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Tuning the Rate and pH Accessibility of a Conformational Electron Transfer Gate

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Methods to fine-tune the rate of a fast conformational electron transfer (ET) gate involving a His–heme alkaline conformer of iso-1-cytochrome *c* (iso-1-Cyt*c*) and to adjust the pH accessibility of a slow ET gate involving a Lys–heme alkaline conformer are described. Fine-tuning the fast ET gate employs a strategy of making surface mutations in a substructure unfolded in the alkaline conformer. To make the slow ET gate accessible at neutral pH, the strategy involves mutations at buried sequence positions which are expected to more strongly perturb the stability of native versus alkaline iso-1-Cyt*c*. To fine-tune the rate of the fast His 73–heme ET gate, we mutate the surface-exposed Lys 79 to Ala (A79H73 variant). This mutation also simplifies ET gating by removing Lys 79, which can serve as a ligand in the alkaline conformer of iso-1-Cyt*c*. To adjust the pH accessibility of the slow Lys 73–heme ET gate, we convert the buried side chain Asn 52 to Gly and also mutate Lys 79 to Ala to simplify ET gating (A79G52 variant). ET kinetics is studied as a function of pH using hexaammineruthenium(II) chloride (a_6Ru^{2+}) to reduce the variants. Both variants show fast direct ET reactions dependent on [a_6Ru^{2+}] and slower gated ET reactions that are independent of [a_6Ru^{2+}]. The observed gated ET rates correlate well with rates for the alkaline-to-native state conformational change measured independently. Together with the previously reported H73 variant (Baddam, S.; Bowler, B. E. *J. Am. Chem. Soc.* **2005**, *127*, 9702–9703), the A79H73 variant allows His 73–heme-mediated ET gating to be fine-tuned from 75 to 200 ms. The slower Lys 73–heme (15–20 s time scale) ET gate for the A79G52 variant is now accessible over the pH range 6–8.

Introduction

Electron transfer (ET) is among the simplest of chemical processes occurring in living organisms, yet certainly one of the most critical. Many biological processes, such as photosynthesis and respiration, are regulated by ET reactions.[1−4] It has become apparent that conformational gating of biological ET reactions can regulate metabolic processes,[3] and an increasing number of examples of gated ET in proteins is being reported.[3,5] Conformational gating also appears to be an important aspect of protein ET at electrode interfaces and thus will be key to developing protein-based molecular electronics devices.[4,6] Therefore, the ability to tune the properties of conformational ET gates will be essential in both manipulating protein function and engineering molecular electronics devices.

Changes in the coordination sphere of a metal can result in significant changes in redox potential and thus can favor ET from one coordination environment versus another.[7] In proteins, changes in coordination environment are expected to require changes in the conformation of the protein. Thus, the rate of a conformational change which controls protein

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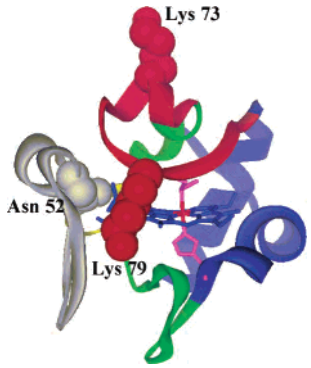

Figure 1. Yeast iso-1-cytochrome *c* in the oxidized state is shown with the substructure classifications of horse cytochrome *c* according to ref 15. The substructures are color-coded gray (N-yellow substructure, residues 40−57), red (residues 71−85), yellow (residues 37−39, 58−61, in back of structure), green (60's helix and 20's−30's loop), and blue (N- and C-terminal helices) in order of increasing stability. The heme (blue, iron in red) and its ligands (magenta), His 18 and Met 80, are shown as stick models. The Lys 73 and Lys 79 heme ligands in the alkaline conformer and Asn 52, which is mutated to Gly in the A79G52 variant, are shown as space-filling models (α-carbon and side-chain atoms) in the color of the substructure in which they are contained. Lys 79 is mutated to Ala in both the A79G52 and A79H73 variants so that there is only one possible heme ligand in the alkaline conformer, Lys 73 and His 73, respectively. The figure was prepared with DS ViewerPro software and the Protein Data Bank file, 2YCC.[12]

ET can be modulated by both the ligands involved and the nature of the conformational change.[8] An early example of conformationally gated protein ET involving a metal−ligand exchange reaction was observed for the alkaline conformer of cytochrome *c* (Cyt*c*).[9] More recently, metal−ligand exchange reactions have been implicated in gated ET for plastocyanin.[5c]

Conformational gates that operate near neutral pH are desirable both for engineered function and as components in molecular electronics devices. The ability to tune the rate of an ET gate over narrow ranges, as well as over orders of magnitude, is also important. Cyt*c* provides a particularly useful scaffold for tuning these properties for a conformationally gated ET reaction. In the alkaline conformer, lysine ligation leads to an ∼0.5 V decrease in the affinity of yeast iso-1-cytochrome *c* (iso-1-Cyt*c*) for an electron,[10] whereas histidine ligation is expected to cause a decrease of ∼0.25 V,[11] relative to the native heme−Met 80 ligation. Thus, conformationally gated ET is expected for both alkaline states. For iso-1-Cyt*c*, the heme ligands are known to be lysines 73 and 79 in the alkaline conformer (see Figure 1),[10a] allowing heme ligation in this state to be readily manipulated. Beyond these convenient properties for manipulating gated ET, a number of studies suggest that the alkaline conformer of Cyt*c* may play a role in its function in mitochondrial electron transport.[13]

In mitochondrial cytochromes *c*, the midpoint pH for formation of the alkaline state of Cyt*c* ranges from 8.5 to 11.[14] To make this conformer accessible near physiological pH, we have exploited the well-understood thermodynamics of this protein. Cyt*c* is known to have five cooperative substructures, as outlined in Figure 1, that unfold sequentially.[15] In other words the thermodynamics of Cyt*c* are hierarchical. The two least stable substructures (shown in gray and red in Figure 1) unfold in the alkaline conformer.[16−18] For either of the alkaline state ligands, Lys 73 or Lys 79, to displace Met 80, the red substructure must be disrupted (see Figure 1). Since the gray substructure is less stable than the red substructure and unfolding of the substructures of Cyt*c* is sequential, mutations which destabilize either of these substructures should destabilize the native state relative to the alkaline state of Cyt*c*. In previous work on yeast iso-1-Cyt*c*, we have shown that stabilizing mutations at position 52 in the gray substructure (see Figure 1) stabilize the alkaline conformer relative to the native state.[18d] Previous studies on the effects of mutations at position 52 on the global stability of iso-1-Cyt*c* show that the stability of the variant proteins depends on the size of the amino acid at this position.[19] In particular, an Asn 52 → Gly mutation significantly decreased the global stability of iso-1-Cyt*c*. On this basis, we created an A79G52 (Lys 79 → Ala and Asn 52 → Gly mutations) variant of iso-1-Cyt*c*. Our thermodynamic and kinetic analyses have verified that A79G52 iso-1-Cyt*c* makes the alkaline conformer accessible near neutral pH (midpoint pH ∼7.4).[20] The Lys 79 → Ala mutation simplifies gating kinetics by making Lys 73 the only heme ligand in the alkaline conformer of this variant. As discussed below, this latter mutation might also be expected to modestly perturb the dynamics of the conformational change from the native to the alkaline state. However, since the kinetics of formation of the lysine 73 and 79 alkaline conformers cannot be determined individually when both are

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present, it is not possible to assess the magnitude of this effect. Thus, the primary goal for ET studies on the A79G52 variant is to make the slower conformational gating due to the lysine−heme alkaline conformer accessible near neutral pH. Here, we use reaction of the Lys 73−heme alkaline state of A79G52 iso-1-Cyt*c* with hexaammineruthenium(II) (a_6Ru^{2+}) to verify that gated ET occurs near neutral pH with this variant. We find that gated ET is observable from pH 6 to 8 on an ∼15−20 s time scale.

The second goal of this report is to develop means of fine-tuning the rate of an ET gate. We have recently reported that replacement of Lys 73 with His (H73 variant) dramatically increases the rate of conformationally gated ET from the alkaline conformer simply by changing the heme ligand in the alkaline conformer from a lysine to a histidine.[8] The rate of the gate could also be increased by a factor of nearly 2.0 by decreasing the pH from 7.5 to 6.[8] Furthermore, the gated ET rates as a function of pH correlated well with the rate of the conformational change from the His 73−heme alkaline conformer to the native state measured independently by pH jump methods.[8,21] As a strategy to further fine-tune the rate of gated ET due to the His 73−heme alkaline conformer, a logical approach is to make mutations of surface residues in the red substructure which is unfolded in the alkaline conformer. Surface mutations are expected to more modestly affect the stability and dynamics of a protein.[22] As a first attempt at this strategy, we have added the Lys 79 → Ala mutation to the H73 variant to create the A79H73 variant. Besides providing for a modest perturbation to the conversion from the alkaline conformer to the native conformer, this variant also removes Lys 79 as an alkaline-state ligand, simplifying the kinetics of ET gating. We have also carried out thorough kinetic and thermodynamic analyses of the alkaline conformational transition of the A79H73 variant which demonstrate that the dynamics of the native to His 73−heme alkaline conformer have been modestly perturbed, as predicted.[23] This previous work will allow for direct correlation of the pH dependence of the conformational change from the His 73−heme alkaline state to the native state measured by pH jump methods with gated ET measured here by reaction with a_6Ru^{2+}. We find that we are now able to modulate gated ET over almost a 3-fold time scale, from 75 to 200 ms using the combined effects of pH and mutagenesis with the A79H73 and H73 variants of iso-1-Cyt*c*.

Experimental Section

Proteins and Reagents. The A79G52 and A79H73 variants of iso-1-Cyt*c* were isolated and purified, as previously described,[20,23] from the *Saccharomyces cerevisiae* cell-line GM-3C-2 (Cyt*c* deficient) transformed with the pRS/C7.8 vector carrying the appropriately mutated iso-1-Cyt*c* gene. Both variants also carry the mutation Cys 102 → Ser to prevent formation of intermolecular disulfide dimers during physical studies.

Commercial $[Ru(NH_3)_6]Cl_3$ (Strem Chemicals) was reduced with zinc by the method of Fergusson and Love.[24] The $[Ru(NH_3)_6]Cl_2$ (a_6Ru^{2+}) was dried in a vacuum desiccator which was then refilled with argon. The product was stored at −20 °C, and care was taken to minimize exposure of $[Ru(NH_3)_6]Cl_2$ to air and moisture to prevent oxidation. The formation of $[Ru(NH_3)_6]Cl_2$ was confirmed by infrared spectroscopy (1217 cm^{-1} band characteristic of Ru(II) complex).[25]

Experiments were done with the following buffers: 10 mM MES in 0.1 M NaCl, pH 6.0 and 6.5; 10 mM Tris buffer in 0.1 M NaCl, pH 7.5 and 8.0. The pH of buffers was adjusted with $NaOH_{(aq)}$ or $HCl_{(aq)}$, as appropriate.

Argon gas (high purity grade, 99.99%) that was further purified with an oxy-trap column (Alltech, Inc.) was used to degas all solutions for anaerobic work.

Anaerobic Stopped-Flow Kinetics Measurements. Stopped-flow mixing of a_6Ru^{2+} with the oxidized form of the A79H73 and A79G52 variants was carried out using an Applied Photophysics π*-180 spectrometer operating in kinetics mode, as previously described.[8] Briefly, the day before experiments were done, the flow cell and the drive syringes were soaked overnight in 50 μM riboflavin, reduced with 1 mM EDTA and light, to ensure anaerobic conditions.[26] Before the experiments were started, the riboflavin solution was driven out of the flow cell and syringes and then the appropriate buffer, degassed with Ar, was used to wash out any remaining riboflavin. Iso-1-Cyt*c* variants were oxidized with $K_3[Fe(CN)_6]$ followed by G-25 chromatography to remove the reagent and change the protein into the appropriate buffer. Oxidized protein was diluted to 10 μM with Ar-degassed buffer and then further degassed on a dual Ar/vacuum manifold. All a_6Ru^{2+} solutions were prepared immediately before use. A known volume of Ar-degassed buffer was cannula-transferred onto solid $[Ru(NH_3)_6]Cl_2$ in an Ar-degassed vessel. The degassed solutions of protein and a_6Ru^{2+} were mixed 1:1 with the π*-180 spectrometer producing final solutions containing 5 μM protein and a_6Ru^{2+} at 1.25, 2.5, or 5 mM in 10 mM buffer containing 100 mM NaCl. Reduction of the heme of the iso-1-Cyt*c* variants was monitored at 550 nm.

At each concentration of a_6Ru^{2+}, a minimum of four trials was done and a total of 1000 points was collected over 2 s on a logarithmic time scale. Data were also collected on a longer time scale for the A79G52 variant (50−100 s, 5000 data points on a logarithmic time scale). The dead time for stopped-flow mixing was measured using ascorbate reduction of 1,6-dichlorophenolindophenol[27] and was found to be 1.0 ms under our mixing conditions. The data were corrected for the dead time of the experiment.

Analysis of the data was done using the curve fitting program, SigmaPlot (v. 7.0). The data were fit to single or double exponential rise to maximum equations depending on the protein and the time scale.

Results

Kinetic Model for Gated ET. The kinetics of conformationally gated ET can be analyzed with a square scheme

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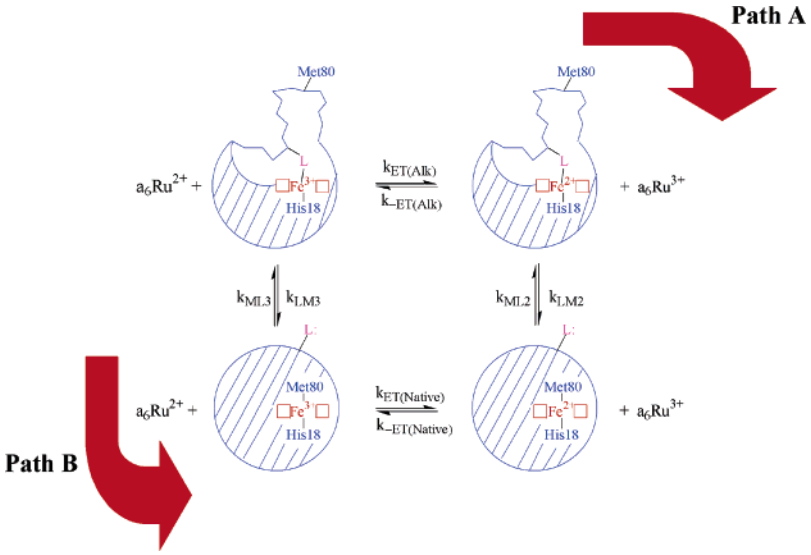

Figure 2. ET square scheme for the reaction of oxidized iso-1-cytochrome *c* with hexaammineruthenium(II) (a_6Ru^{2+}). Horizontal equilibria are ET equilibria, and vertical equilibria are conformational equilibria. The oxidized and reduced alkaline conformers of iso-1-Cyt*c* are shown in the upper row of the ET square scheme. The alkaline state iron ligand, L, is His 73 for the A79H73 variant and Lys 73 for the A79G52 variant. The oxidized and reduced native conformers of iso-1-Cyt*c* are shown in the lower row of the ET square scheme. Reduction of the oxidized alkaline conformer of iso-1-Cyt*c* by a_6Ru^{2+} can occur via either path A or path B as described in the text. The rate constants controlling the conformational change in the oxidized (Fe^{3+}) state of iso-1-Cyt*c* are k_{LM3} and k_{ML3} and in the reduced (Fe^{2+}) state of iso-1-Cyt*c* are k_{LM2} and k_{ML2}. The rate constants controlling ET in the alkaline state are $k_{ET(Alk)}$ and $k_{-ET(Alk)}$ and in the native state are $k_{ET(Native)}$ and $k_{-ET(Native)}$. Path B is expected to be dominant (see text) for the reaction of the oxidized alkaline state of iso-1-Cyt*c* with a_6Ru^{2+}. Therefore, gated ET will be controlled by the rate constant k_{LM3} and direct ET by the rate constant $k_{ET(Native)}$.

model (Figure 2).[7,28] In the square scheme model, the upper left-hand conformer of Cyt*c* is taken to be more stable in the oxidized state (oxidized alkaline state with His 73 or Lys 73 bound to the heme) and the lower right-hand conformer of Cyt*c* is taken to be more stable in the reduced state (reduced native state with Met 80 bound to heme). Thus, in reducing the alkaline conformer of Cyt*c*, two pathways are possible. In path A, the alkaline conformer is reduced first followed by a conformational change to the more stable native reduced state of Cyt*c*. In path B, the conformational change to the native conformer of the oxidized state occurs first followed by reduction of the oxidized native conformer. In the alkaline conformer of the A79H73 variant, His 73 will be bound to the heme whereas Lys 73 will be bound to the heme in the alkaline state of the A79G52 variant. Since the reduction potential of the native state of wild type (WT) iso-1-Cyt*c* is near 290 mV[10a] versus NHE, whereas the reduction potential of the His 73-heme and Lys 73-heme alkaline conformers are near 0 mV[11] and −205 mV versus NHE,[10] respectively, reduction of the A79H73 and A79G52 variants by a_6Ru^{2+} ($E° \approx 50$ mV versus NHE)[29] is expected to proceed from the native conformer. Thus, in Figure 2, reduction of alkaline conformers of these iso-1-Cyt*c* variants should proceed via path B, and the ET reaction should be gated by the conformational change back to the native state (k_{LM3} rate constant in Figure 2). Under pH conditions where both conformers are present in the oxidized state (i.e., $0.1 < k_{LM3}/k_{ML3} < 10$), both direct intermolecular ET to the oxidized native state ($k_{ET(native)}$ in Figure 2) and conformationally gated ET (k_{LM3}) from the oxidized alkaline state should be detectable.

With path B dominant, the rate law for the bimolecular reaction between a_6Ru^{2+} and the Fe^{III} state of the A79H73 and A79G52 variants has the form given in eqs 1 and 2,[7] where the rate

$$-\mathrm{d}[\mathrm{Fe^{III}heme}]/\mathrm{d}t = k_{obs}[\mathrm{Cyt}c(\mathrm{Fe^{III}}-\mathrm{L})][\ a_6Ru^{2+}]$$

$$k_{obs} = \{k_{ET(Native)}k_{LM3}/(k_{ET(Native)}[a_6Ru^{2+}] + k_{ML3})\}$$

constants are defined in Figure 2 and Cyt*c*(Fe^{III}-L) is the alkaline conformer of the A79H73 and A79G52 variants with the alkaline state heme ligand, L, being His 73 and Lys 73, respectively. If $k_{ET(Native)}[a_6Ru^{2+}] \gg k_{ML3}$, then the rate law in eq 1 becomes independent of $[a_6Ru^{2+}]$ and k_{obs} becomes the rate constant of conversion from the alkaline to the native conformer of oxidized Cyt*c* (k_{LM3} in Figure 2, i.e., k_{LM3} is the rate constant for ET gating). Thus, when both conformations are present, a fast phase dependent on $[a_6Ru^{2+}]$ (direct ET with $k_{ET(Native)}$) and a slower gated ET phase (k_{LM3} in Figure 2) independent of $[a_6Ru^{2+}]$ should be observed.

Anaerobic Stopped-Flow Experiments on the A79H73 Iso-1-cytochrome *c* Variant. To distinguish intermolecular ET reactions of the A79H73 variant with a_6Ru^{2+} from conformationally gated ET, reduction of the A79H73 variant was carried out at three different a_6Ru^{2+} concentrations. Our previously reported pH jump stopped-flow studies have shown that the rate of conversion of the His 73−heme alkaline conformer to the native Met 80−heme conformer of the A79H73 variant varies with pH.[23] Therefore, the reduction of A79H73 iso-1-Cyt*c* with a_6Ru^{2+} was carried

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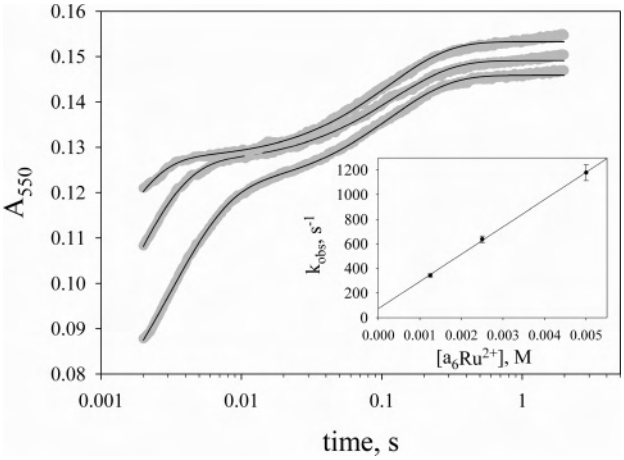

Figure 3. Plot of absorbance at 550 nm versus time (on a logarithmic scale) for the reduction of the A79H73 variant of iso-1-Cyt*c* with hexaammineruthenium(II) chloride at concentrations of, from top to bottom, 5, 2.5, and 1.25 mM. Data at 550 nm were collected for 2 s in 0.1 M NaCl with 10 mM MES, pH 6.0, as buffer. The temperature was 25 °C, and the protein concentration was ~5 μM. The gray dots are the data at 550 nm, and the solid black curves are fits of the data to a double exponential rise to maximum equation. Inset: Plot of k_{obs}(fast) versus [a_6Ru^{2+}] for the reduction reaction of the A79H73 variant at pH 6.0. The solid curve is a fit to $k_{obs}(\text{fast}) = k_{ET(Native)}[a_6Ru^{2+}]$. The error bars are the standard deviation of k_{obs}(fast).

out at three pH values to definitively demonstrate that gated ET is due to the alkaline conformer of this protein. Figure 3 shows data acquired at pH 6 on a 2 s time scale. A very fast phase is complete in less than 10 ms, and a slow phase is observed on a time scale of ~100 ms. These two kinetic phases are analogous to those observed for the H73 variant.[8] The fast phase is clearly dependent on [a_6Ru^{2+}] (Figure 3, inset), as for the H73 variant, and thus can be assigned to direct intermolecular ET to the native state (heme−Met 80 ligation) of the A79H73 variant. The slower time scale phase is independent of [a_6Ru^{2+}] and thus is due to conformationally gated ET. As for the H73 variant,[8] a very slow phase is observed on a 100 s time scale assignable to proline isomerization.[30]

Table 1 summarizes the rate constants for reduction of the Fe(III) state of the A79H73 variant as a function of pH and [a_6Ru^{2+}]. At all pH values, the fast phase is dependent on [a_6Ru^{2+}] and the slow phase is independent of [a_6Ru^{2+}]. Thus, at all pH values, the fast phase can be assigned to intermolecular ET and the slow phase to conformationally gated ET. The observed rate constant for the fast phase, k_{obs}(fast), is ~5-fold faster for a given [a_6Ru^{2+}] for the A79H73 variant than for the H73 variant.[8] Thus, direct ET via path B in the square scheme (see Figure 2) is much faster for the A79H73 variant than for the H73 variant. On the other hand, the rate constant for the slower gated ET (k_{obs}(slow) in Table 1; k_{LM3} in path B of Figure 2) is 50% larger for the H73 variant[8] than for the A79H73 variant studied here. The rate constant for the slow gated ET phase is also dependent on pH, as observed for the H73 variant.[8] This observation is expected on the basis of the pH dependence of the rate of the conformational change from the His 73−heme alkaline conformer to the native state of the A79H73 variant which we have measured independently by pH jump stopped flow methods.[23]

The amplitude of the slow phase (see Table 1) increases with pH in synch with the growth of the population of the His 73−heme alkaline conformer of the A79H73 variant observed in our equilibrium studies over the pH range 6.0−7.5.[23] In contrast, the amplitude of the fast phase decreases over this pH range, consistent with the decreasing population of the native conformer of the A79H73 variant. The match between the amplitude data and the thermodynamics of the conformational change from the native to the His 73−heme alkaline conformer also provide strong support for our assignment of the fast [a_6Ru^{2+}]-dependent kinetic phase to direct bimolecular ET to the native state ($k_{ET(Native)}$ in Figure 2) and the slow [a_6Ru^{2+}]-independent phase to conformationally gated ET (k_{LM3} in Figure 2).

Plots of the fast phase versus [a_6Ru^{2+}] (Figure 3, inset) can be used to extract the bimolecular electron-transfer rate constant for direct ET via path B, $k_{ET(Native)}$ (see Figure 2), for the reaction of a_6Ru^{2+} with the native state of the A79H73 variant. The value of $k_{ET(Native)}$ is $(2.22 \pm 0.05) \times 10^5$ M^{-1} s^{-1} at pH 6.0, $(2.36 \pm 0.01) \times 10^5$ M^{-1} s^{-1} at pH 6.5, and $(1.33 \pm 0.09) \times 10^5$ M^{-1} s^{-1} at pH 7.5. The significance of the decrease in $k_{ET(Native)}$ at pH 7.5 may be questionable given the low amplitude of the fast phase at pH 7.5 and the proximity of its rate to the dead time of the instrument.

Anaerobic Stopped-Flow Experiments on the A79G52 Variant. Our thermodynamic studies on formation of the alkaline state of the A79G52 variant indicate that it converts from the native state to the Lys 73−heme alkaline conformer with an apparent pK_a of ~7.4.[20] Thus, we expect that gated ET from its Lys 73−heme alkaline conformer should be observable near neutral pH. Therefore, we have studied intermolecular ET of the A79G52 variant with a_6Ru^{2+} over the range pH 6−8. Figure 4 shows data obtained at pH 6.5. A fast ET kinetic phase (Figure 4A), dependent on the concentration of the reducing agent, was observed from data collected on a 2 s time scale, consistent with direct intermolecular ET ($k_{ET(Native)}$ in path B of Figure 2) to the heme of the native state of the A79G52 variant. As with the A79H73 variant, this direct ET phase is ~5-fold faster than observed for the H73 variant at a given [a_6Ru^{2+}]. A slow phase (Figure 4B), independent of the concentration of [a_6Ru^{2+}] is seen in the data collected on a 100 s time scale, consistent with conformationally gated ET (k_{LM3} in path B of the ET square scheme in Figure 2).

Rate constants and amplitudes as a function of pH and [a_6Ru^{2+}] are provided in Table 2. Rate constants for the fast phase could not be extracted from the data at pH 7.5 and 8.0 for a_6Ru^{2+} at 2.5 and 5.0 mM concentrations due to the low amplitude of this phase under these conditions. Inspection of the data in Table 2 suggests that there may be a slight dependence of k_{obs} for the slow phase on [a_6Ru^{2+}] at pH 6.5 and 7.5; however, at pH 6.0 and 8.0 no such dependence is observed. In eqs 1 and 2, if direct ET and gated ET phases are not well-separated, the assumption that $k_{ET(Native)}[a_6Ru^{2+}] \gg k_{ML3}$ will not be completely valid and a slight concentra-

(30) Baddam, S. M.S. Thesis, University of Denver, 2005.

Table 1. Rate Constants and Amplitudes for the Reduction of A79H73 iso-1-Cyt*c* by a_6Ru^{2+} at 25 °C as a Function of pH and $[a_6Ru^{2+}]$

pH	$[a_6Ru^{2+}]$, mM	k_{obs}(fast), s^{-1}	amplitude (fast), au	k_{obs}(slow), s^{-1}	amplitude (slow), au
6.0	1.25	343 ± 12	0.065 ± 0.002	8.8 ± 0.2	0.026 ± 0.001
	2.5	638 ± 26	0.065 ± 0.005	8.8 ± 0.4	0.025 ± 0.002
	5.0	1179 ± 64	0.066 ± 0.001	9.0 ± 0.1	0.026 ± 0.001
6.5	1.25	274 ± 8	0.053 ± 0.002	6.3 ± 0.2	0.035 ± 0.001
	2.5	571 ± 10	0.056 ± 0.003	6.3 ± 0.2	0.033 ± 0.001
	5.0	1158 ± 88	0.0586 ± 0.0003	6.6 ± 0.2	0.0352 ± 0.0002
7.5	1.25	255 ± 1	0.0308 ± 0.0004	5.0 ± 0.1	0.0451 ± 0.0004
	2.5	455 ± 11	0.033 ± 0.001	5.1 ± 0.1	0.044 ± 0.001
	5.0	760 ± 92	0.022 ± 0.006	5.18 ± 0.04	0.0434 ± 0.0002

tion dependence will be observed for the gated ET phase. However, the data in Figure 4 clearly demonstrate that the fast and slow ET phases are well separated for the A79G52 variant. The standard deviations in Table 2 at each pH and $[a_6Ru^{2+}]$ are based on multiple stopped-flow experiments acquired immediately sequentially. Thus, the precision for each pH/$[a_6Ru^{2+}]$ experiment for the relatively long data acquisitions required for the slow phase of the A79G52 variant might be expected to be higher than that between

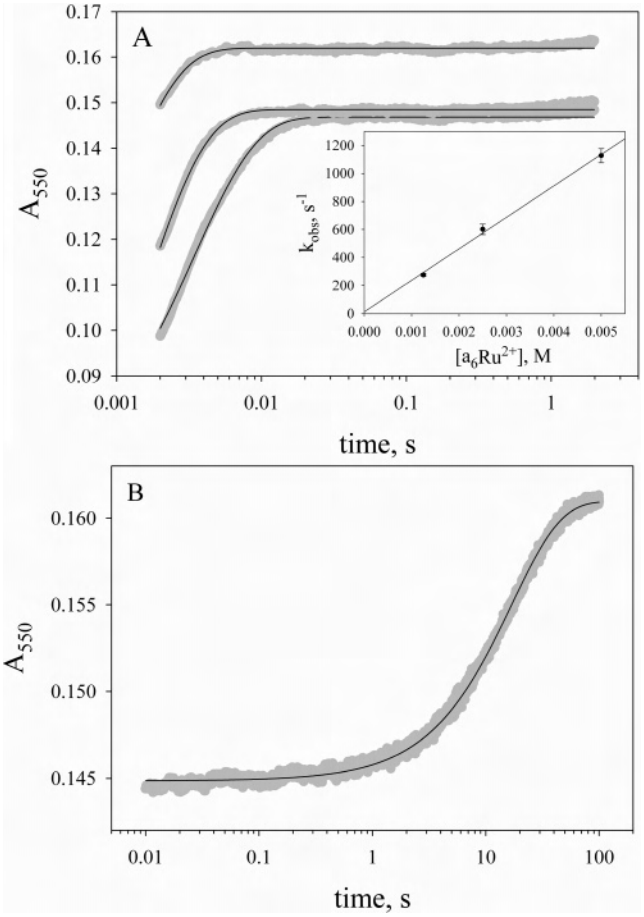

Figure 4. Plot of absorbance at 550 nm versus time (on a logarithmic scale) for reduction of the A79G52 variant of iso-1-Cyt*c* with hexaammineruthenium(II) chloride at pH 6.5. Panel A shows data collected over 2 s with hexaammineruthenium(II) chloride concentration at, from top to bottom, 5, 2.5, and 1.25 mM. The inset to panel A shows a plot of k_{obs}(fast) versus $[a_6Ru^{2+}]$ for the reduction reaction with the A79G52 variant at pH 6.5. The solid curve is a fit to $k_{obs}(\text{fast}) = k_{ET(Native)}[a_6Ru^{2+}]$. The error bars are the standard deviation of k_{obs}(fast). Panel B shows data collected over 100 s with hexaammineruthenium(II) chloride concentration at 2.5 mM. Data were collected in 0.1 M NaCl with 10 mM MES, pH 6.5, as buffer. The temperature was 25 °C, and the protein concentration was ~5 μM. The gray dots are the data at 550 nm, and the solid black curves are fits of the data to a single-exponential rise to maximum equation.

the different pH/$[a_6Ru^{2+}]$ experiments. Therefore, the overall average and standard deviation for k_{obs} for the slow phase at different pH/$[a_6Ru^{2+}]$ values (0.06 ± 0.01 s^{-1}) provide a better estimate of the value of and the precision with which we know the ET gating rate constant for this variant.

The amplitude of the fast phase (Table 2) decreases as pH increases, consistent with the progressive decrease in the population of the native state as pH increases from 6 to 8, as expected on the basis of our thermodynamic studies on the A79G52 variant.[20] Similarly, the slow gated ET phase increases in amplitude over the same pH range, as the Lys 73−heme alkaline conformer becomes the dominate form of the A79G52 variant. As for the ET data for the A79H73 variant, the pH dependence of the amplitudes for the reduction of the A79G52 variant of iso-1-Cyt*c* by a_6Ru^{2+} confirms the assignment of the fast phase to direct bimolecular ET to the native state of the protein and the slow phase to gated ET involving the Lys 73−heme alkaline conformer.

Plots of the fast phase versus $[a_6Ru^{2+}]$ (see Figure 4, inset) can be used to extract the bimolecular ET rate constant, $k_{ET(Native)}$, for the direct ET reaction of a_6Ru^{2+} with the native state of the A79G52 variant. The value of $k_{ET(Native)}$ is (1.6 ± 0.4) × 10^5 M^{-1} s^{-1} at pH 6.0 and (2.2 ± 0.1) × 10^5 M^{-1} s^{-1} at pH 6.5.

Discussion

Comparison of Conformationally Gated ET with the Kinetics of the Conversion of the Alkaline Conformer to the Native Conformer. In studies on the H73 variant of iso-1-Cyt*c*,[8] the pH dependence of the rate constant for gated ET paralleled that for the formation of the native state from the His 73−heme alkaline conformer measured independently. On this basis, it was possible to directly assign the conformational change controlling the rate of ET to the conversion of the His 73−heme alkaline conformer to the native state (k_{LM3} in Figure 2). We have also determined k_{LM3} independently by pH jump stopped-flow mixing for the conversion of the His 73−heme alkaline conformer of the A79H73 variant to the native state.[23] Figure 5 shows a plot of k_{LM3} versus pH obtained by pH jump stopped-flow methods,[23] along with the average values for gated ET for the A79H73 variant at pH 6.0, 6.5, and 7.5 (k_{obs}(slow) in Table 1). The values for k_{obs}(slow) increase as pH decreases, correlating well with the pH dependence for k_{LM3} measured independently. Thus, the His 73−heme alkaline conformer of the A79H73 variant is clearly responsible for ET gating.

In the case of the A79G52 variant, within error, the rate

Table 2. Rate Constants and Amplitudes for the Reduction of A79G52 iso-1-Cyt*c* by a_6Ru^{2+} at 25 °C as a Function of pH and [a_6Ru^{2+}]

pH	[a_6Ru^{2+}], mM	k_{obs}(fast), s^{-1}	amplitude (fast), au	k_{obs}(slow), s^{-1}	amplitude (slow), au
6.0	1.25	231 ± 4	0.101 ± 0.001	0.063 ± 0.002	0.0075 ± 0.0003
	2.5	563 ± 9	0.118 ± 0.009	0.087 ± 0.005	0.0074 ± 0.0001
	5.0	844 ± 27	0.11 ± 0.01	0.063 ± 0.005	0.0080 ± 0.0001
6.5	1.25	276 ± 11	0.081 ± 0.004	0.0549 ± 0.0004	0.0162 ± 0.0003
	2.5	602 ± 37	0.10 ± 0.01	0.059± 0.002	0.0162 ± 0.0002
	5.0	1127 ± 51	0.085 ± 0.013	0.065 ± 0.002	0.0159 ± 0.0001
7.5	1.25	142 ± 3	0.032 ± 0.001	0.0481 ± 0.0004	0.0607 ± 0.0005
	2.5	–	–	0.056 ± 0.001	0.0613 ± 0.0004
	5.0	–	–	0.065 ± 0.001	0.0639 ± 0.0001
8.0	1.25	177 ± 11	0.015 ± 0.001	0.0518 ± 0.0004	0.089 ± 0.001
	2.5	–	–	0.059 ± 0.001	0.0895 ± 0.0001
	5.0	–	–	0.0583 ± 0.0003	0.091 ± 0.001

of gated ET is invariant with pH. The average and standard deviation of k_{obs}(slow) in Table 2 for all pH values and concentrations of a_6Ru^{2+} is 0.06 ± 0.01 s^{-1}. The value of k_{LM3} for the conversion of the Lys 73−heme alkaline conformer of the A79G52 variant to the native state obtained by pH jump stopped-flow measurements is 0.05 ± 0.01 s^{-1} and is also independent of pH.[20] The good agreement between these independently determined values demonstrates that the Lys 73−heme alkaline conformer of the A79G52 variant is responsible for the gated ET observed during its reduction by a_6Ru^{2+}.

There are few cases where conformationally gated ET can be directly correlated with the rate of a conformational change, and these cases are primarily for small molecule systems.[7] Gated ET in proteins is typically assigned on the basis of methods such as the viscosity dependence of the ET rate constant[3] which provide little insight into the nature of the conformational change gating the ET reaction. The ability to directly correlate gated ET with a particular conformational change, as in our model system, provides an entrée to rationale manipulation of gated ET.

Fine-Tuning the Rate of an ET Gate. In previous work,[8] we have demonstrated that the rate of an ET gate can be changed by nearly 3 orders of magnitude by changing the ligand involved in the alkaline state of iso-1-Cyt*c* from Lys to His. While large changes are useful, the ability to fine-tune the rate of a conformational ET gate over small ranges is equally important. Over the pH range 6.0−7.5, the rate constant for the ET gate of the H73 variant varied from 7 to 13 s^{-1}.[8] Over the same pH range, the addition of the Lys 79 → Ala mutation to produce the A79H73 variant shifts the variation in the rate constant of the ET gate to 5−9 s^{-1}. This allows the lifetime of the ET gate to be varied from 75 to 200 ms in discrete steps (H73: pH 6, 75 ms; pH 6.5, 122 ms; pH 7.5, 145 ms. A79H73: pH 6, 113 ms; pH 6.5, 156 ms, pH 7.5, 195 ms.) by varying both pH and the amino acid sequence of the portion of the protein involved in the conformational change. Thus, the strategy of using surface mutations to make modest perturbations to the thermodynamics and kinetics of a conformational change provides a facile means of fine-tuning the flow of an electron through a metalloprotein.

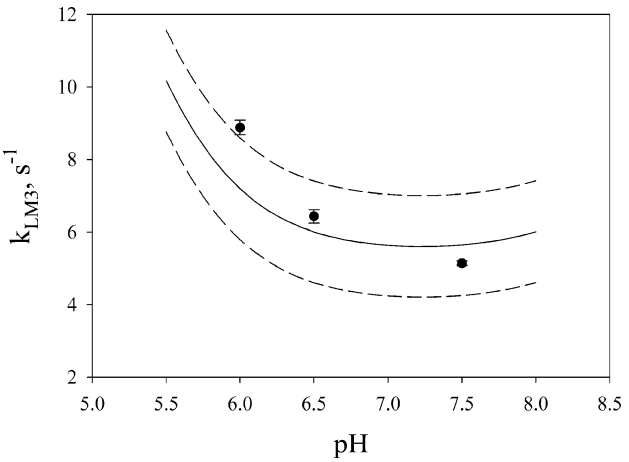

Figure 5. Comparison of k_{obs}(slow) for the reduction of A79H73 iso-1-Cyt*c* by a_6Ru^{2+} to the pH dependence of k_{LM3} for the conformational change from the Cyt*c*(Fe^{III}−His 73) alkaline conformer to the Cyt*c*(Fe^{III}−Met 80) native state. The solid circles and error bars are the average and standard deviation of k_{obs}(slow) for all [a_6Ru^{2+}] at each pH. The solid line is the pH dependence for k_{LM3} measured directly via pH jump stopped-flow methods in 100 mM NaCl and 10 mM buffer.[23] The dashed lines show the average standard deviation of the data points of the pH jump stopped-flow data used to define the dependence of k_{LM3} on pH.

Tuning the pH Accessibility of an ET Gate. As noted in the Introduction, the naturally occurring alkaline conformer of Cyt*c* which is stabilized by lysine−heme ligation generally populates at pH values between 8.5 and 11.[14] For the purposes of engineering protein function related to conformational ET gates in proteins or for developing protein-based molecular electronics,[6] ET gates that operate near neutral pH are preferable. For Cyt*c* from horse heart, it has been demonstrated that the alkaline conformer can act as an ET gate, albeit at high pH.[9] In previous work, ET gating mediated by Lys−heme alkaline conformers is only marginally observable for WT iso-1-Cyt*c* at pH 7.5.[8] Here, we demonstrate that the slow ET gate due to Lys 73−heme ligation can be made accessible at neutral pH and below by using the known substructure hierarchy of Cyt*c* to rationally stabilize the Lys 73−heme alkaline conformer of iso-1-Cyt*c*.[18d,20] Our data on the A79G52 variant (Figure 4, Table 2), show that this ET gate is observable over the pH range 6.0−8.0, becoming the dominant mode of electron flow through this protein at pH 7.5 and 8.0. Therefore, this much slower ET gate, which has a lifetime of 15−20 s can now be used for ET gating near neutral pH. As discussed in the Introduction, the large effect on the stability of the Lys 73−heme alkaline conformer likely results in part from the decrease in the size of the side chain when Asn is mutated to Gly at position 52 which is buried in the native state of

the protein.[19] We have also demonstrated that the effects of mutations at position 52 on the buried hydrogen bond network of iso-1-Cyt*c* can have substantial effects on the cooperative stabilization of this protein.[31] Thus, the destabilization due to the Gly 52 mutation is also likely due in part to its impact on the buried hydrogen-bond network of this protein.

Several variants at position 82 of iso-1-Cyt*c* have previously been reported which shift the alkaline conformational transition to near neutral pH,[32] although no studies on ET gating have been carried out on these variants. Position 82 is buried in the three-dimensional structure of iso-1-Cyt*c* and packs against the heme.[12] Thus, mutations at buried sequence positions in the two least stable substructures of Cyt*c* (shown in gray and red in Figure 1) both appear to be appropriate for manipulating gated ET in iso-1-Cyt*c* as predicted by the hierarchical thermodynamics of Cyt*c* (see Figure 1, caption).

Modulation of the Rate of Direct ET by the Lys 79 → Ala Mutation. In our studies on the reduction of WT and H73 iso-1-Cyt*c* by a_6Ru^{2+},[8] we found that the bimolecular ET rate constants, $k_{ET(Native)}$, were $(4.8 \pm 0.4) \times 10^4$ and $(3.9 \pm 0.5) \times 10^4$ M^{-1} s^{-1}, respectively. Both the A79H73 and A79G52 variants have substantially higher $k_{ET(Native)}$ values of $(1.9 \pm 0.5) \times 10^5$ M^{-1} s^{-1}. Thus, $k_{ET(Native)}$ has increased by a factor of about 3−5. For bimolecular ET between small-molecule reagents and proteins, a minimal mechanism involving a binding equilibrium ($K = k_1/k_{-1}$, where k_1 and k_{-1} are the association and dissociation rate constants, respectively) followed by electron transfer (k_{et}) is generally invoked.[33] For *c*-type cytochromes, the limiting condition $k_{et} \ll k_{-1}$ appears to apply.[33b] Therefore, $k_{ET(Native)}$ is best expressed as the product of the binding equilibrium constant, *K*, and a microscopic ET rate constant, i.e., $k_{ET(Native)} = Kk_{et}$. Thus, a number of factors may be responsible for the increase in $k_{ET(Native)}$ caused by the Lys 79 → Ala mutation.

Since k_{et} will depend on the driving force, it is possible that a change in the reduction potential of iso-1-Cyt*c* caused by the Lys 79 → Ala mutation is a factor. Electrochemical studies on a Lys 79 → Ala variant of iso-1-Cyt*c* demonstrate that the reduction potential does not change within error relative to wild-type iso-1-Cyt*c*.[34] Thus, driving force does not appear to be a factor.

Both variants have in common the mutation of Lys 79 → Ala, which is located at the exposed heme edge of iso-1-Cyt*c*.[12] A number of studies have implicated the exposed heme edge of Cyt*c* as the site of reaction with both protein[35] and small-molecule reagents.[33,36] Changes in the binding site or sites caused by this mutation might be expected to affect both *K* and k_{et} (since perturbations to the binding site will likely affect the ET distance). For inorganic reagents, three sites have been defined around the heme edge where these reagents bind, prior to ET reactions with Cyt*c*.[33a,36d] For positively charged reagents such as $Co(phen)_3^{3+/2+}$ (phen = 1,10-phenanothroline), Site II which includes Val 11, Ala 15, Thr 19, and lysines 7, 25, and 27 in horse heart Cyt*c* has been implicated as the primary binding site in intermolecular ET reactions with Cyt*c*.[36b,c,d] The presence of Glu 21 adjacent to this site is believed to be important for binding positively charged metal complexes.[36d] A prominent enhancement of the oxidation rate of Cyt*c* by $Co(phen)_3^{3+}$ upon chemical modification of Lys 27 with reagents that change its charge is a primary reason for this assignment.[36b,c] Site II is significantly modified in iso-1-Cyt*c* relative to the horse heart protein (Val 11 → Lys, Ala 15 → Leu, Lys 7 → Thr, and Lys 25 → Pro), so changes in the ET binding site for positively charged metal complexes might be expected. The net effect of the mutations relative to the horse protein is to reduce the positive charge near this site, and both Lys 27 and Glu 21 are conserved in yeast iso-1-Cyt*c*. Thus, in principle, one might expect an increase in $k_{ET(Native)}$ relative to the horse protein. However, for WT iso-1-Cyt*c*, we did not observe such an increase in $k_{ET(Native)}$ relative to the horse protein in our previous work.[8]

Lys 79 is not implicated in any of Sites I−III;[33a,36d] however, studies on ferricyanide oxidation of reduced horse Cyt*c* show that blocking the amino groups of either Lys 79 or Lys 72 (Site III) decreases the ET rate by similar amounts.[36a] Thus, Lys 79 clearly affects ET of small-molecule reagents around the heme edge. It is also noteworthy that the differences in binding site preferences are small. For $Co(phen)_3^{3+}$, the ET rate enhancement due to a charge change blocking group at Lys 27 (Site II) is ∼7 whereas at Lys 72 (Site III) it is ∼3 at an ionic strength of 0.1.[36c] Since Lys 79 forms a salt bridge to heme propionate D in iso-1-Cyt*c*,[12] replacement of Lys 79 with Ala would uncover this negatively charged group. Thus, this mutation might be expected to have similar effects on intermolecular ET, as observed for the charge change blocking groups in the $Co(phen)_3^{3+}$ studies.[36b,c] The 3−5-fold enhancement we see in $k_{ET(Native)}$ for the A79H73 and A79G52 variants relative to wild-type iso-1-Cyt*c* for reduction with a_6Ru^{2+} near an ionic strength of 0.1 is certainly in line with the effects of blocking Lys 27 or Lys 72 with charge-change reagents on $Co(phen)_3^{3+}$ oxidation of horse Cyt*c* at the same ionic strength. Thus, a_6Ru^{2+} clearly reacts at the heme edge in a

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manner analogous to $Co(phen)_3^{3+}$. The preference among Sites I, II, and III is unclear from the current work.

Conclusions. We have demonstrated that the rate of an ET gate due to a His 73−heme alkaline conformer of iso-1-Cyt*c* can be fined tuned over the range 75−200 ms by varying pH and introducing mutations at surface-exposed sites in the substructure (red in Figure 1) of the protein involved in this conformational change. By exploiting the known hierarchical conformational thermodynamics of Cyt*c*, we have also designed a variant of iso-1-Cyt*c* that makes the slower Lys 73−heme ET gate accessible at neutral pH. These strategies permit key properties of ET gates to be manipulated to modulate protein function and to aid in the development of tailored components for protein-based molecular electronics devices.

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