Hydrophobic Effect of Trityrosine on Heme Ligand Exchange during Folding of Cytochrome *c*

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Abstract

Effect of a hydrophobic peptide on folding of cytochrome *c* (cyt *c*) is studied with trityrosine. Folding of cyt *c* was initiated by pH jump from 2.3 (acid-unfolded) to 4.2 (folded). The Soret band of the 2-ms transient absorption spectrum during folding decreased its intensity and red-shifted from 397 to 400 nm by interaction with trityrosine, whereas tyrosinol caused no significant effect. The change in the transient absorption spectrum by interaction with trityrosine was similar to that obtained with 100 mM imidazole, which showed that the population of the intermediate His/His coordinated species increased during folding of cyt *c* by interaction with trityrosine. The absorption change was biphasic, the fast phase ($82 \pm 9\ s^{-1}$) corresponding to the transition from the His/H_2O to the His/Met coordinated species, whereas the slow phase ($24 \pm 3\ s^{-1}$) from His/His to His/Met. By addition of trityrosine, the relative ratio of the slow phase increased, due to increase of the His/His species at the initial stage of folding. According to the resonance Raman spectra of cyt *c*, the high-spin 6-coordinate and low-spin 6-coordinate species were dominated at pH 2.3 and 4.2, respectively, and these species were not affected by addition of trityrosine. These results demonstrated that the His/His species increased by interaction with trityrosine at the initial stage of cyt *c* folding, whereas the heme coordination structure was not affected by trityrosine when the protein was completely unfolded or folded. Hydrophobic peptides thus may be useful to study the effects of hydrophobic interactions on protein folding.

Cytochrome *c* (cyt *c*) is frequently used as a model for protein folding studies. The axial ligands of the heme iron for native cyt *c* are Met80 and His18 [1, 2], whereas a non-native histidine instead of Met80 coordinates to the iron when the protein is unfolded with a high denaturant concentration at neutral pH [3, 4]. Both His26 and His33 have been implicated as the non-native histidine [4, 5], but it has been suggested by cyt *c* mutant studies that His33 is the dominant sixth heme ligand in the unfolded state [3]. At low pH, one or both of the axial ligands are replaced by water molecules [6, 7].

The heme and its axial ligands are essential for structural stabilization [8] and function [9] of cyt *c*, and the axial ligands of the heme affect the folding dynamics of cyt *c* [3, 10-16]. Among the three kinetic phases observed as changes in Soret absorbance and tryptophan fluorescence during folding of cyt *c*, the intermediate phase with a time constant of about 240 ms disappeared at low pH, whereas the intermediate and slow phases both disappeared by addition of an excess of an extrinsic imidazole, indicating that the bis-histidine (His/His) coordinated form of the unfolded protein creates a kinetic barrier during folding of the protein [10]. Changes in the heme axial ligands during folding of cyt *c* have been studied in detail for the time scale from 100 μs to 40 ms by resonance Raman (RR) spectroscopy combined with a rapid mixer [13-15]. Replacement of the axial heme ligand of cyt *c* occurs even after a few milliseconds during its folding process, and the native His/Met coordinated species is formed directly from the His/H_2O species.

There are various molecules including charged and hydrophobic molecules in organism, and they could affect the folding process of proteins. Cyt *c* is substantially unfolded with low ionic strength at low pH, whereas the protein cooperatively folds to a compact structure with the properties of the molten globule state by addition of salts, due to binding of anions to the positively charged groups on the protein surface [17, 18]. The anion

size plays a role in determining the overall structure of the molten globule state [19-23]. Hydrophobic interactions have also been known to play an important role on the folded structure of proteins [24-26]. Negatively charged lipid membranes induce an extensive disruption of the native structure of cyt *c* [27]. Apocytochrome *c* undergoes a conformational transition from a random coil to a α-helical structure by interaction with phospholipid vesicles or lysophospholipid micelles, which accounts for both electrostatic and hydrophobic contributions [28]. The effect of 8-anilino-1-naphtalene sulfonic acid (ANS), a charged hydrophobic dye, was found to induce a secondary structure similar to the native protein in the acid-unfolded cyt *c*, while the tertiary structure was disrupted, indicating that ANS stabilizes a molten globule state in the acid-unfolded protein [29].

We have previously shown that charged peptides bind to the protein surfaces and affect the active site structures of cyt *c* and other metalloproteins [30-33]. Although lipids are known to affect the unfolded and folded structures of cyt *c*, the effects of hydrophobic peptides on the folding process of cyt *c* have not been studied. We therefore investigated the hydrophobic effect on the folding character of cyt *c* by using trityrosine and found that it affects the heme coordination structure at its initial folding stage.

Materials and methods

Sample preparation. Purification of oxidized horse heart cyt *c* (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was performed with a CM52 column (Whatman) after oxidizing it by adding a small amount of potassium ferricyanide. Trityrosine was purchased from Bachem, and dityrosine and tyrosinol were purchased from Sigma.

Optical absorption measurements. Folding of cyt *c* from its acid-unfolded state to its native state was measured with an Otsuka Denshi RA601 stopped-flow system. A 42 μM solution of cyt *c* in 10 mM HCl (pH 2.3) was diluted 6-fold with 20 mM acetate buffer, pH 4.3, to a final pH of 4.2. To investigate the effect of trityosine on the axial ligand exchange process of cyt *c* during folding, we added trityrosine (0 ~ 2.7 mM) to the acetic acid buffer solution, the final peptide concentration being 0 ~ 2.3 mM after mixing the two solutions. Dityrosine (4 mM), tyrosinol (8 mM), and imidazole (117 mM) were added to the buffer solution for measurements in the presence of 3.4 mM dityrosine, 6.9 mM tyrosinol, and 100 mM imidazole, respectively. All the kinetic measurements were performed at 5˚C. Least-squares fittings of the time-resolved absorption data were performed with double exponential functions using Igor Pro ver. 4.0 (WaveMetrics).

Resonance Raman measurements. Resonance Raman (RR) scattering was excited at 406.7 nm with a Kr^+ ion laser (Spectra Physics, 2060) and detected with a CCD (PI-CCD, Princeton Instruments) attached to a single polychromator (Ritsu Oyo Kogaku, DG-1000). The slit width and slit height were set to 200 μm and 10 mm, respectively. The excitation laser beam power (at the sample point) was adjusted to 8 mW. Measurements were carried out at room temperature with a spinning cell (3000 rpm). The data accumulation time was 100 s. Raman shifts were calibrated with acetone and the accuracy of the peak positions of the Raman bands was ±1 cm^{-1}. Oxidized cyt *c* (25 μM) was dissolved in 10 mM HCl (pH 2.3) or 17 mM acetate buffer including 1.4 mM HCl (pH 4.2) in the presence and absence of 2.3 mM trityrosine.

Results

Effect of trityrosine on transient absorption spectra of cyt c *folding*

Cyt *c* is unfolded at acidic pH, whereas it can refold by raising the pH. The transient absorption spectra during folding of cyt *c* were obtained by pH jump from 2.3 to 4.2. The Soret band was observed at 397 nm for the 2-ms transient absorption spectrum, and the peak position of the Soret band gradually shifted to 407 nm, which shift accomplished within about 50 ms (Fig. 1A). Although the absorption at 403 nm was almost constant for the transient absorption spectra, a small deviation was detectable around 403 nm for the reaction earlier than 20 ms, indicating contribution of at least two phases during folding of cyt *c*. These absorption changes correspond to the heme axial ligand exchanges during folding of cyt *c*.

By addition of trityrosine, the transient absorption spectra were affected, especially for the spectra earlier than 20 ms (Fig. 1B), although the absorption spectra after completion of folding in the presence and absence of trityrosine were similar (Fig. 1). The Soret band of the 2-ms transient species decreased its intensity and red-shifted for about 3 nm by addition of trityrosine, whereas tyrosinol did not affect the transient absorption spectrum significantly (Fig. 2). Dityrosine caused the Soret band of the 2-ms transient spectrum to slightly decrease its intensity and red-shifted a little (Fig. 2, curve b). The transient absorption spectra during folding of cyt *c*, however, were not affected by charged peptides, such as tetraaspartic acid and tetralysine, or a non-charged peptide without an aromatic residue, tetraglycine (data not shown).

The difference spectrum between the 2-ms transient absorption spectra in the presence and absence of trityrosine exhibited a peak and tough at 412 and 394 nm, respectively (Fig. 3).

The intensities of the peak and trough increased as we increased the concentration of trityrosine, and the difference spectra of the transient absorption spectra between with and without trityrosine were similar to the difference spectrum between with and without imidazole (Fig 3, inset). Addition of imidazole increases the His/His coordinated species [10], and thus some amount of the His/H_2O species should have been converted to the His/His species at the initial stage of folding by interaction with trityrosine.

Kinetics of absorption change during folding of cyt c

The most prominent absorption changes were observed at 394 and 412 nm, and the absorption values were fitted successfully with double exponential functions, $a_0 + a_1\exp(-k_1t) + a_2\exp(-k_2t)$. The obtained rate constants were similar between the fitting at two wavelengths and are listed in Table 1. A fast phase with a rate constant of 82 ± 9 s^{-1} and a slow phase with 24 ± 3 s^{-1} was observed during folding of cyt *c* from the oxidized acid-unfolded to the native form. These phases corresponded to the axial ligand exchange processes, as has been reported previously [10, 11]. The non-native histidine-heme iron ligation is known to create a kinetic barrier for folding, and the associated histidine ligation rate is reported to decrease for about 10^{-3} between before and after the nascent phase [11, 34]. The His/Met native folded species is reported to form directly from the His/H_2O species [15]. The fast phase thus should correspond to conversion from the His/H_2O species to the His/Met species, whereas the slow phase from the His/His species to the His/Met species by way of the His/H_2O species [15].

The absorption difference between the 2-ms intermediate species and the folded species decreased by addition of 2.3 mM trityrosine, since the 2-ms intermediate absorption spectrum changed by addition of trityrosine. The ratio of the slow phase increased for about

40 % by interaction with trityrosine, whereas that of the fast phase decreased for about the same amount. These results show that trityrosine interacts with the folding intermediate of cyt *c* and stabilizes the His/His coordinated species at the initial stage of folding. Tyrosinol, however, did not affect the kinetics of the axial ligand exchange process during folding of cyt *c* (Table 1).

Resonance Raman spectra of cyt c

The RR spectra of acid-unfolded and native oxidized cyt *c* with and without trityrosine are shown in Fig. 4. The RR spectra in the high frequency region (1200–1700 cm^{-1}), where skeletal stretching vibrations of the porphyrin macrocycle appear, reflect the oxidation state, coordination number, and the spin state of the heme iron [35-37]. The ν_3 band is sensitive to the coordination- and spin-state states, and appears at 1502–1507 cm^{-1} for six-coordinate ferric high-spin species (S = 5/2) and at 1480–1483 cm^{-1} for six-coordinate ferric low-spin species (S = 1/2) [35]. It has been shown that five-coordinate, His/H_2O coordinated, and His/His coordinated species exhibit the ν_3 band at 1574, 1571, and 1588 cm^{-1}, respectively, for unfolded cyt *c* [13]. The ν_3 band of acid-unfolded cyt *c* in 10 mM HCl, pH 2.3, was observed at 1573 cm^{-1} (Fig. 4, curve b), and thus the five-coordinate species is the dominant species under this condition, which is consistent with the pK_a value of 3.8 for the transition between the five-coordinate and His/H_2O species. For the folded protein in 17 mM acetate buffer, pH 4.2, including 1.4 mM HCl, the ν_3 band was observed at 1585 cm^{-1} and the RR spectrum was identical with the that of the His/Met coordinated cyt *c* (Fig. 4, curve d) [13]. No significant change was observed in the RR spectra between in the presence

and absence of trityrosine at both pH 2.3 and 4.2, which indicated that trityrosine does not affect the coordination structure of unfolded or folded cyt *c*

Discussion

Axial ligand exchange during folding of cyt c

For the unfolded protein, the pK_a between the His/H_2O and His/His species and between the five-coordinate and His/H_2O species are reported to be 5.2 and 3.8, respectively, [15]. The main species for the acid-unfolded cyt *c* at pH 2.3 thus is the five-coordinate species (Fig. 5). Since the RR spectrum of the folded protein in 17 mM acetate buffer, pH 4.2, including 1.4 M HCl was similar to that of the His/Met coordinated species, the main species of the folded protein was the His/Met coordinated species under the condition used.

During refolding of cyt *c* by pH jump (2.3 → 4.2), non-native ligands were still coordinated to the heme after a nascent phase faster than 100 μs [13]. The Soret maximum wavelength of unfolded cyt *c* at pH 2.3 was observed at 394 nm (data not shown), which red-shifted to 397 nm during the nascent phase (< 100 μs) (Fig. 2, curve a). This wavelength shift is due to conversion of the unfolded five-coordinate species to the molten globule His/H_2O or His/His species or a mixture of these species (Fig. 5) [13]. Actually, the kinetics of the absorption changes during folding was biphasic, which showed that the molten globule state under the experimental condition in this study is a mixture of His/H_2O and His/His coordinated species. It has been pointed out that the His/H_2O and His/His species exist after the fast nascent phase of folding at pH 4.5 with 0.7 M GdnHCl [13].

Effect of trityrosine on the initial transient species during cyt c *folding*

All of the side chains of the tyrosine within trityrosine are protonated at pH 4.3, since the pKa of trityrosine are reported to be 9.56, 9.95, and 10.92 [38]. It has been reported that the tyrosine at the distal histidine position of the H64Y mutant myoglobin coordinates to the heme iron, but dissociates when it is protonated [39]. The tyrosine side chain of trityrosine thus may not coordinate to the heme iron during folding of cyt *c* under acidic conditions. No significant change was detected in the CD spectrum of acid-unfolded cyt *c* by addition of trityrosine (data not shown). These results demonstrate that the protein structure was not affected significantly by trityrosine, although lipids affect the unfolded protein structure. No significant difference was observed in the RR spectra of cyt *c* between with and without trityrosine (Fig. 4), and thus the protein and coordination structures of unfolded and folded cyt *c* are not affected by trityrosine.

The effect of the axial ligands on the Soret absorption band of cyt *c* has been investigated with the use of the N-terminal acetylated microperoxidase 8 (AcMP8), the heme octapeptide obtained by enzymatic digestion of horse cyt *c* [40, 41]. (H_2O)(His18)Fe(III)AcMP8 is a mixed-spin, six-coordinate complex with water or hydroxide coordinating as the sixth ligand (λ_{max} = 394 nm). Imidazole (Im) readily replaces the water axial ligand of (H_2O)(His18)Fe(III)AcMP8, generating the low-spin (Im)(His18)Fe(III)AcMP8 species (λ_{max} = 404 nm) [42]. The (Met)(His18)Fe(III)AcMP8 species was obtained by addition of 2 M methionine to (H_2O)(His18)Fe(III)AcMP8 (λ_{max} = 410 nm) [40]. During folding of cyt *c* by pH jump, the 2-ms transient absorption spectrum red-shifted for about 3 nm by addition of trityrosine (Fig. 2, curve a). The red-shift of the

Soret band suggests that the His/H_2O species converted to the His/His coordinated species by interaction with trityrosine. Actually, the difference spectrum of the 2-ms transient absorption spectra between with and without trityrosine exhibited a similar pattern as the spectrum between with and without 100 mM imidazole (Fig. 3).

Hydrophobic effect of trityrosine on cyt c *folding*

Since cyt *c* is positively charged, charged peptides may affect the folding process of cyt *c*. Actually, negatively charged aspartic acid peptides interacted with cyt *c* at its surface and perturbed its heme site [31]. However, the Soret band of the 2-ms transient absorption spectrum and thus the ligand-exchange process during folding of cyt *c*, which were detected in the time scale of about 10─50 ms during folding of cyt *c*, were not affected significantly by addition of charged peptides, such as tetraaspartic acid and tetralysine. Tetraglycine did not affect the transient absorption spectrum significantly, whereas it red-shifted and decreased its intensity by interaction with trityrosine (Fig. 2). These results show that the aromatic rings of trityrosine may play an important role in the coordination structure during folding of cyt *c*.

Red-shift of the Soret band of cyt *c* by addition of trityrosine was observed for the initial transient species during folding of cyt *c* (Fig. 2), although the RR spectra of the native and acid-unfolded species at pH 4.2 and 2.3, respectively, were not affected significantly (Fig. 4). Trityrosine thus could interact most effectively when the protein forms a loose structure, but may not interact with a completely folded or unfolded protein. When the protein is folded, most of the hydrophobic amino acids, which contain an aromatic group, such as tyrosine, phenylalanine, and tryptophan, are positioned around the heme of cyt *c* [1, 2]. Hydrophobic trityrosine may interact with these hydrophobic amino acid residues when the protein forms a

loose structure, and thus the hydrophobicity around the heme could promote histidine to access to the heme, whereas hydrophobic trityrosine could not interact with the protein when it is completely folded, since the peptides could not access to the hydrophobic residues inside the protein.

Comparison between effects of trityrosine and lipids on cyt c *folding*

Lipid membranes induce an extensive disruption of the native structure of cyt *c* [27]. Lipids also affect the acid-unfolded structure [28], and apocytochrome *c* undergoes a conformational transition from a random coil to a α-helical structure by interaction with phospholipid vesicles or lysophospholipid micelles. Since lipids affect both the acid-unfolded and native folded structures of cyt *c*, the hydrophobic effect of lipids or other molecules on the protein folding character is difficult to investigate. Trityrosine used in this study, however, did not affect the acid-unfolded and native folded structure of cyt *c* significantly and thus it is useful to use trityrosine to study the hydrophobic effects on folding of cyt *c*. We note again that hydrophobic peptides affected the axial ligand exchange process during folding of cyt *c* by increasing the His/His coordinated species at its initial folding stage.

In summary, trityrosine increased the intermediate His/His species during folding of cyt *c* induced by pH jump from 2.3 to 4.2. Trityrosine interacted effectively with cyt *c* when it formed a transient loose structure during folding, but not with the completely folded or unfolded protein. These results show that short hydrophobic peptides could affect the folding properties of proteins and are useful to study the effect of hydrophobic interactions on protein folding.

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Table 1

Kinetic parameters for folding of cyt *c* from the acid-unfolded to the folded state in the presence of trityrosine or tyrosinol[a]

	k_1 (s^{-1})	a_1		k_2 (s^{-1})	a_2	
		394 nm	412 nm		394 nm	412 nm
none	82 ± 9	0.21 ± 0.02	-0.13 ± 0.01	24 ± 3	0.027 ± 0.004	-0.021 ± 0.005
Trityrosine[b]	98 ± 12	0.13 ± 0.02	-0.10 ± 0.01	22 ± 4	0.038 ± 0.003	-0.029 ± 0.005
Tyrosinol[c]	75 ± 8	0.20 ± 0.02	-0.13 ± 0.01	20 ± 4	0.027 ± 0.004	-0.016 ± 0.005

[a] Reaction was performed by pH jump from 2.3 to 4.2 at 5°C. [b] Concentration of 2.3 mM. [c] Concentration of 6.9 mM.

Figure legends

Fig. 1. Transient absorption spectra during folding of oxidized cyt *c* (6 μM) (A) without and (B) with trityrosine (2.3 mM). Spectra are at 2, 5, 10, 20, 50, 100, and 500 ms after the mixing. The reaction was performed by pH jump from 2.3 (10 mM HCl) to 4.2 (17 mM acetate buffer including 1.4 mM HCl) at 5˚C.

Fig. 2. Transient absorption spectra at 2 ms during folding of oxidized cyt *c* (6 μM): With (a, dashed curve) 2.3 mM trityrosine, (b, gray curve) 3.4 mM dityrosine, and (c, dotted curve) 6.9 mM tyrosinol, and (d, dark curve) without them. Other experimental conditions were the same as those for Fig. 1.

Fig. 3. Difference spectra of the 2-ms transient absorption spectra during folding of oxidized cyt *c* (6 μM) between with and without trityrosine (0.6, 1.2, 1.8, and 2.3 mM). Darker spectra correspond to higher trityrosine concentration. Inset: Difference spectrum of the 2-ms transient absorption spectra during folding of oxidized cyt *c* (6 μM) between with and without 100 mM imidazole. Other experimental conditions were the same as those for Fig. 1.

Fig. 4. RR spectra in the 1200–1700 cm^{-1} region for oxidized cyt *c* (25 μM) (a,c) with and (b,d) without 2.3 mM trityrosine at (a,b) pH 2.3 and (c,d) 4.2. The ordinate scales in the spectra are normalized with the intensity of the 1371 cm^{-1} band. Experimental conditions: slit width 200 μm; slight height, 10 mm; excitation wavelength, 406.7 nm; laser power, 8 mW (at

the sample point); room temperature. Cyt *c* was dissolved in 10 mM HCl, pH 2.3, or 17 mM acetate buffer, pH 4.2, including 1.4 mM HCl.

Fig. 5. Schematic view of the effect of trityrosine on the cyt *c* folding process. The boxes represent the major species at each stage.

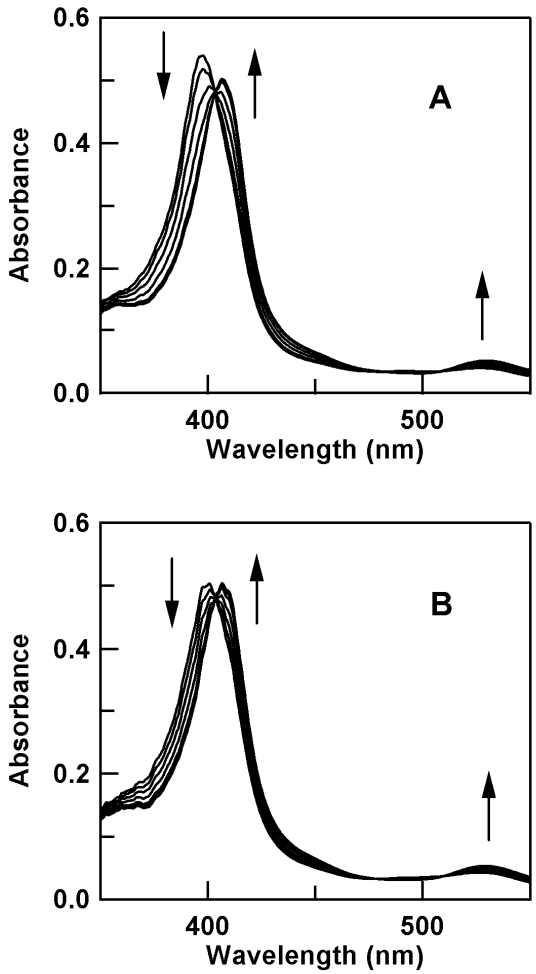

Figure 1

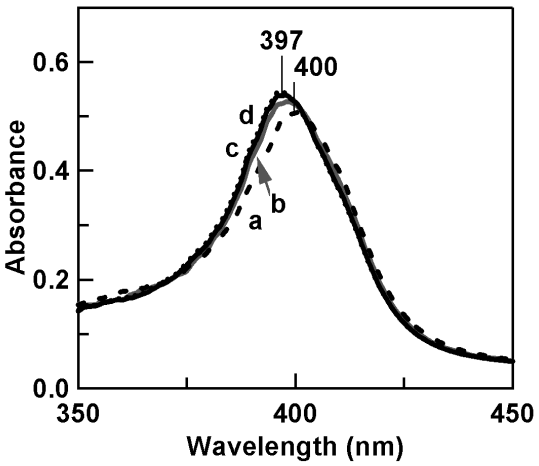

Figure 2

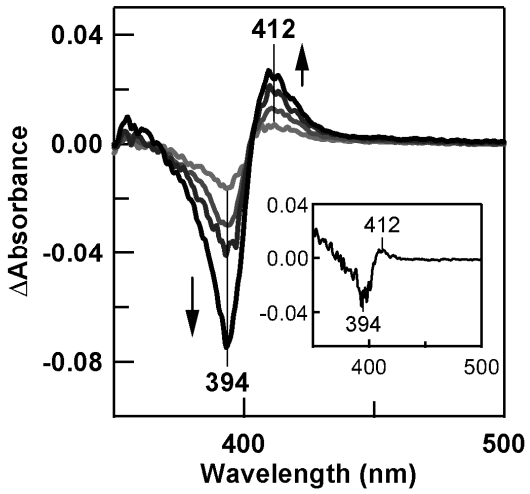
0.04
0.00
-0.04
-0.08
ΔAbsorbance
412
394
400
500
Wavelength (nm)
0.04
0.00
-0.04
412
394
400
500

Figure 3

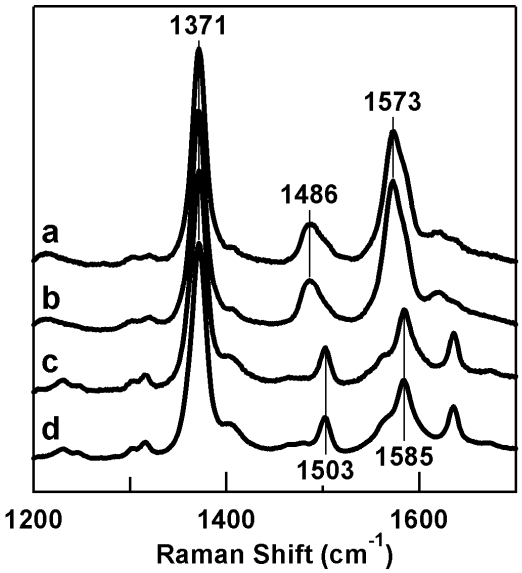

Figure 4

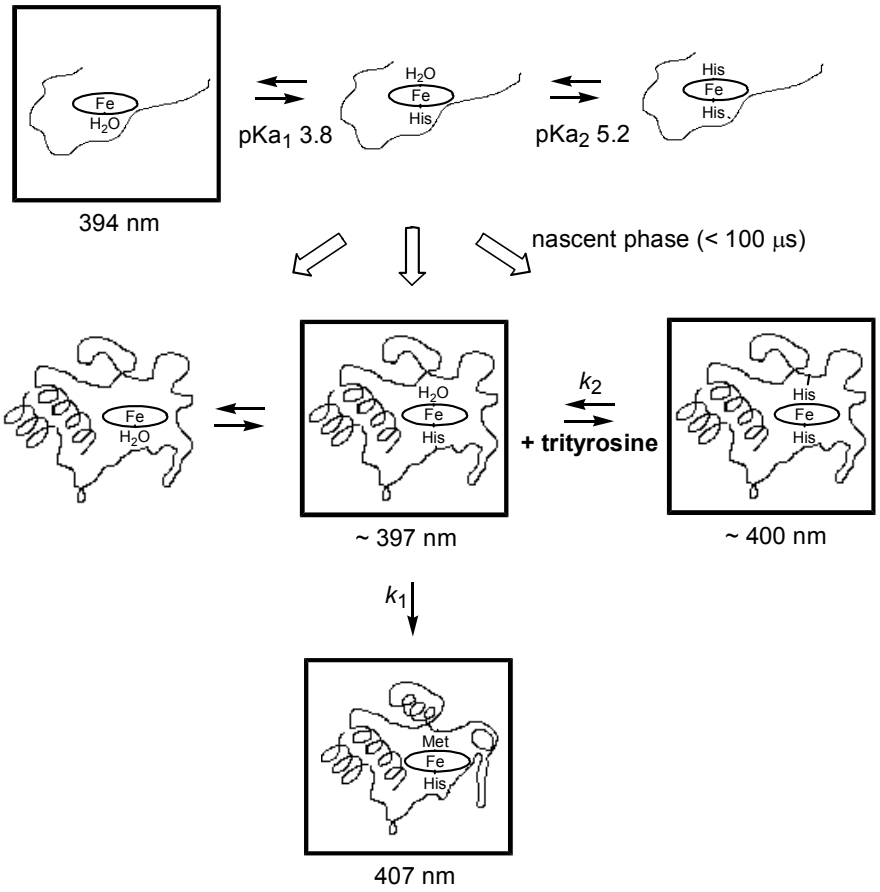
Fe
H2O
394 nm
pKa1 3.8
H2O
Fe
His
pKa2 5.2
His
Fe
His
nascent phase (< 100 μs)
Fe
H2O
H2O
Fe
His
~ 397 nm
k2
+ trityrosine
His
Fe
His
~ 400 nm
k1
Met
Fe
His
407 nm

Figure 5