Direct Measurement of Charge Regulation in Metalloprotein Electron Transfer

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Abstract: *Determining whether a protein regulates its net electrostatic charge during electron transfer (ET) will deepen our mechanistic understanding of how polypeptides tune rates and free energies of ET (e.g., by affecting reorganization energy, and/or redox potential). Charge regulation during ET has never been measured for proteins because few tools exist to measure the net charge of a folded protein in solution at different oxidation states. Herein, we used a niche analytical tool (protein charge ladders analyzed with capillary electrophoresis) to determine that the net charges of myoglobin, cytochrome c, and azurin change by 0.62 ± 0.06, 1.19 ± 0.02, and 0.51 ± 0.04 units upon single ET. Computational analysis predicts that these fluctuations in charge arise from changes in the pK_a values of multiple non-coordinating residues (predominantly histidine) that involve between 0.42–0.90 eV. These results suggest that ionizable residues can tune the reactivity of redox centers by regulating the net charge of the entire protein–cofactor–solvent complex.*

$\boldsymbol{T}$he net electrostatic charge (Z) of a protein, that is, the sum of electrostatic contributions from side chains, tightly bound solvent, co-solvent, buffer ions, and organic or metal cofactors, affects the rate and/or free energy of several chemical processes including molecular recognition,[1–2] catalysis,[3] and possibly electron transfer (ET).[4–6] The long range over which electrostatic forces act in the interior of a protein (and to a lesser extent, in the exterior) provides a qualitative rationale for these effects.[1] A rigorous quantitative understanding remains elusive because the net charge of a folded protein in solution has been measured for just a few proteins.[7] This void is analytical in nature. Biochemistry is flush with tools to measure the mass, catalysis, and structure of proteins, but there are few to measure the net charge of a folded protein in solution at $pH \neq pI$.[1]

The absence of direct measurements of the net charge of proteins at different metal oxidation states has left a few fundamental questions unanswered in bioinorganic chemistry. For example, will the net charge of metalloproteins fluctuate by approximately one unit when cycling between M^{n+} and $M^{(n+1)+}$, or do metalloproteins tightly regulate net charge during ET, that is, reorganize H^+ or OH^- to adjust to the new electrostatic environment? Charge regulation refers to the adjustment in the pK_a value of ionizable functional groups in response to changes in the electrostatic environment of that group. Will the magnitude of charge regulation vary greatly from protein to protein? Could this negative feedback of charge account for a significant fraction of the protein's redox potential ($E°$)?[8] The exact mechanisms by which non-coordinating amino acid residues affect the $E°$ value of metal centers remain poorly understood.[9] Or does the regulation of charge occur with ET—at least for some amino acid residues—and contribute to its reorganization energy (λ_T) according to Marcus theory?[10–12] The kinetic parameters of ET in proteins, that is, the inner-sphere and outer-sphere reorganization energies (λ_i and λ_o), are difficult to measure directly and are inferred from the quadratic relationship (predicted by Marcus theory) between experimentally measured rates and $\Delta G°$ of ET in proteins or through resonance Ramen absorption spectroscopy (for λ_i).[10, 11, 13]

In this study, we used "protein charge ladders"[1] and capillary electrophoresis (CE) to measure the ΔZ values of metalloproteins during single ET. We studied three model systems: 1) holo-myoglobin (Mb) upon ET from sodium dithionite to Fe^{3+} heme$_b$; 2) holo-cytochrome c (Cyt c) upon ET from Fe^{2+} heme$_c$ to potassium ferricyanide; and 3) holo-azurin (Az) upon ET from sodium dithionite to Cu^{2+}.

Protein charge ladders consist of electrostatic isomers of a protein prepared by acetylation of its surface Lys-ε-NH$_3^+$ groups; they possess similar sizes and structures, but systematically altered net charges (Figure 1a).[1] The electrophoretic mobility of each "rung" can be used to determine the net charge of the non-acetylated protein, or "zeroth" rung (Figure 2). A protein charge ladder, when analyzed with CE, is the only self-calibrating, internally consistent tool for rapidly measuring the net charge of a folded protein in solution.[14] The timescale of analysis is typically 2–15 min.

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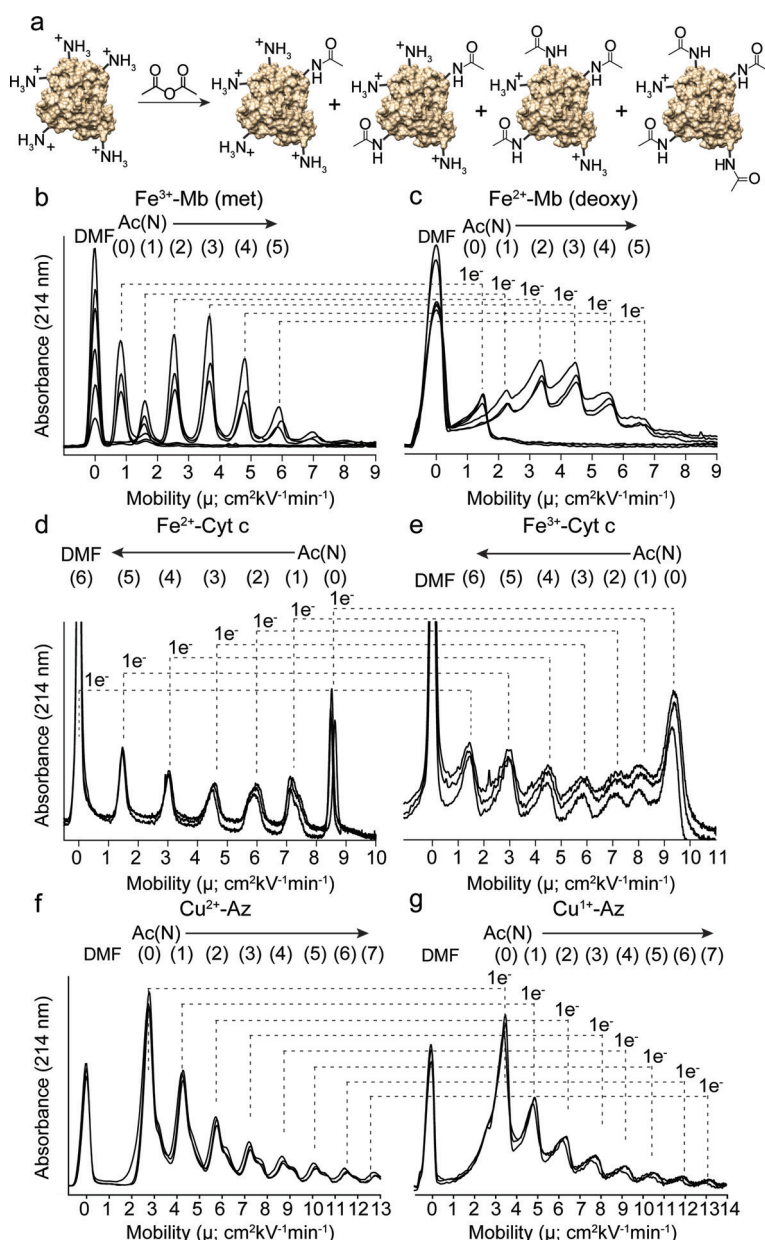1301 S University Parks Dr., Waco, TX, 76706 (USA)

Figure 1. a) Acetylation of lysine to generate protein charge ladders. b–g) Replicate capillary electropherograms of protein charge ladders of oxidized and reduced Mb (b, c), Cyt c (d, e), and Az (f, g). Dimethylformamide (DMF) was added as a neutral marker of electroosmotic flow. Numbers above each peak or "rung" in the electropherogram indicate the number of acetylated lysine residues, Ac(N). Only three replicate electropherograms are shown (for all others, see Figure S4).

Protein charge ladders of Fe^{3+}-Mb (or "met-Mb"), Fe^{2+}-Cyt c, and Cu^{2+}-Az were generated by acetylation of Lys-ε-NH_3^+ residues with acetic anhydride (Figure 1 a), resulting in a distribution of between zero and eight Lys-ε-$NHCOCH_3$ residues (see the Supporting Information, Figure S1). The acetylated residues were shown by tandem mass spectrometry to be a mixture of all Lys-ε-NH_3^+ and N-terminal α-NH_3^+ (Figure S2).[15]

To generate charge ladders of reduced Mb and Az (Fe^{2+}-Mb/"deoxy-Mb" and Cu^{1+}-Az), a charge ladder of each oxidized protein was reduced with 100 molar equivalents of sodium dithionite ($Na_2S_2O_4$).[16] The large molar excess of

$Na_2S_2O_4$ ensures complete reduction, as well as the removal of any Fe^{2+}-Mb species with coordinated oxygen.[17] In the case of Cyt c, transfer of Fe^{3+}-Cyt c to tris-glycine buffer (i.e., CE running buffer for Cyt c; see the Supporting Information) leads to the photoreduction of Fe^{3+}-Cyt c to Fe^{2+}-Cyt c.[18] Thus we generated charge ladders of Fe^{3+}-Cyt c by oxidizing charge ladders of Fe^{2+}-Cyt c using five molar equivalents of potassium ferricyanide (K_3-$[Fe(CN)_6]$).[19] The oxidation state and purity of each protein were confirmed before and after CE by UV/Vis spectrophotometry (Figure S3) based on the characteristic spectra for each species (see the Supporting Information).

We first measured the net charge of Fe^{3+}-Mb and Fe^{2+}-Mb at pH 7.4 (Table 1). To properly identify the zeroth rung, replicate CE electropherograms of Fe^{3+}-Mb charge ladders were overlaid with electropherograms of non-acetylated Fe^{3+}-Mb (Figures 1 b and S4 a); similar overlays are shown for Fe^{2+}-Mb (Figures 1 c and S4 b; see the Supporting Information and Figure S5 for a detailed discussion of peak broadening in electropherograms). Linear plots of the electrophoretic mobility (μ) of each rung versus the number of acetylated lysine residues in that rung (Ac(N)) were extrapolated to the x intercept to determine the net charge of unmodified Fe^{3+}-Mb and Fe^{2+}-Mb (Figure 2a). The x intercept of this plot is equal to the quotient of the net charge of the zeroth rung ($Z_{Ac(0)}$) and the change in charge associated with each acetylation (ΔZ_{Ac}).[7] The net charge of non-acetylated Fe^{3+}-Mb was determined from the protein charge ladders to be $Z_{Fe(III)\text{-}Mb} = -0.59 \pm 0.03$ (Figure 2a and Table 1). The measured net charge of Fe^{2+}-Mb is $Z_{Fe(II)\text{-}Mb} = -1.21 \pm 0.05$ (Figure 2a and Table 1). Thus the measured difference in the net charge of Mb upon reduction of Fe^{3+} to Fe^{2+} is $\Delta Z_{Fe(III)\to(II)}^{Mb} = -0.62 \pm 0.06$ units, instead of the formal value of -1.00 units. The Mb polypeptide therefore regulates its net charge upon the reduction of its iron redox center by $38 \pm 6\%$. To further confirm that the oxidation state did not change as a result of electrophoretic separation of the reducing agent from the protein, we measured the net charge of the most rapidly reoxidizing protein, Fe^{2+}-Mb, with 1 mM dithiothreitol in the capillary running buffer. The net charge did not change significantly compared to Fe^{2+}-Mb without dithiothreitol ($Z_{Fe(II)\text{-}Mb} = -1.23 \pm 0.03$; $n = 3$), indicating that the residual dithionite concentration present in each injected sample was adequate to prevent the reoxidation of Mb during the brief period of electrophoretic separation.

The measured net charge of Fe^{2+}-Cyt c (Figures 1 d, e and S4 c, d) is $Z_{Fe(II)\text{-}Cyt c} = +5.53 \pm 0.01$, and that of Fe^{3+}-Cyt c is $Z_{Fe(III)\text{-}Cyt c} = +6.72 \pm 0.02$ (Figure 2 b and Table 1). The difference in net charge upon single ET of Cyt c is $\Delta Z_{Fe(III)\to(II)}^{Cyt c} = -1.19 \pm 0.02$ units. Therefore, Cyt c does not undergo classical charge regulation per se, but undergoes
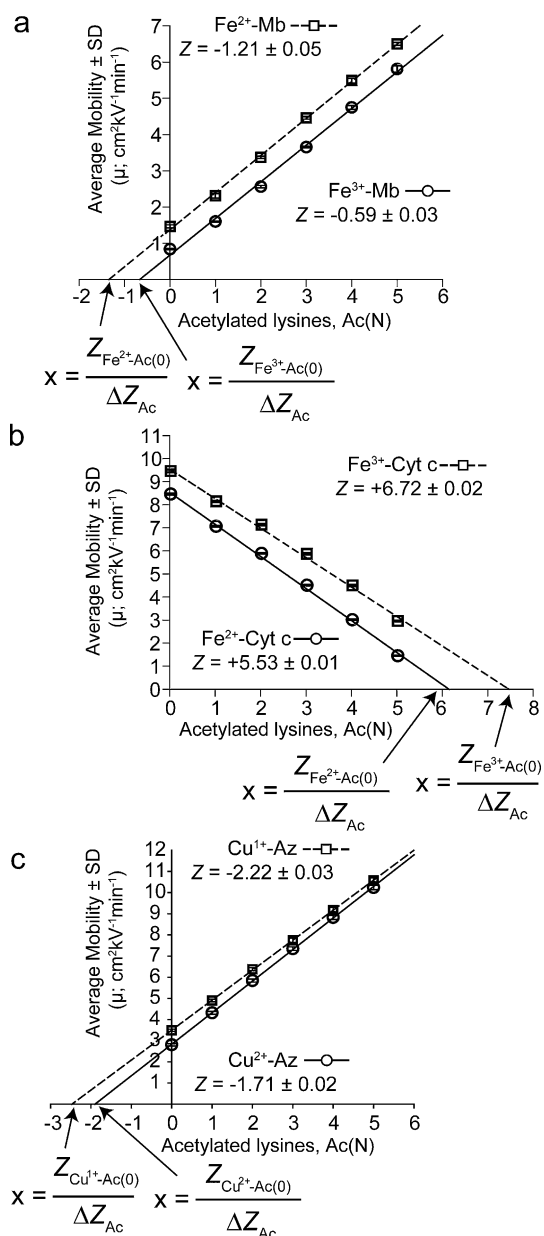
Figure 2. Plots of the average mobility versus Ac(N) for a) Fe^{3+}-Mb ($n=9$) and Fe^{2+}-Mb ($n=9$), b) Fe^{3+}-Cyt c ($n=10$) and Fe^{2+}-Cyt c ($n=10$), and c) Cu^{2+}-Az ($n=10$) and Cu^{1+}-Az ($n=10$).

Table 1: Experimentally and theoretically determined values of the net charge for Fe^{3+}-Mb, Fe^{2+}-Mb, Cu^{2+}-Az, and Cu^{1+}-Az at pH 7.4 and for Fe^{3+}-Cyt c and Fe^{2+}-Cyt c at pH 8.3.

Protein	Z_{CE}	$Z_{Predicted}$[a]
Fe^{3+}-Mb (H_2O/OH^-)	-0.59 ± 0.03	$+4.34$
Fe^{2+}-Mb	-1.21 ± 0.05	$+2.81$
Fe^{3+}-Cyt c	$+6.72 \pm 0.02$	$+10.78$
Fe^{2+}-Cyt c	$+5.53 \pm 0.01$	$+9.95$
Cu^{2+}-Az	-1.71 ± 0.02	-1.34
Cu^{1+}-Az	-2.22 ± 0.03	-2.02

[a] Values of $Z_{Predicted}$ were determined by numerical solutions to the Poisson–Boltzmann equation using finite-difference methods, as described in the Supporting Information.

a larger than expected change in net charge upon ET. Rung (6) in the electropherogram of Fe^{2+}-Cyt c co-migrated with the DMF peak ($Z_{Ac(6)} \approx 0$), and was not included in the calculation of Cyt c net charge. As expected, the relative mobility of the rungs of Cyt c is reversed compared to Mb and Az because Cyt c is a net positively charged protein (unlike Mb and Az). Cytochrome c was therefore separated on a positively charged capillary, reversing the electroosmotic flow (see the Supporting Information). The measured net charge of Az (Figures 1 f,g and S4 e,f) is $Z_{Cu(II)-Az} = -1.71 \pm 0.02$ and $Z_{Cu(I)-Az} = -2.22 \pm 0.03$ (Figure 2c and Table 1). The net charge of Az therefore changes by $\Delta Z_{Cu(II) \rightarrow (I)}^{Az} = -0.51 \pm 0.04$ upon single ET (49 ± 4 % regulation of net charge).

To identify the residues or cofactors that are most likely responsible for charge regulation, that is, those that undergo the largest changes in charge, we calculated the pK_a values of all ionizable residues and cofactors of each protein in both redox states from their corresponding X-ray crystal structures. These calculations utilized numerical solutions of the Poisson–Boltzmann equation based on finite-difference methods.[20] This analysis predicted $\Delta Z_{Fe(III) \rightarrow (II)}^{Predicted} = -1.53$ units for Mb, $\Delta Z_{Fe(III) \rightarrow (II)}^{Predicted} = -0.83$ units for Cyt c, and $\Delta Z_{Cu(III) \rightarrow (I)}^{Predicted} = -0.68$ for Az. These values correspond well with the measured ΔZ values for Cyt c and Az, but not for Mb (Table 1), even though we accounted for the pK_a value of the water molecule coordinated to Fe^{3+}-heme in met-Mb, which dissociates upon reduction.[17]

A structural and biophysical discussion of the residue and cofactor functional groups predicted to undergo significant changes in charge (defined as $\Delta Z > 0.01$ for any individual functional group) upon ET in Mb, Cyt c, and Az can be found in the Results and Discussion Section in the Supporting Information (Figures S6–S9). These mechanisms are summarized in Figure 3. Briefly, in all three proteins, every ionizable functional group experiences at least a minor change in pK_a value upon the change in oxidation state, but only residues with very large changes in pK_a or residues with pK_a values near 7.4 significantly change in ΔZ; these residues are fewer in number. Reduction of Mb results in significant changes in ΔZ (defined as $\Delta Z > 0.01$) of all non-coordinating histidine residues (H24, H36, H48, H64, H81, H82, H97, H113, H116, and H119), α-NH_3^+-G1, and the coordinating H_2O in Fe^{3+}-Mb (Figures S6, S9 a,b and Tables S1 and S2). In Cyt c, oxidation results in significant changes in the ΔZ values of K13, H33, Y48, Y97, and α-NH_3^+-G1 (Figures S7 and S9 c,d and Tables S3 and S4). Most of the ΔZ values associated with ET in Az (ca. 99 %) can be accounted for by the predicted increase in the pK_a values of both non-coordinating histidine residues (H35, H83) and α-NH_3^+-A1 (Figures S8 and S9 e,f and Tables S5 and S6); the protonation of H35 upon ET has been observed experimentally.[8,21]

The free energy required for the predicted adjustments of the pK_a values of all residues upon single ET ($\Delta\Delta G_z$) was calculated to be large: $\Delta\Delta G_z = 0.90$ eV for Mb, $\Delta\Delta G_z = 0.50$ eV for Cyt c, and $\Delta\Delta G_z = 0.42$ eV for Az (calculated from the pK_a values in Tables S1–S6, using the equation: $\Delta G = (2.3\,RT)[pH - pK_a]$). This energy will contribute to the redox potential and/or reorganization energy, depending upon when the protonation occurs relative to ET. It is
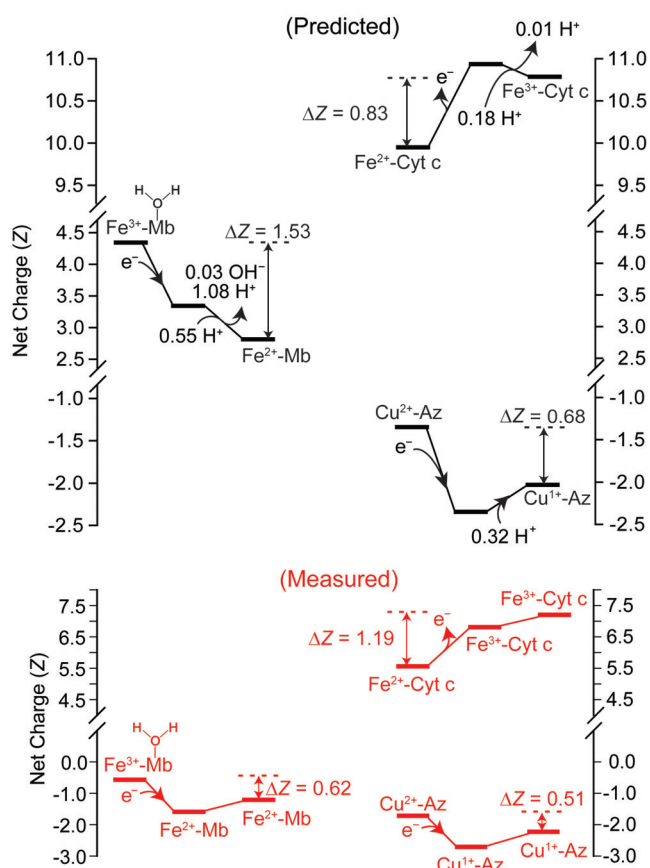
Figure 3. Comparison of experimentally and theoretically determined values of charge regulation.

contributes primarily to λ_o (except for the acidic H_2O bound to Fe^{3+} in Mb, which dissociates upon ET and would contribute to λ_i). According to semi-classical analyses of ET rates for ruthenium-modified Fe^{3+}-Mb, the λ_T associated with its reduction is $\lambda_T = 1.48$ eV.[10] Thus, the values of $\Delta\Delta G_z$ that we calculated for redox cycling in Mb (which are supported by measured values of ΔZ) could account for a maximum of about 61% of λ_T. The $\Delta\Delta G_z$ for Cyt c could account for about 42% of λ_T ($\lambda_T = 1.2$ eV according to semi-classical analyses of rates for ruthenium-modified Fe^{3+}-Cyt c).[10] Previous theoretical predictions suggest that λ_o comprises the majority of λ_T for Mb and Cyt c (87% and 67%, respectively; i.e., $\lambda_o = 1.29$ and $\lambda_i = 0.19$ eV for Mb and $\lambda_o = 0.8$ and $\lambda_i = 0.4$ eV for Cyt c).[10] Therefore, the lower value of $\Delta\Delta G_z$ for Cyt c, compared to Mb, could at least partially explain the lower value of λ_T for Cyt c compared to Mb.

In as much as λ_o comprises a majority of λ_T for other proteins—and contributions of λ_i are a minority—it is reasonable to predict that the $\Delta\Delta G_z$ values might correlate roughly (and linearly) with experimentally determined values of λ_T (if $\Delta\Delta G_z$ contributes to λ_T). This correlation would not hold for proteins whose λ_i values comprise larger proportions of λ_T or for proteins where a majority of $\Delta\Delta G_z$ contributes to $E°$. Although λ_T has been experimentally inferred (using ruthenium donors) for Az, Mb, and Cyt c, the relative contributions of λ_o and λ_i have only been theoretically predicted for Mb and Cyt c. Nevertheless, this trend is apparent from a comparison of predicted values of $\Delta\Delta G_z$ and measured λ_T for six ET proteins (Figure S10a), where $\Delta\Delta G_z$ correlates linearly with λ_T ($R^2 = 0.76$). There was only a weak correlation between $\Delta\Delta G_z$ and $E°$ for the same proteins (Figure S10b; $R^2 = 0.22$), as expected when considering that $E°$ is controlled by multiple bioinorganic and biophysical factors, including ligand-field effects and protein net charge.[9,23] If $\Delta\Delta G_z$ does not contribute significantly to λ_T in a protein, it would then contribute to $E°$, and the magnitude of $\Delta\Delta G_z$ suggests that these contributions will be large. However, if changes in protonation occur long after ET, there is a possibility that they are entirely disconnected from ET parameters.

In conclusion, measurements of ΔZ_{ET} with protein charge ladders and CE provide a rapid method for quantifying how proteins regulate their net charge during ET. The experimental and theoretical results of this study on Az, Cyt c, and Mb suggest that non-coordinating, ionizable residues (predominately histidine and N-terminal α-NH_3^+) can tune the reactivity of redox centers by charge regulation. The range of $\Delta\Delta G_z$ values associated with ET in these proteins is large (0.42 to 0.90 eV), and will significantly affect rates of ET by altering $E°$ and/or λ_T. Future studies will be required to discern which parameter of ET, that is, $E°$ or λ_T, is predominantly affected by the ΔZ_{ET} of specific ionizable residues during electron transfer.

beyond the scope of this current study to determine the certainty with which fluctuations in the protonation of Mb, Az, and Cyt c occur after ET (affecting $E°$) or with ET (affecting λ_T). Previous studies of Az suggested that the protonation of H35, which we predict to account for approximately 43% of charge regulation, occurs after reduction, and is coupled to slow conformational changes.[8,21] Moreover, substituting H35 in Az with Phe, Leu, or Gln lowered the bimolecular ET rate constant by a factor of up to two, and diminished $E°$ by up to 50 mV.[8] These results suggest that protonation of H35 does not contribute to λ_T, but occurs after ET, and therefore contributes to $E°$ of Az. For Mb and Cyt c, far more residues were predicted to be involved in charge regulation, and it is possible that changes in protonation of some residues might occur in concert with ET. Similar mutagenesis studies on all non-coordinating residues that we found to be implicated in charge regulation are not available for Cyt c and Mb; however, replacing H64 in Mb (the "distal" histidine, which we predict to account for ca. 7% of ΔZ_{ET}) with non-ionizable residues increased the rate of ET by a factor of > 10 and $E°$ by 6–50 mV.[22]

Considering the importance of solvent reorganization in λ_T,[5] we suspect that changes in the protonation of some residues might occur with ET, and make non-negligible contributions to λ_T. Insofar as the fluctuation in the pK_a value of a residue might contribute to λ_T, we presume that it

Conflict of interest

The authors declare no conflict of interest.

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