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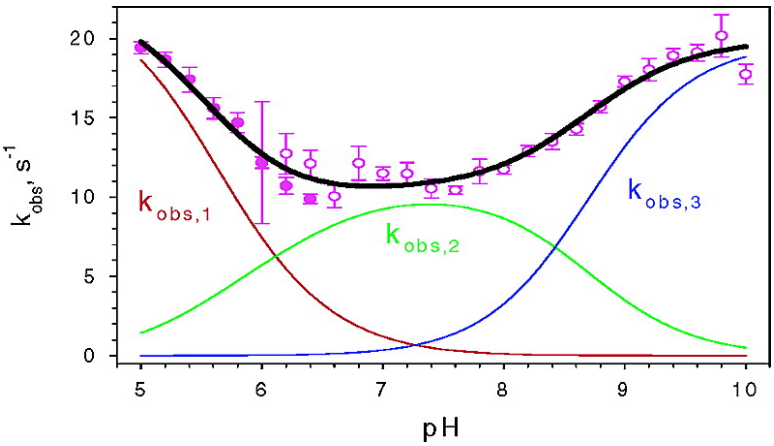

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Proton-Mediated Dynamics of the Alkaline Conformational Transition of Yeast Iso-1-Cytochrome *c*

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Abstract: The kinetics of the alkaline conformational transition of a Lys 73→His variant of iso-1-cytochrome *c* have been investigated using pH jump stopped-flow methods to probe the nature of the ionizable "trigger" group for this conformational change. This mutation moves the pK_a of the ligand replacing Met 80 from about 10.5 to approximately 6.6 and has unmasked two other ionizable groups, besides the ligand replacing Met 80, that modulate the kinetics of this process. The results are discussed in terms of the impact of ionization equilibria on protein folding mechanisms.

Introduction

At moderately alkaline pH values of 8.5 to 10, cytochrome *c* undergoes a small conformational change that is manifested by substitution of the methionine 80 (Met 80) ligand in the sixth coordination site of the heme.[1] Much progress has been made in understanding the properties of this conformational transition. Using site-directed mutagenesis, lysines 73 and 79 have been identified as the ligands which replace Met 80 in the alkaline state of yeast iso-1-cytochrome *c*.[2] NMR structural data for the lysine 73 liganded conformational state of yeast iso-1-cytochrome *c* have also recently been published.[3] However, despite much research, there is still significant debate about the identity of the ionizable group which triggers this conformational transition.[1,2b] Numerous kinetics studies have been undertaken to provide insight into the triggering process.[2b,4,5] Typically, kinetic data provide acid constants for the triggering group, pK_H, which are consistent with lysine. Thus, although there have been many proposals for the triggering group,[1,2b] it has been difficult to demonstrate a triggering deprotonation that is distinct from deprotonation of the incoming ligand.

Besides the long time interest in the alkaline conformational state of cytochrome *c*,[1] recent studies suggest that this conformational state of cytochrome *c* may coincide with a late intermediate along the folding pathway of this protein.[5c,6,7] Thermodynamically accessible partially unfolded states of proteins, similar in nature to the alkaline state of cytochrome *c*, have also been implicated as precursors in the nucleation of protein aggregation, which can lead to chronic physiological disorders.[8] A number of studies also suggest that the alkaline state of cytochrome *c* may play a role in modulating the electron-transfer dynamics of cytochrome *c* in the electron transport chain.[2b,9] Thus, a deeper understanding of the dynamics of the alkaline state of cytochrome *c* should provide insight into fundamental aspects of protein folding and misfolding and may potentially lead to greater understanding of the functional properties of this protein.

If deprotonation of groups other than the ligand replacing Met 80 is involved in triggering the alkaline conformational transition, one way to unmask such "trigger" groups is to move the pK_a of the ionizable protein side chain that replaces Met 80 in the alkaline state. Recently, we have characterized the equilibrium partial unfolding of iso-1-cytochrome *c* driven by a histidine 73 variant of the protein.[7] Equilibrium analysis of the alkaline conformational transition of this variant showed that the equilibrium pK_H is 6.6 in 0.1 M NaCl solution for the His 73-ligated alkaline state, consistent with the His 73 side chain pK_a controlling this conformational transition. Here, we analyze the kinetics of formation of the alkaline conformational state of His 73 iso-1-cytochrome *c* using pH jump stopped-

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flow methods. The data provide a direct demonstration that deprotonation of two other ionizable groups besides the ligand replacing Met 80 influence the dynamics of formation of the alkaline state of cytochrome *c*.

Materials and Methods

Protein Purification. The His 73 variant of iso-1-cytochrome *c* was isolated and purified from *Saccharomyces cerevisiae* GM-3C-2 cells (deficient in cytochrome *c*) carrying the pRS425/CYC1 phagemid vector using previously described methods[7,10] and was stored at −70 °C. Protein was thawed and purified by HPLC with a cation exchange column (Waters ProteinPak SP 8HR)[10b] just prior to use in stopped-flow experiments. The His 73 variant also has the Cys 102 replaced with a serine residue to avoid complications related to dimerization of protein molecules resulting from intermolecular disulfide cross-links.

Stopped-Flow Measurements. The His 73 variant was oxidized with $K_3Fe(CN)_6$ for 1 h at 4 °C. The protein was then run down a sephadex G-25 column using 100 mM NaCl as the eluant to separate the oxidizing agent from the protein. The concentration of the protein was determined spectrophotometrically, as previously described,[10a] and the volume was adjusted using 100 mM NaCl, until the desired concentration was obtained.

Oxidized His 73 protein at a final concentration of 20 μM and an initial pH of 5.0 was used in all upward pH jump experiments (pH adjusted with HCl). The buffers used to achieve the final pH were all 20 mM in buffer and contained 100 mM NaCl. Buffers used were as follows: MES (pH of 6.0 to 6.6), $NaH_2PO_4 \cdot H_2O$ (pH 6.8 to 7.6), Tris (pH 7.8 to 8.8), and H_3BO_3 (pH 9.0 to 10.0). For downward pH jumps, the pH of the starting protein solution was adjusted to pH 7.65 with NaOH solution. The buffers used to obtain the final pH were all 20 mM with 100 mM NaCl as for upward pH jumps. Buffers used were as follows: acetic acid (pH 5.0 to 5.4) and MES (pH 5.6 to 6.4). The pH was adjusted with NaOH for all buffers. Protein at pH 5.0 (upward jumps) or 7.65 (downward jumps) was mixed in a 1:1 ratio with the appropriate buffer to achieve the desired final pH, using an Applied Photophysics PiStar 180 spectrometer operating in kinetics mode. After mixing, the final protein concentration was 10 μM and the buffer concentration was 10 mM with 100 mM NaCl. Measurements of the pH of solutions after mixing indicated that shifts in the pH of the buffers upon mixing were negligible.

All pH jump experiments were carried out at 25 ± 0.1 °C (Thermo Neslab circulating water bath). The conformational change between the native and alkaline states was monitored by absorption spectroscopy at 406 nm, the wavelength of maximum change in absorbance for the conversion between the native and His 73-ligated alkaline state.[7c] The dead time of the stopped spectrometer was measured by mixing dichloroindole phenol with ascorbate at pH 2.0,[11] giving a value of 1.6 ms under the mixing conditions used in the experiments reported here. A minimum of five kinetic traces was collected at every pH. A total of 5000 points was collected on a logarithmic time scale for each kinetic trace.

Analysis of Kinetic Data. Analysis of the kinetic data obtained was performed using the curve fitting program SigmaPlot (v. 7.0). For upward pH jump data, kinetics traces were fit using equations for either a double (equation 1, data for pH 6.0 to 7.6) or triple exponential (equation 2, data for pH 7.8 to 10.0) rise to maximum. For downward pH jump data,

$$A_{406}(t) = A_{406}(\infty) + a_1(1 - \exp(-k_1 t)) + a_2(1 - \exp(-k_2 t)) \quad (1)$$

$$A_{406}(t) = A_{406}(\infty) + a_1(1 - \exp(-k_1 t)) + a_2(1 - \exp(-k_2 t)) + a_3(1 - \exp(-k_3 t)) \quad (2)$$

kinetic traces were fit using the equation for a triple exponential decay (eq 3).

$$A_{406}(t) = A_{406}(\infty) + a_1\exp(-k_1 t) + a_2\exp(-k_2 t) + a_3\exp(-k_3 t) \quad (3)$$

In these equations, $A_{406}(t)$ is the absorbance as a function of time at 406 nm; $A_{406}(\infty)$ is the absorbance at 406 nm at infinite time; a_1, a_2, and a_3 are amplitudes; and k_1, k_2, and k_3 are rate constants.

Results

General Features of Upward and Downward pH Jump Kinetics. Equilibrium data on His 73 iso-1-cytochrome *c* indicate that His 73 begins to displace Met 80 in the sixth coordination site of the heme near pH 6.0 reaching maximal population near pH 7.8.[7c] Above this pH, lysine 79 begins to displace His 73 as the alkaline state heme ligand, becoming essentially the sole ligand near pH 10. NMR data confirm this progression.[7c] Thus, separate kinetic phases might be expected for the native to His 73-ligated and native to Lys 79-ligated alkaline transitions. Upward pH jumps were initiated at pH 5.0 where equilibrium data indicate that His 73 iso-1-cytochrome *c* is fully native. Data were collected at final pH values from 6.0 to 10.0 to provide data that progress from a region dominated by His 73 ligation to one dominated by Lys 79 ligation. Representative kinetic traces for upward pH jump experiments are shown in Figure 1. Two obvious kinetic phases are observed, one which occurs on an approximately 100 ms time scale and the other on an approximately 25 s time scale. Below pH 7.8, the data are adequately fit by two first order rate processes. At pH 7.8 and above, significantly better fits to the data are obtained using three first-order rate processes to fit the data, the third phase occurring on an intermediate time scale. At pH 8.0, the fast phase is the dominant phase with a lower amplitude observed for the two slower phases. At pH 10, clearly the two slower phases have become dominant; however, the fast phase still persists.

For upward pH jumps, the amplitude of the fast phase slowly increases with pH, leveling off above pH 8 and remaining constant up to pH 10 (Figure 2). The amplitude of the slowest phase begins to increase near pH 8, leveling off near pH 9.4 (Figure 2). The growth of the amplitude of the fast phase is qualitatively consistent with the displacement of Met 80 by His 73 previously observed under equilibrium conditions, as pH is increased from 5 to 8.[7c] Similarly, the growth in amplitude of the slowest phase parallels the pH dependence of Lys 79 binding to heme observed for His 73 iso-1-cytochrome *c*[7c] and for Ala 73 iso-1-cytochrome *c*[2b] under equilibrium conditions. A fit of the slow phase amplitude to the Henderson−Hasselbalch equation gives an apparent pK_a of 8.7 ± 0.1, within error of the value of 8.8 observed for the Lys 79 alkaline state.[2b,7c] Based on the behavior of the amplitude of the fast and slow phases as a function of pH, we assign the fast phase to the formation of the His 73-ligated alkaline state from the native state and the slow phase to the formation of the Lys 79-ligated alkaline state from the native state. Equilibrium analysis indicates that the His 73-ligated alkaline state reaches a maximum population near pH 7.6 and then decreases as it is replaced by the Lys 79-ligated alkaline state.[7c] However, in Figure 2, the amplitude of the fast kinetic phase assigned to formation of the His 73-ligated alkaline

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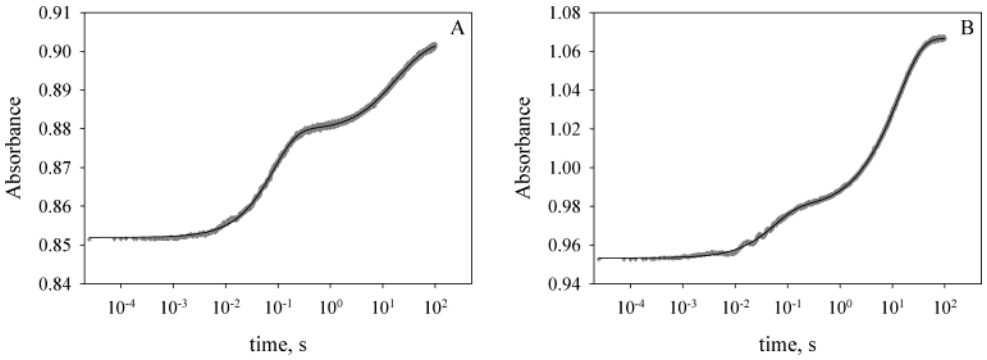


Figure 1. Plot of A_{406} vs time (on a logarithmic scale) for His 73 iso-1-cytochrome *c*. Data at 406 nm (gray dots) as a function of time were collected in 0.1 M NaCl at 25 °C with the protein concentration near 10 μM. The buffers in the final mixtures were (A) 10 mM Tris, pH 8.0 and (B) 10 mM H_3BO_3, pH 10.0. The pH jump experiments were carried out as described in Materials and Methods. The solid black curves are fits of the data to a triple exponential rise to maximum (eq 2, Materials and Methods) obtained with nonlinear least-squares methods using SigmaPlot (v. 7).

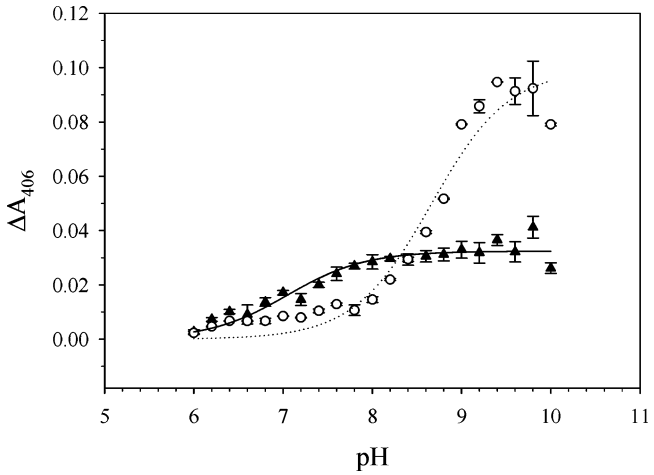


Figure 2. Plot of change in amplitude, ΔA_{406}, vs pH for the fast (▲) and slow (○) phases for upward pH jumps with His 73 iso-1-cytochrome *c*. Data were collected at 406 nm in 0.1 M NaCl at 25 °C with the protein concentration near 10 μM. Amplitudes for each kinetic phase were determined from fits to kinetics traces using eq 1 and 2 in Materials and Methods. Error bars represent the standard deviation of at least five separate kinetics traces. The solid curve is a fit of the amplitude of the fast phase to the kinetic model described in the Discussion section (eq 6). The dotted line is a fit of the slow phase amplitude to the kinetic model described in the Discussion section (eq 8).

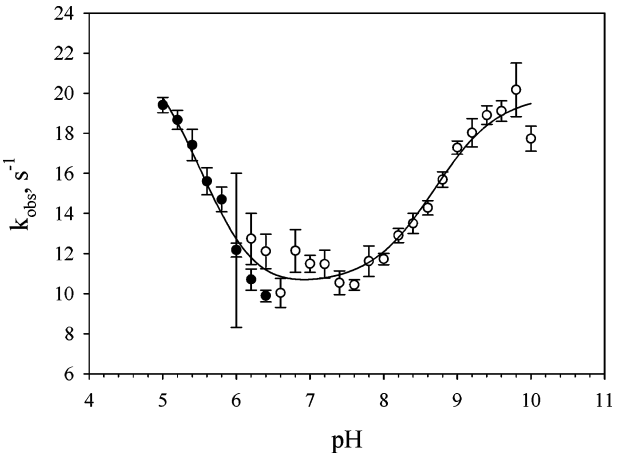
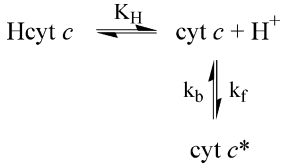


Figure 3. Plot of k_{obs} vs pH for the fast phase of the alkaline conformational transition of His 73 iso-1-cytochrome *c*. Data were collected at 406 nm in 0.1 M NaCl at 25 °C with protein concentration near 10 μM. Data for this plot were collected and analyzed as described in Materials and Methods. Data points for downward jumps are shown with solid circles. Data for upward pH jumps are shown with open circles. Error bars represent one standard deviation. The larger error bar at pH 6 is for the upward pH jump data point at this pH. The solid curve is a fit of the data to the kinetic model described in the Discussion section (eq 5) with the conformational equilibrium constant for the interconversion between the native and His 73-ligated alkaline states assumed to be 0.5 (see ref 7c).

state persists at a constant level all the way out to pH 10. Evidently, the faster kinetics of formation of the His 73-ligated alkaline state allow it to reach its maximal equilibrium population ($K_{eq} \approx 0.5$ at pH's where His 73 is fully deprotonated[7c]) above pH 8 before being displaced by the more favorable Lys 79-ligated alkaline state on a longer time scale. The amplitude of the intermediate phase observed above pH 7.8 shows no trend as a function of pH, having a value of 0.008 ± 0.002 absorbance units (au) throughout the pH range where it is observed (data not shown).

From equilibrium data,[7c] the amplitude for upward pH jumps was expected to be too small to make reliable measurements below pH 6.0. To obtain kinetic data for the alkaline conformational transition below pH 6, downward pH jumps from pH 7.65 to pH values from 5.0 to 6.4 were carried out to obtain k_{obs}. Again three kinetic phases, fast (50−100 ms time scale), intermediate (10 s time scale), and slow (~50 s time scale) were observed. Within error, the amplitude of each phase was invariant with final pH (fast, 0.013 ± 0.001 au; intermediate, 0.008 ± 0.001 au; slow, 0.017 ± 0.001 au). The summed amplitude was 0.038 ± 0.003 au, consistent with the summed amplitude observed for upward jumps from 5 to 7.6 or 7.8.

Scheme 1. Standard Kinetic Model for the Alkaline Conformational Transition



pH Dependence of the Rate Constant, k_{obs}, for the Fast Phase. A prime interest of this work was to observe the pH dependence of k_{obs} for the alkaline conformational transition between the native state of iso-1-cytochrome *c* and the His 73-ligated alkaline state with the intent of unmasking other ionizable "trigger" groups which modulate this transition. The amplitude data indicate that the fast phase occurring on the 50−100 ms time scale corresponds to this conformational transition. Figure 3 shows the variation in k_{obs} as a function of pH for the fast phase. In the standard model for the alkaline transition of cytochrome *c*, a rapid protonation equilibrium precedes the

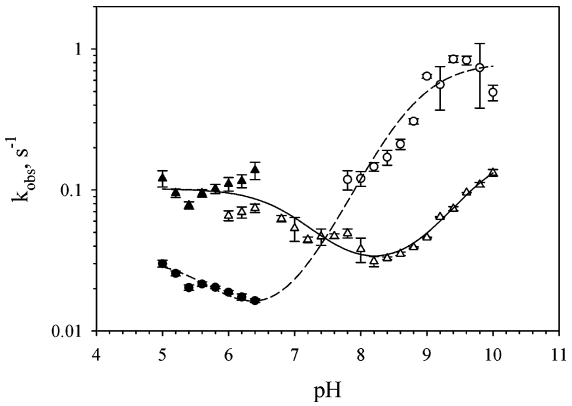


Figure 4. Plot of k_{obs} vs pH for the slow and intermediate phases of the alkaline conformational transition of His 73 iso-1-cytochrome *c*. Data were collected at 406 nm in 0.1 M NaCl at 25 °C with the protein concentration near 10 μM. Data for this plot were collected and analyzed as described in Materials and Methods. Data points for downward jumps are shown with solid triangles (intermediate phase) and solid circles (slow phase). Data for upward pH jumps are shown with open circles (intermediate phase) and open triangles (slow phase). Error bars represent one standard deviation. The solid curve is a fit of the data for the equilibrium between the native and Lys 79-ligated alkaline states (open and solid triangles) to the kinetic model described in the Discussion section (eq 7). The dashed curve is a fit of the data for the slow phase for the equilibrium between the native and His 73-ligated alkaline state (solid and open circles) to the model described in the Discussion section (eq 7) with pK_{H1} set equal to 5.6, k_{b1} set equal to 0.033 s^{-1}, and k_{b2} set to 0.01 s^{-1}.

conformational change (Scheme 1). This model predicts that the k_{obs} for the alkaline transition will follow eq 4.

$$k_{obs} = k_b + k_f\left(\frac{K_H}{K_H + H^+}\right) \quad (4)$$

At low pH, k_{obs} is predicted to be coincident with k_b, rising to a value equal to the sum of k_b and k_f with a pK_a consistent with the acid ionization constant, K_H (or pK_H), of the "trigger" group. Clearly, the behavior of the His 73-mediated alkaline transition of iso-1-cytochrome *c* is more complex. The k_{obs} for this transition decreases from pH 5 to 6, appears to level out from approximately pH 6 to 8, and then rises again from pH 8 to 10. The increase in k_{obs} occurring from pH 8 to 10 is qualitatively consistent with the simple model shown in Scheme 1. However, if this portion of the pH dependence follows the model in Scheme 1, the pK_H for the "trigger" group is inconsistent with the pK_a of the His 73 ligand replacing Met 80 in the alkaline conformer. The thermodynamics of the His 73-mediated transition are controlled by an ionizable side chain with a pK_a of 6.6 in 0.1 M NaCl, consistent with His 73 ionization.[7c] Thermodynamic control of the fast phase with a pK_a in the 6.5 to 7 range is also evident from the growth in the amplitude of this phase shown in Figure 2. Evidently, proton mediation of the alkaline conformational transition involving His 73 ligation is complex.

pH Dependence of the Rate Constants for the Intermediate and Slow Phases. Slow and intermediate time scale events are also associated with the alkaline conformational transition of the His 73 variant of iso-1-cytochrome *c*. As noted above, the growth in the amplitude of the slow phase for upward pH jumps is consistent with equilibrium data for formation of the Lys 79-ligated alkaline state.[7c] Figure 4 shows the pH dependence of k_{obs} for these two phases for both upward and downward pH jump experiments. For upward jumps, as noted above, it is not possible to distinguish distinct intermediate and slow phases below pH 7.8. The intermediate phase k_{obs} values for downward jumps do not change with pH, within error. The amplitude for this phase is consistent with the amplitude expected for the conversion of the Lys 79-ligated alkaline state to the native state, for a downward jump from pH 7.65. In Figure 4, the magnitude of k_{obs} for the slow phase for upward pH jumps and the intermediate phase for downward jumps appears to merge in the pH range 6.0 to 6.5. The k_{obs} data therefore indicate that the rate of interconversion of the native and Lys 79-ligated alkaline states decreases as pH increases from 6 to 8 and then increases again above pH 8. The k_{obs} values for the native state to Lys 79-ligated alkaline state interconversion are still increasing at pH 10 indicating that the pK_a of the group causing k_{obs} to increase is greater than the pK_a of the group affecting the fast phase above pH 8.

The k_{obs} for the slow phase in the downward jumps, appears to increase as the pH drops from 6.4 to 5.0 in analogy to the behavior of the fast phase (Figure 3). The intermediate phase for upward jumps increases from pH 7.8 to 10, also similar to the behavior of the fast phase. The pH behavior of these two phases suggests that both are associated with the interconversion between the native and His 73-ligated alkaline states, since they respond to pH in a similar manner.

Discussion

Assignment of Kinetic Phases Observed for the Alkaline Conformational Transition of His 73 Iso-1-Cytochrome *c*. As discussed above, the behavior of the amplitude for the fast phase (Figure 2) observed for interconversion between the native and alkaline states of His 73 iso-1-cytochrome *c* is consistent with its assignment to formation of the His 73-ligated alkaline state of this protein. Similarly, the amplitude data for the slow phase for upward pH jumps and the intermediate phase for downward pH jumps are consistent with the assignment of these two phases to the interconversion between the native and Lys 79-ligated alkaline states. The observation that k_{obs} for these two phases merges (Figure 4) strengthens this assignment.

The intermediate phase for upward jumps has an amplitude that is invariant, within error, over the pH range where it can be distinguished. It is noteworthy for upward pH jumps that from pH 7.8 to 10, where the intermediate phase is detected, the amplitude of the fast phase associated with formation of the His 73-ligated alkaline state is also invariant. For downward jumps from pH 7.65, the amplitude of the fast phase (0.013 ± 0.001 au) is less than might be expected based on the amplitude of the fast phase for upward pH jumps to pH 7.6 to 7.8 (0.024 to 0.027 au). The amplitude for the slow phase in downward jumps (0.017 ± 0.001 au) is large relative to the amplitude for the intermediate phase for upward pH jumps (0.008 ± 0.002 au). However, the sum of the amplitude for the phases we assign to the interconversion between the native state and His 73-ligated alkaline state for upward (0.032 to 0.035) and downward jumps (0.030 ± 0.002) is similar. Thus, it appears that partitioning between the fast and slow phases associated with the equilibrium between the native state and the His 73-ligated alkaline state changes depending on the direction of the pH jump.

The time scale of the slow phases associated with the native to His 73-ligated alkaline state interconversion is in the range expected for cis/trans proline isomerization,[12] and all prolines

are trans in the native state of iso-1-cytochrome *c*.[13] Moreover, proline isomerization provides a plausible explanation for the large redistribution between the amplitude of the fast and slow phases for the native to His 73-ligated alkaline state interconversion. In upward jumps, the equilibrium constant for the His 73-ligated alkaline state at high pH relative to the native state is 0.5, giving a limiting fractional population of the His 73-ligated alkaline state of 0.33 above pH 8.[7c] Fluorescence-detected folding of iso-2-cytochrome *c* to the alkaline state indicates that slow phases associated with proline isomerization are not observed,[14] in contrast to the observation of slow proline isomerization phases during folding to the native state.[12] This observation suggests that the alkaline state does not change the distribution of proline isomers relative to the unfolded state. Thus, approximately one-third of the His 73 iso-1-cytochrome *c* molecules will be able to isomerize away from the all trans proline conformation of the native state after pH jumps to pH 8 or above. Two-thirds of these isomerized molecules will equilibrate back into the native state (equilibrium constant for formation of the His 73-ligated alkaline state is 0.5) leading to the observed intermediate phase at high pH. Thus, the fraction of molecules responsible for the slow phase will be ~0.22 for upward pH jumps. At equilibrium, at pH 7.6 to 7.8, the fractional population of the native state is 0.6 for His 73 iso-1-cytochrome *c*, the remainder being a combination of the His 73-ligated and Lys 79-ligated alkaline states. Thus, the fractional population of molecules where proline can isomerize away from the all trans state will be ~0.4. For downward pH jumps from pH 7.6−7.8 to pH 5−6, all these molecules will return to the native state and will contribute to a proline-mediated slow phase. Thus, the amplitude of the slow phase for downward pH jumps should approximately double relative to the amplitude of the intermediate phase for upward pH jumps, as is observed (intermediate phase for upward pH jumps, $\Delta A_{406} = 0.008 \pm 0.002$ au; slow phase for downward pH jumps, $\Delta A_{406} = 0.017 \pm 0.001$ au). Kinetic folding data for a Pro 76→Gly variant of iso-2-cytochrome *c* suggest that Pro 76 could be responsible for the slower phases associated with the native state to His 73-ligated alkaline state interconversion.[12,14]

For wild-type iso-1-cytochrome *c*, no proline isomerization kinetic phase is observed for the alkaline conformational transition.[2b] Near neutral pH, the time scale of the phase we assign to proline isomerization is similar to k_{obs} for the native to alkaline conformer interconversion for wild-type iso-1-cytochrome *c*.[2b] Above pH 8, proline isomerization is always faster than k_{obs} for the native to alkaline interconversion for wild-type iso-1-cytochrome *c*.[2b] So, proline isomerization should not be observable in the kinetics of the isomerization from the native to alkaline state of wild-type iso-1-cytochrome *c*.

Mechanistic Implications of the pH Dependence of the Fast Phase for Interconversion between the Native and His 73-Ligated Alkaline Conformations of His 73 Iso-1-cytochrome *c*. The standard mechanistic scheme for the alkaline conformational transition of cytochrome *c* (Scheme 1) is clearly inadequate to describe the interconversion between the native and His 73-ligated alkaline conformers of the His 73 variant of iso-1-cytochrome *c*. The amplitude of the fast phase associated with this interconversion grows in over a pH range consistent with the involvement of the ionization of His 73 (Figure 2), as was demonstrated for the equilibrium thermodynamic analysis of this conformational interconversion.[7c] The pH dependence of k_{obs} for the fast phase (Figure 3) indicates that two other ionizable groups affect the kinetics of this interconversion. Thus, three ionizable groups control or "trigger" the kinetics of formation of the His 73-ligated alkaline state.

Since three ionizable groups affect the kinetics of the native state to His 73-ligated alkaline state conformational change, the mechanism for this process must involve three ionizable groups. A plausible mechanism is presented in Scheme 2, where C and C(FeL) represent native and alkaline cytochrome *c*, LH^+ and L represent the protonated and deprotonated forms of the ligand that replaces Met 80 in the alkaline state, and XH+/X and YH+/Y are the two ionizable groups that affect the rate of the conformational interconversion. The mechanism assumes that the ligand, L, must be ionized for it to bind to the iron of the heme replacing Met 80. This assumption is used because the amplitude of the His 73 alkaline state grows in over the pH range where His 73 is expected to ionize (see Figure 2), and so the His 73-ligated alkaline state does not populate without ionization of the His 73 ligand.

The derivation of the expressions for the pH dependences of k_{obs} and the amplitude of the reaction (see Supporting Information) is similar to the derivation of these dependencies for the simpler scheme for the alkaline transition (Scheme 1 and eq 4) presented by Davis et al.[4a] Equations 5 and 6 describe the pH dependences for k_{obs} and the amplitude at 406 nm for upward pH jumps, ΔA_{406}, respectively, due to the mechanism in Scheme 2.

$$k_{obs} = \left[\left(\frac{K_{HL}}{K_{HL} + [H^+]}\right) \times \left(\frac{k_{f1}[H^+]^2 + k_{f2}K_{H1}[H^+] + k_{f3}K_{H1}K_{H2}}{K_{H1}K_{H2} + K_{H1}[H^+] + [H^+]^2}\right)\right] + \left(\frac{k_{b1}[H^+]^2 + k_{b2}K_{H1}[H^+] + k_{b3}K_{H1}K_{H2}}{K_{H1}K_{H2} + K_{H1}[H^+] + [H^+]^2}\right) \quad (5)$$

$$\Delta A_{406} = [C_T] \times \left\{\frac{\left(\frac{K_{HL}}{K_{HL} + [H^+]}\right)}{\left(\frac{K_{HL}}{K_{HL} + [H^+]}\right) + \left(\frac{k_{b1}[H^+]^2 + k_{b2}K_{H1}[H^+] + k_{b3}K_{H1}K_{H2}}{k_{f1}[H^+]^2 + k_{f2}K_{H1}[H^+] + k_{f3}K_{H1}K_{H2}}\right)}\right\} \quad (6)$$

In eq 5, the first term expresses the pH dependence of the forward rate for the native to alkaline state interconversion, and the second term, that of the back rate. The $(K_{HL}/(K_{HL} + [H^+]))$ factor in the forward rate constant term has two important consequences. One consequence is that the effect of ionizable groups on the forward rate of the alkaline transition will be masked by the pK_a of the ligand replacing Met 80 (pK_{HL}) if the pK_a of the group is lower than that of the ligand replacing Met

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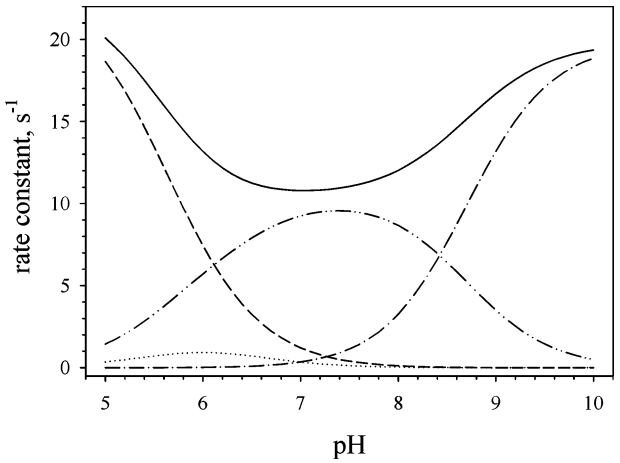
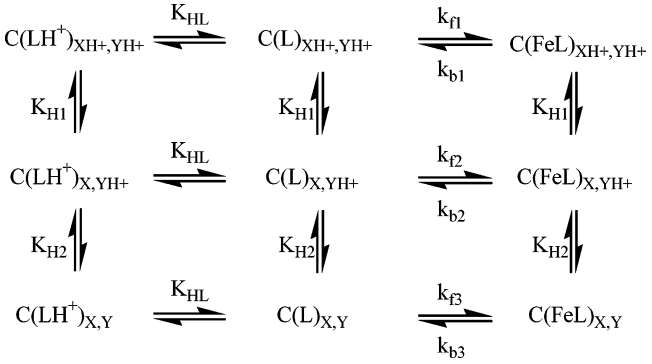


Figure 5. pH dependence of the components of k_{obs} for the fast phase of the alkaline conformational transition of His 73 iso-1-cytochrome *c*. The solid line is the fit of the data in Figure 3, using eq 5 with the parameters from Table 1. The dashed line shows the pH dependence of $k_{obs,1}$ ($k_{f1} + k_{b1}$), the dash−dot-dot−dashed line shows the pH dependence of $k_{obs,2}$ ($k_{f2} + k_{b2}$), and the dash−dot−dashed line shows the pH dependence of $k_{obs,3}$ ($k_{f3} + k_{b3}$). The dotted line shows the pH dependence of k_{f1}, demonstrating that its contribution to the fit is minimal, as discussed in the text.

Scheme 2. Proposed Mechanism for Formation of the His 73 Alkaline State



80 (see Figure 5). The second consequence of the (K_{HL}/(K_{HL} + [H^+])) term is that changes in k_{obs} at pH values below pK_{HL} will reflect changes in the rate constant for the back reaction. Thus, if pK_{HL} is greater than pK_{H1}, k_{obs} may increase or decrease depending on the relative magnitudes of k_{b1} and k_{b2}. Whether or not k_{obs} for the alkaline transition increases or decreases as pH increases has been used as a measure of whether deprotonation of a "trigger" group precedes or follows the conformational change, respectively. Scheme 2 and eq 5 demonstrate that effects on k_{obs} at pH values below the pK_a of the ligand replacing Met 80 (pK_{HL}) cannot necessarily be used to inform on this aspect of the mechanism when multiple ionizations affect the mechanism.

When rate constants for the alkaline conformational transition change, as a result of an ionization event, the ionization event may be affecting the stabilities of the transition state, the native state, or the alkaline state. For the pH effect on k_{obs} in Figure 3 occurring above pH 8, it is clear that the relative stabilities of the native and alkaline state do not change, since the amplitude of the fast phase (Figure 2) is invariant. Thus, we can assume the ratio of the forward and back rate constants is unchanged by this ionization, simplifying curve fitting. From thermodynamic studies,[7c] we know that $k_f/k_b = 0.5$ for the native to His 73-ligated alkaline transition. In Figure 3 the solid line is a fit

Table 1. Rate and Ionization Constants Associated with the Fast Phase of the Alkaline Transition of His 73 Iso-1-cytochrome *c*

Rate Constants, s^{-1}	
k_{f1}	11.4 ± 0.9
k_{b1}	23 ± 2
k_{f2}	3.5 ± 0.2
k_{b2}	7.0 ± 0.4
k_{f3}	6.6 ± 0.2
k_{b3}	13.2 ± 0.4
Ionization Constants	
pK_{H1}	5.6 ± 0.2
pK_{HL}	6.4 ± 0.5
pK_{H2}	8.7 ± 0.2

of k_{obs} to eq 5 where k_f/k_b is assumed to be 0.5 at all pH values. The parameters from this fit are collected in Table 1. The effects of the pK_{H1} and pK_{H2} ionizations are substantial leading to changes in the rate constants for this conformational interconversion of 2- to 3-fold.

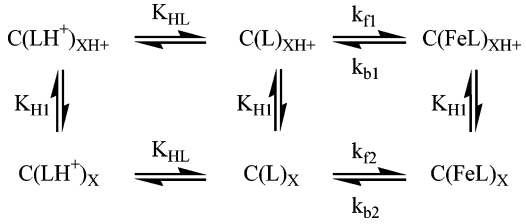
To fit the amplitude of the fast phase, the rate constants in Table 1 and the ionization constants pK_{H1} and pK_{H2} were input into eq 6, so that the only adjustable parameter in the fit was pK_{HL}. The solid line in Figure 2 is the fit of the amplitude to eq 6, yielding pK_{HL} = 7.2 ± 0.1. The values obtained for pK_{HL} by fitting amplitude and k_{obs} data (see Table 1) are both reasonably close to the value of 6.6 ± 0.1 obtained from thermodynamic analysis of the alkaline transition of His 73 iso-1-cytochrome *c* and attributed to the ionization of His 73.[7c]

A simple visual inspection of the data in Figure 3 might suggest that only two ionizable groups affect k_{obs}. However, as noted above, the amplitude of this phase grows in over a pH range consistent with the ionization of His 73, the ligand which controls the thermodynamics of formation of the His 73-ligated alkaline state.[7c] As can be seen in Figure 5, the decrease in k_b (k_{b1} changing to k_{b2}) due to pK_{H1} over the pH range 5 to 7 masks the increase in k_{obs} due to ionization of His 73 (pK_{HL}) that is occurring in an overlapping pH range. As noted above, forward rate constants do not contribute significantly below the pK_a of the ligand replacing Met 80 (pK_{HL}). The dotted line in Figure 5 demonstrates the minor contribution of k_{f1}. It is important to note that, due to the minor contribution of k_{f1} to our fit, its magnitude relies primarily on the assumption that $k_f/k_b = 0.5$, which may not be true at lower pH values. Thus, the magnitude of k_{f1} is not well-determined.

From the parameters in Table 1 and the trends seen in Figure 5, it is evident that pK_{H1}, pK_{HL}, and pK_{H2} all have important effects on the rate of the alkaline conformational transition to the His 73-ligated state of the His 73 iso-1-cytochrome *c* variant. Although, it is clear that His 73 deprotonation (pK_{HL}) is essential from the alkaline state to populate, the roles of the other deprotonation reactions (pK_{H1} and pK_{H2}) are also important in triggering this process. The pK_{H1} ionization slows the back rate, k_b, for the reaction by a factor of 3. Without this drop in k_b, population of the His 73-ligated alkaline state would be much lower. Thus, both pK_{H1} and pK_{HL} should be viewed as important thermodynamic and kinetic triggers of this conformational change. The triggering action of the pK_{H2} ionization is solely through the kinetics. It enhances the rate at which the conformational change achieves equilibrium.

The pK_{H1} and pK_{H2} values of 5.6 and 8.7, respectively, which modulate k_{obs} for the fast phase associated with formation of the His 73-ligated alkaline state do not correspond to any ion-

Scheme 3. Proposed Mechanism for Formation of the Lys 79 Alkaline State of His 73 Iso-1-cytochrome *c*



izable groups in native iso-1-cytochrome *c*.[15−18] Since the pK_{H2} ionization does not affect the relative stabilities of the native state and His 73-ligated alkaline state, and since native iso-1-cytochrome *c* has no ionizable group with a pK_a near 8.7, the increase in k_{obs} due to the pK_{H2} ionization likely results from selective stabilization of the transition state or a transient intermediate by an ionization event. It has been pointed out by Mauk and co-workers that minimally an intermediate with neither Met 80 nor a lysine (His 73 in our case) ligand must exist.[5b] Englander and co-workers[5c] have recently provided evidence that transient intermediates involving unfolding of the two least stable substructures (residues 40 to 57 and 71 to 85, respectively)[6a] of cytochrome *c* are involved in promoting the alkaline transition. Assignment of pK_{H2} to a specific group is not possible at this time; however, its association with the transition state or a transient intermediate is clear. Assignment of the ionizable group corresponding to p$K_{H1} = 5.6$ to a transient intermediate versus the alkaline state cannot be done, since we have no definitive evidence that the k_f/k_b ratio for the native state to His 73-ligated alkaline state conformational change remains at 0.5 below pH 6.

Previously reported kinetic data for the alkaline isomerization of cytochrome *c* have not provided evidence for more than a single ionization affecting the kinetics below pH 10, although there is evidence for a fast phase affected by other ionizations above pH 10.[2b,4,5] For example, only one ionization affects k_{obs} for the alkaline conformational transition of a Lys 79→Ala variant of iso-1-cytochrome *c*. However, in the model in Scheme 2, the increase in k_{obs} would act primarily through k_b at pH values below the ionization of the incoming ligand (Lys 73 for the Lys 79→Ala variant). The 2-fold increase in k_b which would result from the ionization of a group with a pK_a near 8.7 would likely be obscured by the onset of the growth in k_f observed for this variant in this pH regime (see Figure 10 in ref 2b). With few exceptions,[5a,d] kinetic data on the alkaline transition of cytochrome *c* have been collected above pH 7, so an ionization below pH 6 affecting the kinetics of the alkaline conformation has not be observed previously.

Mechanistic Implications of the pH Dependence of the Rate of Interconversion between the Native and the Lys 79-Ligated Alkaline Conformations of His 73 Iso-1-cytochrome *c*. The data in Figure 4 (open and solid triangles) show the pH dependence of the interconversion between the native and Lys 79-ligated alkaline states of His 73 iso-1-cytochrome *c*. There appear to be two ionizable groups affecting the kinetics of this interconversion. The data are consistent with the mechanism shown in Scheme 3. This mechanism yields eq 7 for k_{obs} and eq 8 for the amplitude of k_{obs} for upward pH jumps.

$$k_{obs} = \left(\frac{K_{HL}}{K_{HL} + [H^+]}\right)\left(\frac{k_{f1}[H^+] + k_{f2}K_{H1}}{K_{HL} + [H^+]}\right) + \left(\frac{k_{b1}[H^+] + k_{b2}K_{H1}}{K_{HL} + [H^+]}\right) \quad (7)$$

$$\text{Amplitude} = [C_T]\left\{\frac{\left(\frac{K_{HL}}{K_{HL} + [H^+]}\right)}{\left(\frac{K_{HL}}{K_{HL} + [H^+]}\right) + \left(\frac{k_{b1}[H^+] + k_{b2}K_{H1}}{k_{f1}[H^+] + k_{f2}K_{H1}}\right)}\right\} \quad (8)$$

The dotted line in Figure 2 shows the fit of eq 8 to the observed amplitude for upward pH jumps. In fitting the data in Figure 4 to eq 7, the $K_{HL}/(K_{HL} + [H^+])$ term is expected to make the fit insensitive to the forward rate below the pH where the incoming Lys 79 ligand ionizes (see Figure 5), so the second bracket in the first term has been simplified to its high pH limit, k_{f2}. The fit is reasonable and yields p$K_{H1} = 7.0 \pm 0.3$, p$K_{HL} = 9.8 \pm 0.4$, $k_{b1} = 0.10 \pm 0.01$ s^{-1}, $k_{f2} = 0.18 \pm 0.08$ s^{-1}, and $k_{b2} = 0.026 \pm 0.011$ s^{-1}. The magnitude of pK_{H1} is consistent with the pK_a of His 73 (6.6 ± 0.1),[7c] suggesting that its protonation lowers the barrier to the back reaction to the native state. The pK_{HL} value is low compared to p$K_H = 10.8 \pm 0.1$ observed for a Lys 73→Ala variant of iso-1-cytochrome *c*.[2b] The conformational equilibrium constant, $K_c = k_f/k_b$, is also about an order of magnitude low compared to data for a Lys 73→Ala variant of iso-1-cytochrome *c*.[2b] The errors in pK_{HL}, k_{f2}, and k_{b2} are large, however. We see no clear evidence of an ionization with a pK_a of 5.6 modulating the k_{obs} for the native state to Lys 79-ligated alkaline state conformational transition in His 73 iso-1-cytochrome *c*. Lack of observation of an effect on k_{obs} in this pH regime could be due to the scatter in the data or might indicate that this ionization does not influence the native state to Lys 79-ligated alkaline state conformational transition.

pH Dependence of the Proline Isomerization Associated with the Native State to His 73-Ligated Alkaline State Isomerization for His 73 Iso-1-cytochrome *c*. The k_{obs} data we have tentatively assigned to isomerization of Pro 76 in conjunction with the native state to His 73-ligated alkaline state conformational change shows significant pH dependence. The k_{obs} data in Figure 4 (open and solid circles) were fit to a kinetic model with two ionizations analogous to that shown in Scheme 3. However, due to the lack of data between pH 6.4 and 7.8, the fit (dashed line) was constrained to have pK_{H1} equal to 5.6 and k_{b1} and k_{b2} were set to values providing for a good fit to the data for downward pH jumps (solid circles). Only pK_{H2} and k_{f2} were allowed to vary in the fit, yielding p$K_{H2} = 8.8 \pm 0.1$ and $k_{f2} = 0.79 \pm 0.07$ s^{-1}. The data are, thus, consistent with the ionizations pK_{H1} and pK_{H2} observed for the interconversion between the native state and His 73-ligated alkaline state being important for the isomerization of Pro 76. For pK_{H2}, this observation suggests that the isomerization of Pro 76 to the all trans conformation observed in the native state is promoted by a dynamic equilibrium with a transient intermediate.

Implications for Protein Folding and the Function of Iso-1-Cytochrome *c*. A number of recent studies have indicated

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that the alkaline conformer of cytochrome *c* may be related to a late folding intermediate on the folding pathway of this protein.[5c,7] The data presented here indicate that ionization of groups in non-native states of a protein can have a large influence on the rate of conformational interconversions, since the pK_a values of the observed ionizations do not correspond to ionizations observed in the native state of the protein. In both cases, the ionizations are in pH regimes that do not correspond to "normal" pK_a values for any of the amino acid side chains, suggesting that the ionizable groups involved are ones that have abnormal pK_a values in the native state. Ionization of such groups in non-native states at their "normal" pK_a values or at partially shifted pK_a values is expected to have important differential effects on the relative stabilities of native and non-native states, and thus pH is expected to have significant effects on the rate of conformational interconversion.

It is also interesting to note that the slowest rate of interconversion between the native and His 73-ligated alkaline states of iso-1-cytochrome *c*, indicating the highest kinetic barrier to formation of non-native states or transient intermediates, occurs in the pH range 6 to 8 (see Figure 3 and Table 1). Many studies indicate that proteins are more likely to aggregate at nonphysiological pH values due to the greater accessibility of non-native states.[8b,19] For cytochrome *c*, the barriers toward the interconversion of native and the most accessible non-native state are evidently at a maximum around physiological pH. For cytochrome *c* this would inhibit possible pathological aggregation. Maximizing kinetic barriers to partial unfolding near physiological pH may be one means of preventing pathological aggregation of proteins in living organisms.

The pH dependence observed for the kinetic phase tentatively assigned to Pro 76 isomerization has interesting implications for the mechanism of proline isomerization during protein folding. Our data are consistent with access to a non-native state promoting isomerization to the correct native state proline isomer, since the rate of this isomerization increases when barriers to non-native states are lowered. This mechanism is reminiscent of the folding mechanism proposed recently by Gray and Winkler and their co-workers[20] which suggests that access to expanded states is necessary to allow trapped compact states to fold productively to the native state.

With regard to the function of cytochrome *c*, the pH dependence of k_{obs} seen in Figure 3 could provide a mechanism by which cytochrome *c* could act to gate the flow of electrons along the electron transport chain. It has been proposed that the lower potential alkaline form of cytochrome *c* could provide a more efficient means to transfer electrons into cytochrome *c* oxidase.[2b,9] When the proton gradient is small across the inner mitochondrial membrane (pH high in the intermembrane space), the kinetic accessibility of the low potential alkaline form could be enhanced by pK_{H2}, increasing the efficiency of electron flow along the electron transport chain and speeding recovery of the proton gradient. On the other hand when the proton gradient is high (pH low in the intermembrane space), the increased k_b seen in Figure 3 could decrease the accessibility of the low potential alkaline state and slow electron flow along the electron transport chain, when energy stored in the proton gradient is plentiful.

Conclusion

The pH dependence of the kinetics of the conformational change between the native state and His 73-ligated alkaline state of a His 73 variant of iso-1-cytochrome *c* demonstrates that three ionizable groups affect this process. Thus, the kinetics of the alkaline conformational transition of iso-1-cytochrome *c* appear to involve more than a single trigger group.

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Supporting Information Available: Tabulated rate constant and amplitude data; derivations of the pH dependence of k_{obs} and the amplitude for alkaline conformational transitions involving two and three ionizations. This material is available free of charge via the Internet at http://pubs.acs.org.

JA0494454

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