (4). The heme–heme pKₐ is calculated to be 9.8 and measured to be 9.6 (83), close to that of sperm whale myoglobin. The heme I and myoglobin have similar structures with 23% identity. A larger cavity in the heme distal pocket leads to a smaller desolvation energy. As His 64 does in myoglobin, Gln 64 changes the pKₐ of the amino acid near the metal center and CuB reduced, the ferric aquo–heme pKₐ has been measured to be 9 (53). Using the full CE interaction between CuB and the aquo–heme, the calculated pKₐ is 3. The DFT correction reduces the interaction from 6.4 to 3.2 ΔpK units. Since the aquo–heme titration now occurs at higher pH, the protein net charge is more negative than when the titration is at pH 3. This raises the pKₐ further to 8.6, in good agreement with experiment (43). The cytochrome c oxidase heme is more deeply buried than in the other proteins, losing more reaction field energy, which shifts the pKₐ down. Within the membrane-embedded protein, pairwise electrostatic interactions with other residues are quite long range. However, the net interaction of the fully ionized propionic acids and the rest of the protein shifts the pKₐ up by only 2 pH units.

HORSE RADISH Peroxidase. The multihelical peroxidase uses heme to reduce hydrogen peroxide to water. The aquo–heme pKₐ is calculated to be 10.1, in reasonable agreement with the measured 10.9 (52). The aquo–heme has a similar reaction field energy loss as the globins. Unlike the globins, interactions with the backbone amides near the metal center are small and somewhat negative, raising the pKₐ by 0.6 pH unit. Interactions with other residues raise the pKₐ by 1.5 ΔpK units. His 42 and Asp 43 raise the pKₐ by 6 pH units while Arg 38 shifts the pKₐ down by 4.5 pH units (assuming a 60% DFT correction). His 42 sits in the distal pocket in a position similar to His 42 in myoglobin. However, this His is fixed in the δ tautomer. The multihelical peroxidase uses heme to reduce hydrogen peroxide to water. The aquo–heme pKₐ is calculated to be 10.1, in reasonable agreement with the measured 10.9 (52). The aquo–heme has a similar reaction field energy loss as the globins. Unlike the globins, interactions with the backbone amides near the metal center are small and somewhat negative, raising the pKₐ by 0.6 pH unit. Interactions with other residues raise the pKₐ by 1.5 ΔpK units. His 42 and Asp 43 raise the pKₐ by 6 pH units while Arg 38 shifts the pKₐ down by 4.5 pH units (assuming a 60% DFT correction). His 42 sits in the distal pocket in a position similar to His 42 in myoglobin. However, this His is fixed in the δ tautomer. The multihelical peroxidase uses heme to reduce hydrogen peroxide to water. The aquo–heme pKₐ is calculated to be 10.1, in reasonable agreement with the measured 10.9 (52). The aquo–heme has a similar reaction field energy loss as the globins. Unlike the globins, interactions with the backbone amides near the metal center are small and somewhat negative, raising the pKₐ by 0.6 pH unit. Interactions with other residues raise the pKₐ by 1.5 ΔpK units. His 42 and Asp 43 raise the pKₐ by 6 pH units while Arg 38 shifts the pKₐ down by 4.5 pH units (assuming a 60% DFT correction). His 42 sits in the distal pocket in a position similar to His 42 in myoglobin. However, this His is fixed in the δ tautomer.
MCCE allows protonation of the aquo—heme to be coupled to heme reduction (20). The relevant $E_m$ for water—heme of $-120$ mV (72, 73) and for hydroxyl—heme of $-200$ mV (72) is included in the microstate energy (eq 1) so the pH dependence of the $E_m$ is properly treated in the redox titration. This degree of freedom could be important for Aplysia myoglobin and heme oxygenase, which have $pK_a$s near 7. However, since their ferric $pK_a$s are above 7 and ferrous $pK_a$s are higher, the aquo—heme ligand remains predominately water at pH 7.

Electrostatic interactions that favor the ferric heme with its net charge of $+1$ over the neutral ferrous heme shift the $E_m$ down. The same electrostatic interactions favor the cationic ferric water—heme over the neutral hydroxyl—heme, raising the $pK_a$. While a higher $pK_a$ is correlated with a lower $E_m$, the connection is by no means exact (Figure 5). In most proteins, the $\Delta G_{\text{protein}}$ for the redox reaction is larger than for the protonation reaction so the $E_m$ is shifted from $E_m$ by more than the $pK_a$ is shifted from $pK_a$ (Figure 5, eqs 2 and 3). One difference is the desolvation energy is on average 2.4 $\pm$ 0.3 $\Delta pK$ units larger for the redox reaction than for the protonation reaction (Table 1). Because of its significant dipole the solvated neutral ferric hydroxyl—heme has a solvation energy that is not much smaller than the positively charged ferric water—heme. Thus, both reactant and product of the protonation reaction have similar $\Delta G_{\text{rel}}$. With the exception of cytochrome $c$ oxidase, the desolvation penalty, which always favors the neutral species, shifts the $pK_a$ down by at most 2 pH units. In contrast, when compared to the neutral, ferrous water—heme, the cationic, ferric water—heme has much larger interactions with water. The impact of the desolvation energy on the redox reaction when the heme is buried in the protein is thus much larger.

Hemoglobin I has a $pK_a$ which is only 0.2 pH unit higher than the $pK_{a,\text{sol}}$, while the $E_m$ is 223 mV more positive than $E_m$ (3.8 $\Delta pK$ units). The difference in desolvation energy for the two reactions contributes 3 $\Delta pK$ units. In addition, Gln 64 in the distal pocket changes conformation in the aquo—heme $pK_a$ titration but does not in the $E_m$ titration. The rigid Gln 64 raises the water—heme $E_m$ by 1 $\Delta pK$ unit (58 meV) more than it shifts the ferric aquo—heme $pK_a$ down. The reorientation of the Gln is part of the protein dielectric response that is captured by the explicit motions available in MCCE (30, 55). The Gln motion coupled to aquo—heme protonation thus diminishes the effective pairwise interaction.

In horseradish peroxidase, $\Delta G_{\text{protein}}$ shifts the $E_m$ down more than it shifts the $pK_a$ up. Besides the 2.2 $\Delta pK$ unit difference in the desolvation energy, $\Delta G_{\text{res}}$ favors heme reduction 3.3 $\Delta pK$ units more than water deprotonation. Although both ferrous water—heme and ferric hydroxyl—heme have a net charge of 0, they interact differently with nearby residues. The hydroxyl—heme has a large dipole with its negatively charged hydroxide pointing toward the distal pocket. Arg 38 in the distal pocket and Asp 247 on the opposite side form a dipole stabilizing the hydroxyl—heme. The difference in interactions with hydroxyl—heme and water—heme is relatively small, leading to a small $\Delta G_{\text{res}}$, contributing to the $pK_a$ shift. On the other hand, the neutral ferrous water—heme is not stabilized by the electrostatic dipole generated by these two amino acids while interactions of the ferric water—heme with nearby negative charges shift the $E_m$ down.

As in horseradish peroxidase, the favorable interaction of Cu$_b$ with the hydroxyl—heme in cytochrome $c$ oxidase makes the contribution of $\Delta G_{\text{res}}$ to the in situ $pK_a$ smaller than found for in situ $E_m$. Assuming the $E_{m,\text{sol}}$ of heme $a$ is 100 (1) to 160 (74, 75) mV more positive than that of heme $c$, the $E_m$ of heme $a$ is predicted to be 9 to 69 mV in cytochrome $c$ oxidase when Cu$_A$ is oxidized and heme $A$ and Cu$_B$ are reduced.

There are no experimental data available for the $E_m$ in heme oxygenase 1. These calculations predict an $E_m$ of 40 mV. A run of four backbone dipoles lowers the $pK_a$ more than the $E_m$. All of the other proteins studied here have similar values of $\Delta G_{\text{pol}}$ for the redox and protonation reactions.

CONCLUSIONS

The $pK_a$s of ferric aquo—heme at pH 7 in sperm whale myoglobin, Aplysia myoglobin, hemoglobin I, heme oxygenase 1, horseradish peroxidase, and cytochrome $c$ oxidase were calculated with MCCE. The calculations reveal a breakdown in the classic continuum electrostatic analysis of pairwise interactions. Large errors in calculated $pK_a$s were found in horseradish peroxidase and cytochrome $c$ oxidase. DFT calculations suggest that CE calculations overestimate pairwise interactions between ferric water—heme and a positive charge above the heme plane. This could be due to significant out-of-plane charge density, which would be ignored in the atom-centered charges used in the CE analysis. Alternatively, these external charges could polarize ferric hydroxyl—heme significantly more than the amount accounted for by the $\epsilon = 4$ used in the MCCE calculation. Using a correction that brings the DFT and CE interactions into agreement, the calculated $pK_a$s in six proteins are within 1 pH unit of the experimental values. The calculated $E_m$s using the correction derived from DFT calculations are within 35 mV of the experimental values.

Proteins with higher ferric aquo—heme $pK_a$s have lower $E_m$s since both arise from the protein stabilizing a positively
charged heme. However, the proteins shift the free energy of the redox reaction ($\Delta G_{protein}$) by an amount that would shift a pK$_a$ by >6 pH units (375 mV), while the pK$_a$s span only 3.3 pH units (eqs 2 and 3). The difference in $\Delta G_{protein}$ shows that there is a larger effective dielectric response for the protonation than the redox reactions. In sperm whale myoglobin and hemoglobin I protonation is coupled to conformational changes while the redox reaction is not. These conformation changes allow a residue in the distal pocket to stabilize both protonation states, reducing the energy difference between them. In contrast, when the group is rigid as it is for the redox reaction, the pairwise interactions are larger, shifting the $E_a$ more. This difference in flexibility for the two reaction types found in MCCE is supported by the good match between calculation and experiment for both pK$_a$s and $E_a$s.

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SUPPORTING INFORMATION AVAILABLE

Interactions between heme $a_1$ and Cu$_b$ in different aqua protonation and cofactor redox states calculated in a vacuum by Coulomb’s law and by DFT using Gaussian 98 and in cytochrome c oxidase embedded in a low dielectric slab surrounded by water using DelPhi. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES


