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pH Dependence of Heme Electrochemistry in Cytochromes Investigated by Multiconformation Continuum Electrostatic Calculations

Abstract: *Cytochromes belong to a diverse family of heme-containing redox proteins that function as intermediaries in electron transfer chains. They can be soluble, extrinsic, or intrinsic membrane proteins, and are found in different structural motifs (globin, 4-helix bundles, αβ roll, β sandwich). Measured electrochemical midpoint potentials vary over a wide range even though the basic redox reaction at the heme is the same for all cytochromes. The perturbation of the heme electrochemistry is induced by the protein structure. Also, the pH dependence varies since it depends on the strength of interaction between the heme and surrounding residues as well as the ionization states of these groups. Multiconformation continuum electrostatics (MCCE) has been used to investigate the pH dependence of heme electrochemistry in cytochromes with different folds. Often propionates are the primary contributors for pH dependence especially if they are partially protonated in the reduced heme as it is shown for globin cytochrome c551* P. aeruginosa *and cytochrome b5* R. norvegicus *(αβ roll). However, if the propionates are already fully ionized at a certain pH they do not contribute to the pH dependence even if they have big interaction with the heme. At pH 7 there is no propionate contribution for cytochrome f* C. reinhardtii *(β sandwich) and the 4-helix bundle c′* R. palustris. *Other residues can also change their ionization significantly during heme oxidation and therefore be involved in proton release and pH dependence. These residues have been identified for different cytochrome types.*

Keywords: *cytochrome; heme electrochemistry; pH dependence; electrostatic calculations*

INTRODUCTION

Cytochromes are redox proteins that transport electrons. Their redox active site is a heme group containing a porphyrin ring with two propionates as ring substituents and a central iron atom axially coordinated to the ligands. C-type hemes are bound via thioether linkages to two cysteines of the protein lacking in other heme types. During the electron transport the heme iron changes its valence from Fe(II) to Fe(III). Although the heme redox reaction is basically the same for all cytochrome types there is a big variation of the electrochemical midpoint potentials [$\pm$400 mV vs standard hydrogen electrode (SHE)][1] due to different stabilization of the oxidized heme by the protein structure. Many factors like the axial ligands, the heme solvent exposure, surface and buried charged groups, the protein backbone and side-chain dipoles, changes in protein conformation, and residue protonation have been considered to be important.

In a previous analysis, multiconformation continuum electrostatics (MCCE) was used to study how the heme redox potentials were modulated by different structural motifs (globin, 4-helix bundles, $\alpha\beta$, β sandwich).[2] Using the same parameter set for all cytochromes and Protein Data Bank (PDB) data from crystal as well as NMR structures in different oxidation states and at different pH or ionic strength conditions, MCCE calculations of midpoint potentials (E_m) have been in good agreement with experimental data. Factors controlling the midpoint potential have been calculated previously[2] and it was shown that the loss of reaction field energy raises whereas heme propionates lower the E_m. The contribution of the protein backbone as well as of buried polar and charged side chains was also evaluated for different cytochrome types.

An interesting question to ask is whether the electron transfer reaction is coupled by proton transfer that can be addressed by the pH dependence of the redox reaction. The pH dependence arises not from the heme oxidation itself since the heme is not protonatable. The redox potential becomes pH dependent when acid and base pK_as of surrounding residues are shifted by the heme oxidation.[3] MCCE is an appropriate tool to investigate the pH dependence of the redox potential as it has been shown for one type of cytochrome, the four-helix bundle b562, where comparable experimental results have been available.[2]

In this study, representatives of cytochromes with different fold (globin, four-helix bundle, $\alpha\beta$, β sandwich), different ligand, and different midpoint potentials have been chosen to conclude general principles of the pH dependence of cytochrome heme electrochemistry. Focus was set to monitor significant ionization changes with pH of propionates and other residues. Knowledge of these residues may allow a specific design of mutants with altered electrochemical properties.

METHODS

MCCE is a hybrid method combining continuum electrostatics with molecular mechanics.[4–6] Traditional continuum electrostatic calculations consider different ionization states of residues. Conformational changes of the protein and solvent responses to charge changes are included implicitly in the dielectric constant.[7]

MCCE adds explicit conformers for polar and ionizable side chains, the cofactor, and buried water molecules. The side-chain rotamer sampling provides a position-dependent response to changes in charge that allows MCCE to employ a low protein dielectric constant of 4. The value ε=4 accounts for the electrostatic response of the protein medium that MCCE does not treat explicitly such as bond stretching, bond twist, and charge-induced polarization. The value ϵ=80 is used for the solvent. Each propionic acid is treated as an independent ionizable residue. The protein backbone and nonpolar side chains are fixed.

The MCCE method starts from a protein coordinate file. Here, calculations have been performed with 4 cytochromes, the globin c551 *P. aeruginosa* (PDB entry 451c), the 4-helix bundle c′ *R. palustris* (PDB entry 1a7v, chain a), the $\alpha\beta$ roll b5 *R. norvegicus* (PDB entry 1b5a), and the β sandwich f *C. reinhardtii* (PDB entry 1cfm, chain b) (Table I). Only buried water molecules are treated in atomic detail

Table I Investigated Cytochromes Varying in Folds, Ligands, and Midpoint Potentials

Cytochrome	Source	Fold	Ligand	Exp./calc. F_m (mV)[2]	PDB Entry
c551	*P. aeruginosa*	Globin	His–Met	270/234	451c
c′	*R. palustris*	4-Helix bundle	His	94/98	1a7v:a
b5	*R. norvegicus*	$\alpha\beta$ Roll	bis–His	−102/−10	1b5a
f	*C. reinhardtii*	β Sandwich	His–Ntr	350/353	1cfm:b

whereas those with more than 10% solvent exposure are deleted. Hydrogens are added after side-chain rotamers have been created.

MCCE calculates the free energy of a conformer considering its reaction field energy, torsion energy, pairwise electrostatic, and Lennard–Jones interactions with fixed groups of the protein and with other conformers. DelPhi using the finite difference Poisson–Boltzmann procedure[9] is part of the MCCE method and calculates all electrostatic terms. PARSE atomic charges and radii are used.[10] When groups are close together interaction energies are very sensitive to small changes in position and can become unrealistically strong. Other factors than electrostatics may take effect in these cases. The unrealistic strong pairwise electrostatic interactions are reduced with the empirical function SOFT.[11] The nonelectrostatic torsion and Lennard–Jones parameters have been previously reported.[11] Heme parameters can be found in Ref. 2. Protein microstates are defined by using one conformer for each residue, cofactor, and buried water molecule. Reference values for the chemistry of the group in solution (pK_a and E_m) and solvent conditions are accounted in the free energy calculation of a protein microstate. For the propionates, the pK_a in solution of 4.9[8] has been chosen as reference pK_a. Monte Carlo sampling yields the conformer occupancies in a Boltzmann distribution of states as a function of pH. Conformation and ionization degrees of freedom come to an equilibrium. A detailed description of the method can be found in Ref. 11.

RESULTS AND DISCUSSION

Cytochromes representing four different folds [globin (c551 *P. aeruginosa*), 4-helix bundle (c′ *R. palustris*), $\alpha\beta$ roll (b5 *R. norvegicus*), and β sandwich (f *C. reinhardtii*)], different ligand types (His–Met, His, bis-His, His–Ntr), and midpoint potentials varying between −100 and 350 mV (Table I) have been used to investigate the pH dependence of cytochrome heme electrochemistry by MCCE calculations.

The residue contribution to the pH dependence is a function of the strength of its interaction with the heme and its ionization state in the reduced protein. If residues show significant pH-dependent ionization change during the heme oxidation, they take part in proton transfer. To identify these residues their conformer occupancies have been calculated at various pHs with heme oxidized and reduced respectively. Figure 1 shows the proton release at oxidation in a pH range from 3 to 10. Only residues with ionization change more than 5% are shown.

In general, propionates are good candidates for pH dependence since usually they show a large interaction with the heme. Especially if the propionates are not fully ionized in the reduced protein, the heme oxidation is coupled to some proton loss. At pH 7

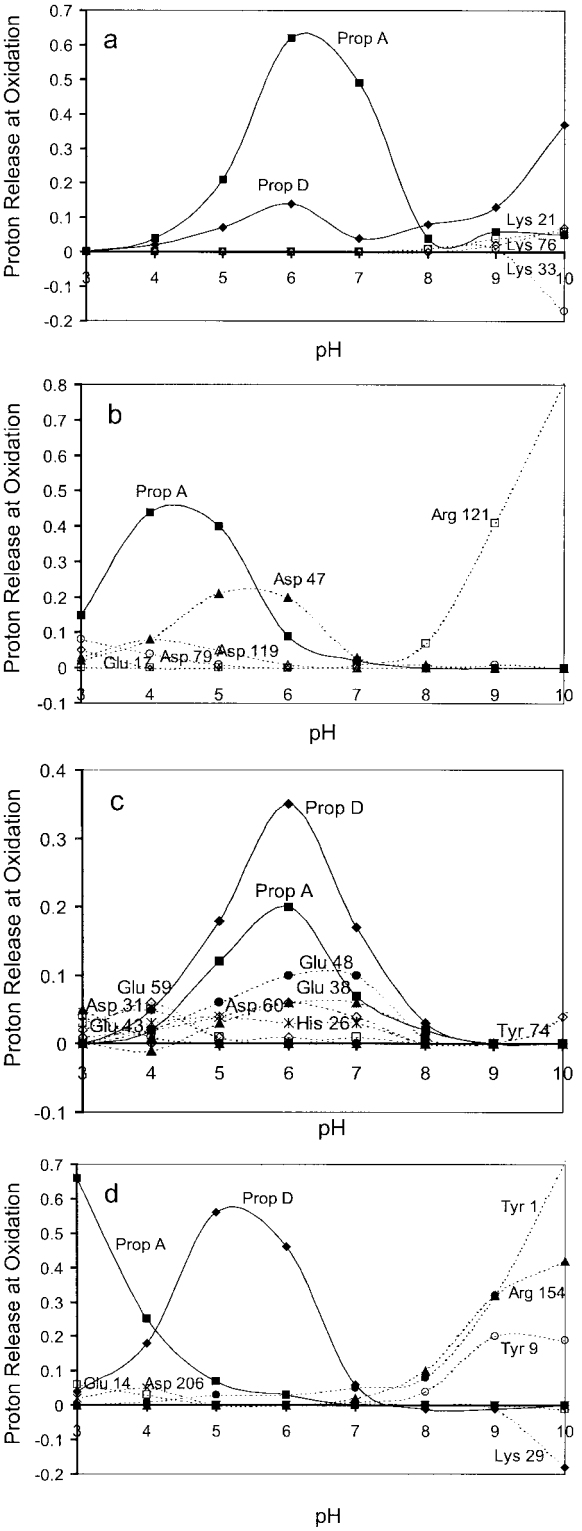

FIGURE 1 Residue contribution to pH dependence of heme electrochemistry in cytochromes. (a) c551 *P. aeruginosa* (451c.pdb); (b) c′ *R. palustris* (1a7v.pdb, chain a); (c) b5 *R. norvegicus* (1b5a.pdb); (d) f *C. reinhardtii* (1cfm.pdb, chain b). Only residues that change ionization by more than 5% are shown.

propionates are contributing to the pH dependence in the globin c551 *P. aeruginosa* (Figure 1a) and the $\alpha\beta$ roll b5 *R. norvegicus* (Figure 1c) but not in the 4-helix bundle c′ *R. palustris* (Fig. 1b) and β sandwich cytochrome f *C. reinhardtii* (Figure 1d). This agrees with previous calculations,[2] which showed that at pH 7 the propionates of c551 and b5 are partially ionized (50 and 81% in the reduced protein respectively). When the propionates are already fully ionized in the reduced protein as in cytochromes c′ and f (98 and 100% respectively), they cannot contribute to the pH dependence although they have a large interaction with the heme.

Other residues also contribute to E_m-pH dependence. Thus, in b5 of *R. norvegicus* Glu 38 and Glu 48 become more ionized on heme oxidation. Sometimes residues respond to ionization changes of other residues—for example, Lys 33 in c551 responds to propionate ionization and Lys 29 in f responds to ionization of tyrosines. It is not possible to generalize what residues will be involved since this depends on the respective primary sequence. For example, b562 *E. coli* and c′ *R. palustris* are both 4-helix bundle. At pH 7, propionates His 63 and Glu 4 contribute to the pH dependence in b562[2] while no amino acids contribute significantly in c′ (Figure 1b).

These calculations show that propionates are the primary contributors to pH dependence. The strong pairwise interaction between the two propionates in many cytochromes makes their full ionization difficult and this leads to pH dependence. If residues other than propionates are involved in pH dependence, their ionization changes are often linked to the change in propionic acid ionization rather than to the heme oxidation. When ionizable residues are found in clusters with interdependent charge states, small differences in the structure can lead to significant difference in the assignment of ionization states.[7] Rotamer sampling in MCCE reduces but does not eliminate the dependence on the initial protein structure. However, this study shows that MCCE is an appropriate tool to indicate individually for any cytochrome with an available coordinate file which residues are involved in pH dependence of heme electrochemistry.

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