Alkaline Conformational Transitions of Ferricytochrome *c* Studied by Resonance Raman Spectroscopy

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Abstract: The pH-dependent conformational equilibria of iso-1-ferricytochrome *c* that occur between pH 7 and pH 12 have been studied by resonance Raman (RR) spectroscopy. Detailed analysis of the RR spectra provides the number and the spectra of the conformational species that occur over this range of pH as well as their relative concentrations at each pH. Between pH 7 and pH 12, the wild-type protein exhibits five conformers which differ with respect to the axial ligands of the heme iron. In the medium alkaline pH range, the neutral form undergoes two parallel conformational transitions with similar pK_as (8.7 and 8.9) to those of the states IVa and IVb. Comparative studies with protein variants in which either Lys79 or Lys73 or both lysyl residues were replaced by alanine(s) confirm previous conclusions (Rosell et al., preceding paper in this issue) that Lys73 and Lys79 are the axial ligands replacing Met80 in the states IVa and IVb, respectively. On the basis of isotopic shifts caused by H/D exchange, the Fe−N(Lys) stretching vibrations of these species were identified at 385 cm^{-1}. In the high alkaline pH range, the wild-type protein forms two further conformers with pK_as of 10.5 and 11. In these states, Va and Vb, which are also detected in the Lys73Ala, Lys79Ala, and the Lys73Ala/Lys79Ala variant proteins, another strong-field ligand, presumably a hydroxide, occupies the sixth axial coordination site. Comparison of the RR spectra demonstrates far-reaching similarities between state Va and the conformational state B2 that cytochrome *c* forms in electrostatically stabilized complexes at neutral pH where, inter alia, Lys73 and Lys79 are involved in intermolecular interactions. This finding as well as the dramatically lowered pK_as (<10) for formation of Va and Vb in the double variant (Lys73Ala/Lys79Ala) suggest that the residues 73 and 79 are part of a switching mechanism by which conformational changes at the heme pocket are induced to regulate the function of the protein in biological electron-transfer reactions.

Between pH 7 and pH 12, ferricytochrome *c* undergoes a series of reversible conformational transitions that involve the substitution of the axial methionine ligand (Met80) of the heme iron.[1,2] State III (the native conformation) prevails at neutral pH and is converted to state IV with pK_a values between 8.5 and 9.5 that depend on the species of the cytochrome. At more alkaline pH, state V is formed. The transitions between these conformational states have been studied by a variety of physical and spectroscopic techniques, but the structural changes involved have resisted unambiguous definition.[1]

Understanding the conformational pH dependence of ferricytochrome *c* is of interest for several reasons. First, an increasing number of proteins is now recognized as exhibiting pH-linked conformational diversity.[3] Ferricytochrome *c* serves as an excellent model of such systems owing in part to the variety of informative spectroscopic techniques that can be used to characterize these conformational states and the mechanisms by which they interconvert and to extensive structural characterization of the native protein by X-ray diffraction analysis.[4] Second, as the structure of cytochrome *c* changes with pH, the functional properties of the protein also change. For example, in previous work, we demonstrated that, as native cytochrome *c* is titrated to alkaline pH, the reduction potential of the protein decreases by nearly 500 mV.[5] For this reason, the interconversion of native and alkaline cytochrome *c* can be regarded as an efficient binary switch that has a dramatic effect on the activity of the protein in electron-transfer reactions. Third, spectroscopic

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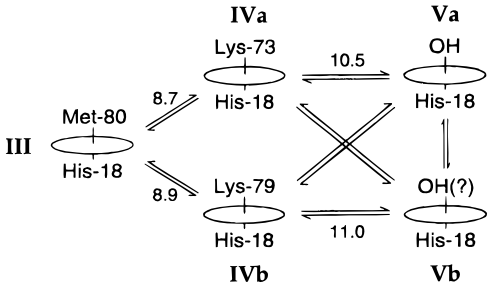
Scheme 1. Conformational Equilibria of Wild-Type Ferricytochrome *c* in the pH Range between 7.0 and 12.0



studies indicate that pH-linked conformational changes of cytochrome *c* result in both local structural changes in the heme pocket and more global changes of the protein matrix.[1,6] Finally, MCD and CD studies have provided evidence that the structural changes occurring at high pH may be related to those associated with the electron-transfer process of cytochrome *c* in the complex with its natural reaction partners.[7]

In the present work, we have employed resonance Raman (RR) spectroscopy to analyze the alkaline equilibria of wild-type and variant forms of yeast iso-1-ferricytochrome *c*. This technique offers the advantage of focusing selectively on the vibrational spectrum of the heme to provide detailed information about changes of the ligation state as well as changes in protein−heme interactions.[8] The primary goal of the current study was to analyze the conformational equilibria in the pH range between 7 and 12 (Scheme 1) quantitatively and to characterize the heme structure of the species involved. While for the transitions between states III and IV these investigations complement the previous spectroscopic studies through use of NMR and EPR spectroscopic techniques,[9] extending the experiments to ferricytochrome *c* at high alkaline solutions (i.e., pH > 10) allows the identification and structural characterization of the state V. In this way, a comprehensive picture of the alkaline conformational transitions is obtained which provides insight into the structural flexibility of ferricytochrome *c*. Finally, our findings are discussed in the context of the previously observed relationship between the alkaline species and the physiologically reactive species of cytochrome *c*.[7]

Experimental Section

Sample Preparation. The wild-type and variant forms of yeast iso-1-cytochrome *c* used in this work were constructed, expressed, and purified as described previously.[9,10] For the RR experiments, ferricytochrome *c* samples (15 μM) were studied in sodium or potassium phosphate buffer (150 mM), which controlled the pH within ±0.1 pH units during the RR experiments (i.e., 6−8 h). Lower buffer concentrations resulted in time-dependent pH changes of ≤0.3 pH units. pH values in D_2O were not corrected for the deuterium isotope effect. A catalytic amount of cytochrome *c* oxidase (<100 nM) was added to each sample to maintain the cytochrome in the oxidized state.

Spectroscopic Measurements. RR spectra were measured with the output of a Kr^+ laser with a double monochromator equipped with a photon counting system. For all the spectra reported in this work, the 413-nm excitation line was used (~25 mW focused by a 10-cm lens onto the sample). The spectral resolution was 2.8 cm^{-1}, and the step width (increment per data point) was 0.2 cm^{-1}. The signal-to-noise (S/N) ratio was improved upon repetitive scanning so that the total accumulation time was ~10 s per data point. The reproducibility of the monochromator during the experiments was ±0.1 cm^{-1} as checked by repeated calibration against the position of the laser line. The sample was placed in a rotating cell to avoid photoinduced damage. Under these conditions and at pH < 11, the protein was stable for at least 8 h as required to achieve an acceptable S/N ratio. At higher pH, time-dependent changes in the RR spectra revealed a decreased stability that necessitated replacement of the sample after 4−6 h. All measurements were carried out at ambient temperature. Further details of the experimental setup as well as of the divided-cell technique that was employed in some experiments are described elsewhere.[11,12]

Analysis of Spectra. The single-scan spectra of each experiment were carefully compared and combined only if no spectral differences were noted. The spectra obtained in this way included a continuous and structureless background that was removed by polynomial subtraction. The spectra were analyzed by a specialized component analysis developed for this purpose.[13] The principle of this approach is that a series of spectra (i.e., pH-dependent RR spectra) can be simulated adequately by a superposition of the invariant (pH-independent) spectra of all components that exist in the pH range under consideration. Spectra of individual species that are not known a priori are approximated iteratively. In this way, it is possible to determine the number of components involved, their spectra, and their relative contributions to the individual, experimentally determined spectra. Reliable criteria for the goodness of the fits and the validity of the underlying assumption (i.e., number of components) are the relative standard deviation (Δrms) and the average maximum errors of the spectroscopic parameters of the unknown species as discussed in detail previously.[13]

Results and Discussion

The RR spectroscopic investigations were focused on two frequency regions that provide complementary information about the heme pocket structure. The region between 1300 and 1700 cm^{-1} includes a number of bands that are known to be diagnostic markers for the oxidation, spin, and coordination state.[8,14] Furthermore, these bands may provide information regarding the geometry of the porphyrin.[11b,15] The mode assignment in this frequency range is straightforward and discussed extensively elsewhere.[16]

The region between 200 and 500 cm^{-1} can be regarded as the fingerprint region for the characteristic heme−protein interactions of each cytochrome conformer, although there are no unique correlations between specific structural properties of

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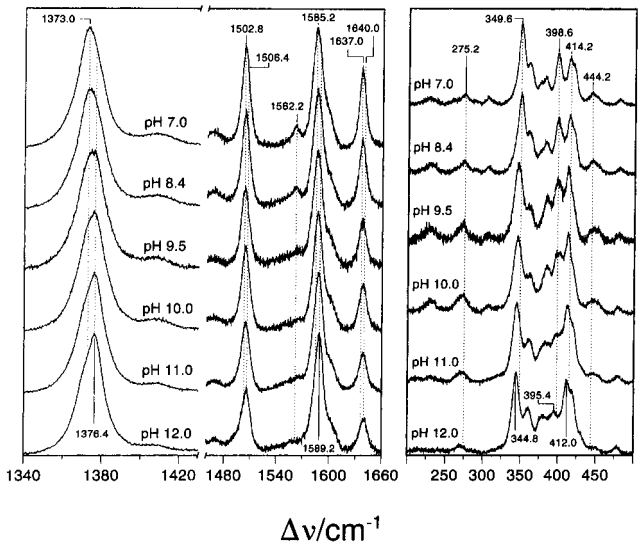

Figure 1. RR spectra of wild-type ferricytochrome *c* in the marker band (1340−1660 cm^{-1}) and low-frequency (200−500 cm^{-1}) regions measured at various pH values with 413-nm excitation.

the heme pocket and spectral parameters of individual bands.[17,18] Hence, both regions are appropriate for identifying and characterizing the various conformers of cytochrome *c* involved in the pH-dependent equilibria.

Dependence of Wild-Type Cytochrome *c* RR Spectra on pH. RR spectra of wild-type cytochrome *c* were obtained over the pH range 7−12. A selection of spectra that includes the marker band and the low-frequency regions is shown in Figure 1. The spectra reveal systematic changes for most of the bands over the entire pH range. For example, the positions of the peaks in the marker band region (1340−1660 cm^{-1}) shift up to higher frequencies with increasing pH. In the low-frequency region, we note that the intensities of the peaks at 275 and 444 cm^{-1} increase from pH 7 to pH 9.5 and decrease again as the pH is increased above 10. Thus, visual inspection of the spectra supports earlier findings[1,2] that cytochrome *c* exhibits two main alkaline conformational transitions with well-separated pK_as, i.e., below and above pH 9.5. Consequently, the separate analysis of the RR spectra measured below and above this pH value appears to be a justified approximation.

Alkaline Conformers of Wild-Type Ferricytochrome *c* (pH 7.0−9.5). Previous studies have indicated the existence of two alkaline conformers of wild-type ferricytochrome *c* in this pH range.[9,19] However, immediate evaluation of this conclusion is not possible by simple visual inspection of the RR spectra. For this reason, detailed analysis of the spectra was undertaken through implementation of the component analysis in which a series of spectra is simulated by a preset number of component spectra.[13] In the present case, we have initiated analysis by assuming the smallest possible number of components, i.e., the neutral form and one alkaline form.[20]

It is reasonable to assume that the RR spectrum of the wild-type protein measured at pH 7.0 represents the spectrum of neutral form III exclusively. This approximation is justified because the pK_a associated with the disappearance of the neutral form is 8.7 at the ionic strength used in the RR experiments[9b] so that at pH 7.0 the alkaline conformer(s) do(es) not exceed 2%. Hence, we tried to fit the RR spectra of the wild-type protein in the pH range between 7.0 and 9.5 using the spectrum of III and an unknown component spectrum. In the component analysis, the spectroscopic parameters of the unknown species are iteratively optimized so that the resultant spectrum, together with the known spectrum, yield the best possible global fit for all measured spectra under consideration. However, the goodness of this fit, as expressed by $\Delta rms = 25.0\%$, is clearly below those of a true two-component system ($\Delta rms < 10\%$),[13] implying that there is a second alkaline component. These findings independently confirm the results obtained from NMR, EPR, and UV−vis absorption spectroscopic techniques.[9,19]

In previous studies, it was shown that the Lys79Ala and Lys73Ala variants exhibit only a single alkaline conformer, IVa and IVb, respectively.[9] In fact, the component analysis of the RR spectra of these single variants, measured in the range between pH 7 and pH 9.5, afforded excellent fits based on two-component spectra, i.e., state III and state IVa (IVb) (spectra not shown here; for Lys79Ala, see ref 13). Hence, it is reasonable to analyze the RR spectra of the wild-type protein with the component spectrum of the neutral form III and those of the alkaline conformers of both single variants, denoted as IVa (coordination by Lys73) and IVb (coordination by Lys79), respectively. For this approach, it was necessary to determine whether the substitutions at position 73 or 79 affect the heme pocket structure of the native conformation and thereby alter its RR spectra. For this reason, we have carefully compared the RR spectra of the states III of the wild-type protein and those of the single variants and established that the spectroscopic parameters of the native forms of these variants exhibit differences no greater than 0.3 cm^{-1}, 0.6 cm^{-1}, and 7% for the frequencies, half widths, and relative intensities, respectively.[13] Thus, it is highly probable that this agreement would also hold for the alkaline conformers. In fact, using the same spectroscopic parameters for IVa and IVb as determined from the variants, a good global fit of the RR spectra of the wild-type protein was obtained (Figure 2). Further refinement was achieved by minor adjustments of the parameters of IVa ($\leq$0.5 cm^{-1} for the frequencies). The resultant fits yielded a Δrms of 5.9% and maximum errors for the frequencies, half widths, and relative intensities of $\pm$0.7 cm^{-1}, $\pm$1.2 cm^{-1}, and $\pm$14.5%, respectively, which is significantly better than those values obtained for the two-component analysis of the wild-type cytochrome. Consequently, we conclude that in this pH range the wild-type protein exhibits two alkaline conformers and that Lys73 and Lys79 are the residues that substitute for the Met80 ligand in IVa and IVb, respectively.[9]

pH-Dependent Equilibria above pH 9.5. Upon increasing the pH above 9.5, distinct changes are noted in the RR spectra of wild-type ferricytochrome *c* (Figure 1). For instance, the marker frequencies shift to higher values, and the intensities of the ν_3 (1505 cm^{-1}) and ν_{10} (1640 cm^{-1}) bands decrease relative to the ν_2 (1587 cm^{-1}) peak. The changes in the low-frequency region are more pronounced as exemplified by the marked intensity reduction of the bands at ~230, 274, 306, 398, and 442 cm^{-1}. Qualitatively, the same observations are made for the Lys79Ala, Lys73Ala (spectra not shown here), and Lys73Ala/Lys79Ala variants (see below), implying that the underlying conformational changes are largely independent of the identity of the amino acid at the positions 79 and 73. Thus, we conclude

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(20) As discussed elsewhere,[13] the component analysis in the marker band region was restricted to the modes[16] ν_4, ν_3, ν_2, ν_{37}, and ν_{10}, which are expected to be most dependent on the coordination environment of the heme iron.[14] All other bands in this region were not specifically assigned to the individual conformers and were fitted independently in the analysis. Using simplified component spectra does not affect the accuracy of the procedure, but it greatly facilitates the data analysis. In the low-frequency region, all bands between 330 and 460 cm^{-1} were attributed to the individual component spectra.

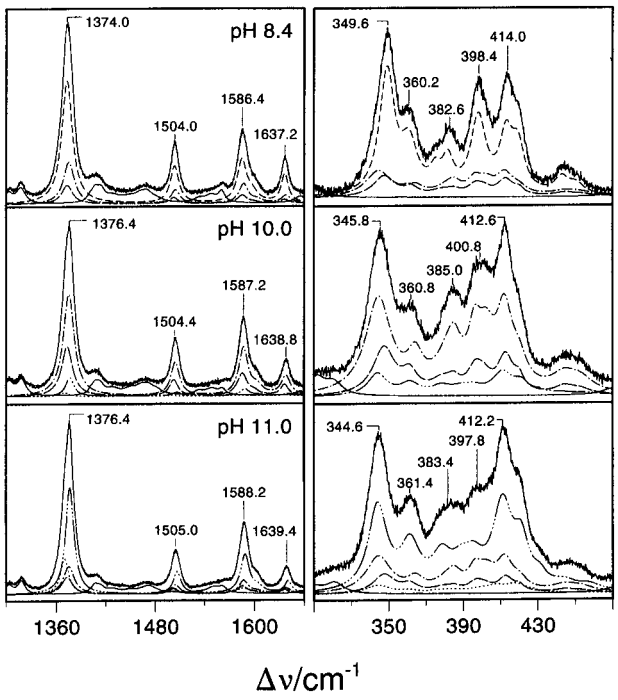

Figure 2. RR spectra of wild-type ferricytochrome *c* in the marker band (1340−1660 cm^{-1}) and low-frequency (200−500 cm^{-1}) regions measured at various pH values with 413-nm excitation. The upper (solid) line in each panel is the experimentally recorded spectrum after background correction. The contributions from the various conformational species as determined by component analysis are indicated by different line types: III, - - -; IVa, −•−; IVb, − − −; Va, −•••−; Vb, •••. The sum of the bands that were fitted independently of the component spectra (−).

that at pH > 9.5 the alkaline forms IVa and IVb are converted into (an)other species.

In the marker band region of the RR spectrum of wild-type cytochrome *c* measured at pH 12.0, the peak positions at ∼1506, 1588, and 1640 cm^{-1} also indicate that, at high pH, the six coordinate, low-spin configuration prevails (Figure 1). Moreover, close inspection of the spectrum reveals asymmetric band shapes. This characteristic can be seen most clearly in the 1375 cm^{-1} peak (ν_4), which exhibits a distinct shoulder at ∼1370 cm^{-1}. This feature implies that there are (at least) two different six coordinate, low-spin species involved. Hence, it was necessary to analyze the RR spectra measured at pH > 9.5 on the basis of two unknown species as well as the two known alkaline species IVa and IVb. In general, such an analysis is associated with lower accuracy than if only one component is unknown so that the proper choice of the initial spectroscopic parameters for the unknown species was of utmost importance.[13]

In related cases, good estimates close to the true values were obtained by subtracting the various measured RR spectra from each other so that crude component spectra were generated.[21] This procedure, however, requires that the ratio between both unknown species varies in the RR spectra under consideration which, unfortunately, did not appear to be the case here. On the other hand, this ratio (as reflected by the shape of the ν_4 peak) was significantly altered when glycine buffer was used instead of sodium or potassium phosphate buffer (spectrum not shown). Thus, crude spectra of the high alkaline species, denoted as Va and Vb, were obtained by mutual subtraction of the RR spectra measured at pH 12 in both buffer systems. The resultant spectra were used as initial component spectra and were further refined during the component analysis. The final results for the spectra between pH 9.5 and pH 12 (Figure 2) were satisfactory with respect to the Δrms value and the maximum errors of the spectral parameters, albeit somewhat worse than for the analysis of the spectra below pH 9.5. This increased uncertainty arises primarily from the poorer quality of the RR spectra above pH 9.5, where the protein has been found to be less stable. For instance, at pH 12, a slow increase in intensity of bands attributable to a five-coordinated high-spin configuration has been observed [e.g., a shoulder at 1490 cm^{-1} (ν_3)],[14] that is ascribed to an irreversibly denatured form of the protein. To minimize the contributions from this species to the RR spectra, fresh samples were used after a considerably shorter period of time (4−6 h) than required for the experiments at pH < 10.0. In the component analysis, any five-coordinate, high-spin marker bands detected were fitted independently rather than grouped to a third unknown component spectrum. As expected, the RR spectra of the wild-type, Lys79Ala, and Lys73Ala proteins in this pH range could be analyzed well in terms of the spectra of Va and Vb and with only subtle adjustments of the spectroscopic parameters.

Quantitative Analysis of the pH-Dependent Equilibria of the Wild-Type Cytochrome. As it has been shown that the RR spectra of wild-type cytochrome *c* between pH 7.0 and pH 12.0 can be adequately described by a superposition of five independent component spectra, it is now possible to propose a model for the pH-dependent conformational transitions. As IVa and IVb are formed in the same pH range at the expense of III, it is reasonable to assume that there are two parallel reactions of III, each associated with the release of one proton. Correspondingly, Va and Vb should also be formed from IVa and IVb in parallel single proton reactions.

This reaction scheme is adequately described by four pK_a values (eq 1).

$$\begin{aligned} pK_{III,IVa} &= pH - \log\frac{c_{IVa}}{c_{III}} \\ pK_{III,IVb} &= pH - \log\frac{c_{IVb}}{c_{III}} \\ pK_{IVa,Va} &= pH - \log\frac{c_{Va}}{c_{IVa}} \\ pK_{IVb,Vb} &= pH - \log\frac{c_{Vb}}{c_{IVb}} \end{aligned} \quad (1)$$

To determine these pK_a values, it is necessary to convert the relative contributions g_i of the individual species *i* to a given RR spectrum, as derived from the component analysis, into relative concentrations c_i according to

$$c_i = \frac{f_i g_i}{\sum_i f_i g_i} \quad (2)$$

where f_i is a proportionality factor that is characteristic for the component spectrum I_i of each species *i*. These factors can be obtained readily for normalized RR spectra that were measured quasi-simultaneously with an external standard (acetone) by the divided-cell technique.[11a] For these spectra, the relative contributions g_i obey eq 3.

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$$1 = \sum_i f_i g_i \qquad (3)$$

The underlying assumption of this approach is that self-absorption of the Raman scattering by the solution is weak and independent of the sample composition. This approximation is justified for the scattering geometry used in our experiments, particularly since the changes of the Soret band are small between pH 7 and pH 12.

Once the f_i factors ($f_{i,HF}$, marker band region; $f_{i,LF}$, low-frequency region) have been determined, the relative concentrations are evaluated according to eq 2. The data, averaged over the results obtained from the analysis of the marker band and the low-frequency regions, are plotted in Figure 3. In the next step, we calculated the pH dependence of the various species according to eq 1 to provide a fit to the experimental data. In this process, we employed a fitting algorithm with appropriate weighting factors that accounted for the significant decrease in accuracy of the experimental data that resulted from the small relative concentrations. The concentrations of the ferricytochrome *c* conformers were determined in this manner with an uncertainty of ~10%, and the pK_a values derived from these concentrations have a resulting uncertainty of ±0.1 pK_a unit. These pK_a values are listed in Table 1.

Comparison with Previous Results. Many of the previous studies of the alkaline transitions of ferricytochrome *c* have involved monitoring the 695-nm absorption band in the electronic absorption spectrum of the protein that is a characteristic feature of state III (i.e., of the Met80-iron bond) but not found in the corresponding spectra of any of the alkaline conformers.[1] Hence, this method probes the disappearance of III rather than a well-defined conformational transition. Consequently, the equilibrium constants obtained from such titration experiments correspond to eq 4 as defined by the reaction scheme depicted in eq 1.

$$K_{app} = \frac{(c_{IVa} + c_{IVb})c_{H^+}}{c_{III}} = K_{III,IVa} + K_{III,IVb} \qquad (4)$$

The corresponding pK_a, i.e., pK_{app}, can be derived from eq 5.

$$pK_{app} = -\log(10^{-pK_{III,IVa}} + 10^{-pK_{III,IVb}}) \qquad (5)$$

Because the individual pK_as of the transitions III ⇌ IVa and III ⇌ IVb are quite similar, it is easy to understand why titration experiments that monitor the electronic spectrum of the protein cannot distinguish between the two transitions and can produce only an apparent pK_a (i.e., pK_{app}). The individual pK_a values determined in the present RR experiments correspond to a pK_{app} of 8.5 as defined by eq 5 which is within experimental error of the value determined in the current study (Table 1).

Within the accuracy of the experiments and the data analysis, the individual pK_a values for the III ⇌ IVa and III ⇌ IVb transitions derived for the wild-type ferricytochrome from the RR experiments agree with the corresponding values obtained for the Lys79Ala and Lys73Ala variants, respectively. For these proteins, pK_a values have also been determined by electronic spectroscopy.[9] The pK_a values determined for these equilibria by RR spectroscopy and by electronic absorption spectroscopy are in good agreement if care is taken to compare these results under conditions of comparable ionic strength (Table 1).

The Alkaline Transitions of the Lys73Ala/Lys79Ala Variant. The RR spectrum of state III of the double variant measured at pH 7.0 is quite similar to that of the wild-type protein (Figure 4). Upon increasing the pH, there are significant spectroscopic changes at pH 9.0, although the alkaline conformers IVa and IVb observed in the RR spectra of the wild-type cytochrome at this pH cannot be formed by this protein. In fact, any attempts to simulate the RR spectrum on the basis of the component spectra of IVa and IVb failed. Instead, the RR spectrum of the double variant at pH 9.0 can be simulated readily by including the component spectra of the species Va and Vb with only minor adjustments of the spectroscopic parameters. These two conformers are the only detectable species at pH > 10.5. These findings imply that, in the Lys73Ala/Lys79Ala double variant, Va and Vb are formed with significantly lower pK_as than in the case of the wild-type protein.

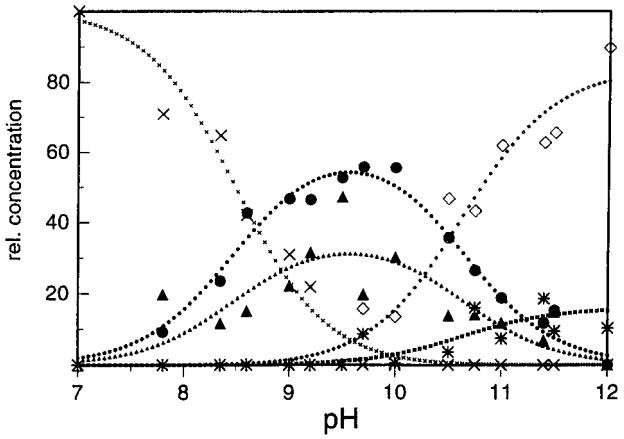

Figure 3. Dependence of the conformational equilibria of wild-type ferricytochrome *c* on pH between 7.0 and 12.0. The relative concentrations of the individual species were obtained from the component analysis of the RR spectra according to eq 2. The relative f_i factors ($f_{i,HF}/f_{III,HF}$) that refer to the ν_4 mode were determined to be 0.87 (IVa), 0.83 (IVb), 0.56 (Va), and 0.35 (Vb), and those that refer to the ν_8 mode ($f_{i,LF}/f_{III,HF}$) are 1.86 (III), 3.76 (IVa), 4.91 (IVb), 6.68 (Va), and 4.74 (Vb). The experimental data and the calculated curves of the various species are represented by the following symbols and line types: × × × (III); ● ● ● (IVa); ▲ ▲ ▲ (IVb); ◇ ◇ ◇ (Va); *** (Vb). Further details are given in the text.

Table 1. pK_a Values for the Alkaline Transitions of Ferricytochrome *c*

		pK_a[a]	
protein	transition	RR	UV−vis
wild-type	III → IVa	8.7 ± 0.2	
	III → IVb	8.9 ± 0.2	
	III → IVa, III → IVb	8.5 ± 0.3[b]	8.7[22]
	IVa → Va	10.5 ± 0.2	
	IVb → Vb	11.0 ± 0.2	
Lys79Ala[c]	III → IVa	8.9 ± 0.3	8.9[9a]
Lys73Ala[c]	III → IVb	8.8 ± 0.3	9.0[9b]
Lys73Ala/Lys79Ala	III → Va	9.1 ± 0.3	
	III → Vb	9.4 ± 0.3	
	III → Va, III → Vb	8.9 ± 0.5[b]	10.5[9b]
Tyr67Phe	III → IVa	10.2 ± 0.3	
	III → IVb	10.3 ± 0.3	
	III → IVa, III → IVb	9.9 ± 0.5[b]	10.3[23,24]

[a] pK_a values determined by RR spectroscopy (this work) refer to an ionic strength of 0.4 M. The uncertainties of the quantitative RR spectra analysis of wild-type cytochrome *c* are estimated to 10%, which corresponds to ±0.1 pK_a units. Taking into account experimental errors the total accuracy of the pK_a determination is assumed to be ±0.2 pK_a units. The uncertainty is greater for the other variants because fewer spectra were used for the analysis. The error of apparent pK_{app}s is assessed on the basis of eq 5. The constants determined by electronic absorption spectroscopy are based on our previous work[9b] and are interpolated to the ionic strength used in the RR experiments. [b] Apparent pK_a as defined by eq 5. [c] The pK_a values for the formation of Va and Vb are similar to the those obtained for the wild-type protein.

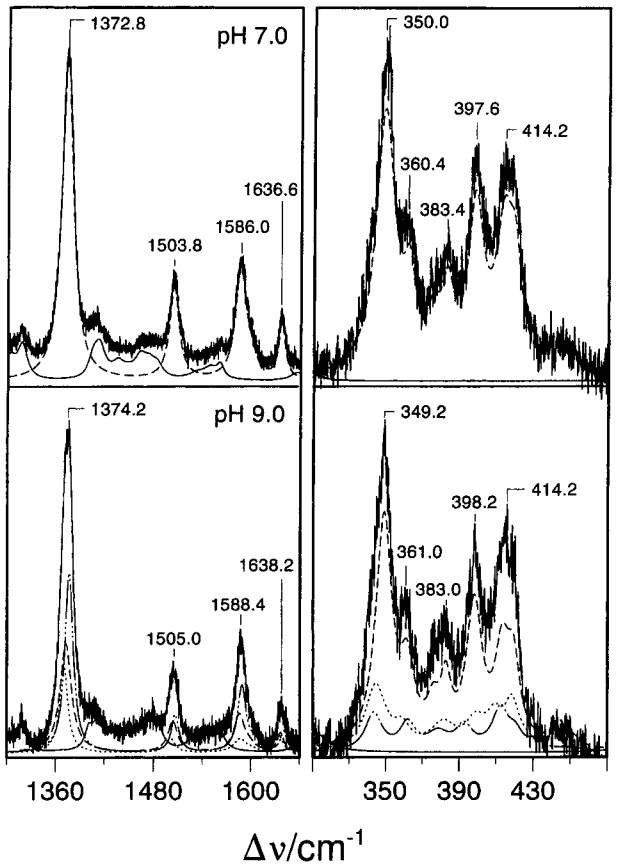

Figure 4. RR spectra of the Lys73Ala/Lys79Ala double variant measured in the marker band (1340−1660 cm^{-1}) and the low-frequency (200−500 cm^{-1}) regions at pH 7.0 and 9.0 with 413-nm excitation. The upper (solid) line in each panel is the experimentally recorded spectrum after background correction. The contributions from the various conformational species determined by component analysis are indicated by different line types: III, - - -; Va, —•••—; Vb, •••. The sum of the bands that were fitted independently of the component spectra (—).

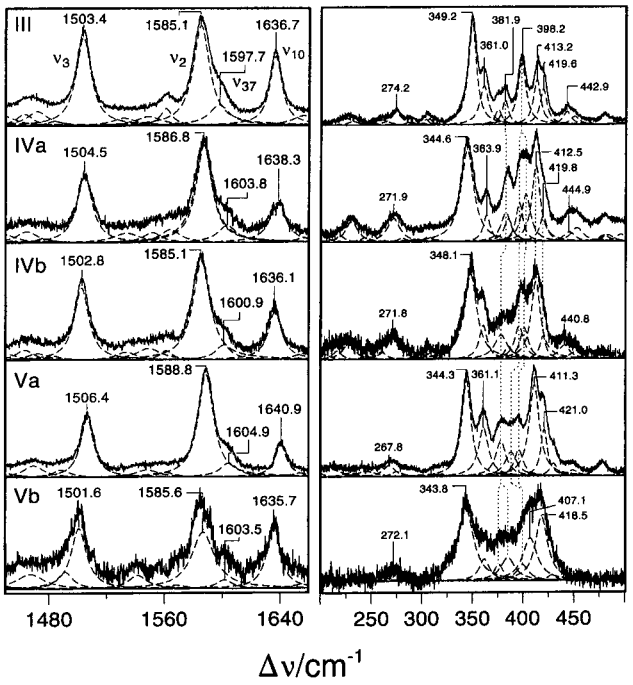

Figure 5. RR spectra of the various conformers of wild-type ferricytochrome *c* in the marker band (1450−1660 cm^{-1}) and low-frequency (200−500 cm^{-1}) regions with excitation at 413 nm. The spectra were obtained by the component analysis as described in the text. The dashed lines represent the Lorentzian band shapes fitted in a conventional band analysis.

A rough approximation of the pK_as for the transitions III ⇌ Va and III ⇌ Vb suggests values between 9.0 and 9.5 that correspond to an apparent pK_{app} of ∼9.0 (eqs 4 and 5). For this double-variant protein, however, there is a considerable discrepancy with that pK_{app} obtained from titration experiments in which the disappearance of the 695-nm absorption band is monitored (10.5 ± 0.3).[9b] It is possible that the ionic strength dependence of formation of states Va and Vb is different from the ionic strength dependence of formation of states IVa and IVb so that these values cannot be compared. Alternatively, the Met80−iron bond in the double variant may become photolabile at higher pH to result in an artifically lower pK_a in the RR experiment.

Alkaline Transitions of Tyr67Phe. An unusually high pK_{app} (≥10.3) has been reported for cytochrome *c* variants in which Tyr67 is replaced by Phe.[23,24] This observation is confirmed by RR spectra of the Tyr67Phe variant in which state III dominates the spectrum up to pH 9.5. At pH 10.0, the RR spectrum includes significant contributions from the alkaline species IVa and IVb, as shown by the component analysis (spectra not shown). In this case, slight modifications of the spectroscopic parameters compared to the wild-type cytochrome were required to optimize the fits. This requirement is not surprising in view of the structural changes at the active site of the protein that result from replacement of Tyr67 with Phe[24] and that in turn affect the RR spectrum. The pK_as determined from the RR experiments for the transitions III ⇌ IVa and III ⇌ IVb of this variant are ∼10.2 and 10.3, respectively. At pH 11.5, the conformers Va and Vb are the dominant species. These states of the variant exhibit similar but not identical spectroscopic parameters as the corresponding states of wild-type cytochrome *c*. These observations rule out Tyr67 as an axial ligand in any of the alkaline conformer(s) of the wild-type protein.

The Resolved RR Spectra of Native and Alkaline Cytochrome *c* Conformers. So far the analysis of the RR spectra was carried out with component spectra that include several characteristic bands of the individual conformers.[13,20] While this approach is sufficiently accurate for the quantitative analysis of measured spectra, the structural characterization of the individual conformers is only possible on the basis of the complete (real) RR spectra, which also include those bands not originally assigned to the individual species. Based on the relative contributions of the individual components to the measured spectra, i.e., the g_i factors (cf. eq 2) that are known from the component analysis, the measured RR spectra can be subtracted from each other such that a specific component *i* is eliminated in the resultant difference spectra. Using these difference spectra, the procedure is repeated until a spectrum including only a single component is obtained. In our experiments, the number of measured spectra exceeds the number of components, so there are several different subtractive combinations of the measured spectra that can yield the spectrum of a specific component. Such spectra were ultimately added to

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(23) (a) Wallace, C. J. A.; Mascagni, P.; Chait, B. T.; Collawn, J. F.; Paterson, Y.; Proudfoot, A. E. I.; Kent, S. B. H. *J. Biol. Chem.* **1989**, *264*, 15199−15209. (b) Luntz, T. L.; Schejter, A.; Garber, E. A. E.; Margoliash, E. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 3524−3528. (c) Frauenhoff, M. A.; Scott, R. A. *Proteins* **1992**, *14*, 202−212.

(24) Berghuis, A. M.; Guillemette, J. G.; Smith, M.; Brayer, G. D. *J. Mol. Biol.* **1994**, *235*, 1326−1341.

Table 2. Assignment of the RR Bands in the Marker Band Region of the Various Conformers of Ferricytochrome *c*[a]

mode	III ν (I)	IVa ν (I)	IVb ν (I)	Va ν (I)	Vb ν (I)
$\delta_{(CH)}$ (T)[b]	1302.9 (0.057)	1303.8 (0.038)	1303.3 (0.056)	1300.7 (0.066)	1301.6 (0.004)
$\delta_{(CH)}$ (T)[b]	1317.9 (0.093)	1317.3 (0.081)	1317.9 (0.009)	1318.9 (0.078)	1319.8 (0.082)
ν_4	1373.0 (1.000)	1375.1 (1.000)	1373.1 (1.000)	1376.5 (1.000)	1370.2 (1.000)
ν_{20}	1400.6 (0.035)	1401.6 (0.011)	1400.7 (0.039)	1400.5 (0.018)	1398.9 (0.023)
ν_{29}	1406.8 (0.068)	1406.8 (0.067)	1407.7 (0.059)	1407.6 (0.021)	1405.0 (0.009)
	1413.6 (0.073)	1413.2 (0.043)	1412.8 (0.047)	1411.8 (0.034)	1411.6 (0.064)
	1428.5 (0.041)	1428.4 (0.044)	1427.5 (0.030)	1428.9 (0.029)	1437.7 (0.054)
ν_{28}	1463.0 (0.039)	1464.9 (0.054)	1463.5 (0.047)	1469.6 (0.041)	1468.0 (0.043)
	1471.7 (0.047)	1476.2 (0.022)	1472.1 (0.031)		
ν_3	1503.4 (0.298)	1504.5 (0.296)	1502.8 (0.340)	1506.4 (0.211)	1501.6 (0.155)
	1531.4 (0.024)	1533.5 (0.045)	1533.3 (0.036)	1533.1 (0.009)	1540.4 (0.031)
ν_{38}	1548.6 (0.029)	1551.3 (0.048)	1549.0 (0.052)	1548.0 (0.026)	
ν_{11}	1560.7 (0.056)	1563.9 (0.061)	1561.2 (0.042)	1560.9 (0.017)	1555.7 (0.012)
ν_2	1585.1 (0.338)	1586.8 (0.464)	1585.1 (0.448)	1588.8 (0.372)	1585.6 (0.215)
ν_{37}	1597.7 (0.068)	1603.8 (0.069)	1600.9 (0.067)	1604.9 (0.052)	1603.5 (0.027)
ν_{10}	1636.7 (0.233)	1638.3 (0.221)	1636.1 (0.221)	1640.9 (0.111)	1635.7 (0.140)

[a] Mode numbering refers to that of Hu et al.[16] Frequencies are given in cm^{-1}, and relative band intensities (in parentheses) refer to the mode ν_4. [b] Mode involving the C−H in-plane bending of the thioether bridge.

Table 3. Assignment of the RR Bands in the Low-Frequency Region of the Various Conformers of Ferricytochrome *c*[a]

mode	III ν ($\Delta\nu_{HD}$)	IVa ν ($\Delta\nu_{HD}$)	IVb ν ($\Delta\nu_{HD}$)	Va ν ($\Delta\nu_{HD}$)	Vb ν ($\Delta\nu_{HD}$)
γ_{24}	224.4 (nd)	219.3 (−1.1)	216.5 (−0.1)	211.2 (nd)	
	231.9 (nd)	230.5 (+0.5)	227.7 (+1.5)	229.5 (nd)	
ν(Fe−N_H)[b]		244.1 (−3.6)	245.4 (−5.4)		
	260.3 (0.0)	261.1 (+1.3)	260.2 (−1.6)	245.5 (nd)	263.4 (nd)
ν_9	274.2 (+0.2)	271.9 (−0.4)	271.8 (−1.5)	267.8 (nd)	272.1 (nd)
	288.1 (nd)	280.6 (−2.4)			
ν_{51}	303.7 (−0.8)	306.2 (−0.8)	306.3 (−0.8)	313.8 (nd)	302.4 (nd)
	307.1 (nd)	313.7 (+0.8)		320.3 (nd)	
ν_8	349.2 (−0.4)	344.6 (+1.4)	348.1 (−1.7)	344.3 (+0.4)	343.8 (+0.2)
ν_{50}	361.0 (−0.9)	363.9 (−7.0)	360.7 (−2.9)	361.1 (−0.7)	362.0 (0.0)
δ(p)[c]	375.1 (−0.4)	382.2 (−1.9)	378.3 (+3.2)	378.3 (+0.7)	376.2 (−1.0)
δ(p)[c]	381.9 (0.0)				385.3 (+1.0)
ν(Fe−N_L)[b]		385.0 (−14.7)	385.7 (−16.6)		
δ(CCS)[c]	398.2 (−0.9)	396.2 (−3.9)	396.3 (−2.8)	388.4 (+0.3)	399.8 (0.0)
δ(CCS)[c]		402.6 (−2.4)	401.2 (+0.4)	396.0 (−0.2)	
δ(CCC)[c]	413.2 (+0.5)	412.5 (+0.8)	412.6 (+1.0)	411.3 (+0.5)	407.1 (−1.0)
δ(CCC)[c]	419.6 (+0.8)	419.8 (+2.1)	419.7 (+2.0)	421.0 (0.0)	418.5 (−0.6)
				430.3 (+1.0)	428.5 (−0.5)
γ_{22}	442.9 (+0.5)	444.9 (−0.9)	440.8 (+1.7)	448.5 (nd)	
γ_{22}	450.5 (−2.3)	453.6 (−5.1)	452.0 (−2.7)		
ν_{33}	480.5 (nd)	479.4 (−1.7)	478.9 (−1.8)	478.0 (nd)	

[a] Mode numbering refers to Hu et al.[16] Frequencies are given in cm^{-1}; shifts produced by H/D exchange ($\Delta\nu_{HD}$) are included in parentheses. For some of the weak and overlapping bands, small shifts could not be determined satisfactorily (nd). [b] Modes involving the Fe−N stretching vibrations of the Lys [ν(Fe−N_L)] and His ligands [ν(Fe−N_H)] in IVa and IVb. [c] Modes involving the propionate bending, δ(p), and bending vibrations of the thioether bridges, δ(CCS) and δ(CCC).

improve the S/N ratio. The resultant spectra of conformers IVa, IVb, Va, and Vb as well as the spectrum of III, obtained at pH 7.0, are displayed in Figure 5. The relatively poor S/N ratio of the spectra of conformers IVb and Vb results from low concentrations of these components relative to those of IVa and Va over the pH range of the experiment. These resolved spectra for the individual protein conformers were analyzed by conventional band fitting.

Vibrational Assignments for the Various States of Cytochrome *c*. In the marker region, the assignment of the prominent bands to the modes ν_4, ν_3, ν_{11}, ν_2, ν_{37}, and ν_{10} is straightforward and has been discussed for the neutral form in detail elsewhere (Table 2).[16,17] The bands at 1303 and 1317 cm^{-1} in the RR spectrum of III have recently been assigned to modes involving the C−H in-plane bending of the thioether bridges.[16] Other weaker peaks exhibit asymmetric band shapes but are generally difficult to resolve into individual bands owing to their low intensities. This difficulty arises for the peaks at ∼1407 and 1465 cm^{-1} that include the B_{2g} modes ν_{29} and ν_{28}, respectively.

Considerable progress in the assignment of the bands in the low-frequency region has been achieved by a recent study of cytochrome *c* reconstituted with isotopically labeled hemes.[16] In the present work, we have adopted these results to revise assignments suggested in our previous studies (Table 3).[25] Hence, the bands at 274 and 349 cm^{-1} are assigned to the A_{1g} modes ν_9 and ν_8. One of the bands at 224 and 232 cm^{-1} should correspond to the out-of-plane mode γ_{24}. The bands at 361, 304/307, and 481 cm^{-1} are presumably attributable to E_u modes ν_{50} and ν_{51} and the B_{2g} mode ν_{33}, respectively. Modes involving bending vibrations of the thioether bridges are attributed to the bands at 398, 413, and 420 cm^{-1}. The two modes involving the bending of the heme propionates are assigned to the bands at 375 and 382 cm^{-1} instead of the bands at 443 and 451 cm^{-1} as suggested previously.[25] As in some other cases, the latter

(25) Hildebrandt, P.; Vanhecke, F.; Heibel, G.; Mauk, A. G. *Biochemistry* **1993**, *32*, 14158−14164.

pair of bands was not resolved in the RR spectra presented by Hu et al.[16] Thus, the proposed assignment to the out-of-plane mode γ_{22} is not unambiguous. It might be that one of the band components corresponds to this mode while the second one has a different origin (e.g., an overtone or a combination mode). On the other hand, both bands reveal a similar behavior in several conformational transitions of ferricytochrome *c*. For instance, both bands disappear or lose intensity in the conformers Va and Vb, favoring the idea that the nature of the underlying modes is the same.

Structural Characterization of Conformers IVa and IVb. It is surprising that in the marker band region the RR spectra of IVa and IVb do not differ greatly from that of III despite the exchange of the axial ligand. This observation is in particular true for the RR spectrum of IVb. In this case, only subtle frequency changes of the marker bands are observed relative to the spectrum of conformer III, and the most pronounced spectroscopic difference is the increase in the relative intensities of the modes ν_3 and ν_2 with respect to the ν_4 mode. Thus, it can be concluded that the transition III $\rightleftharpoons$ IVb does not involve geometric changes of the porphyrin. For IVa, the changes in the RR spectrum are somewhat more apparent, with frequency upshifts of the marker bands as great as 3 cm^{-1} and changes of the relative intensities of the modes ν_2 and ν_{10}. Apparently, the binding of Lys73 (IVa) to the heme iron and the concomitant rearrangement of the peptide segment 70−80 requires some structural adjustments of the heme. According to the core-size frequency relationships,[14] the observed frequency upshifts of the marker bands would correspond to a slight contraction of the porphyrin core. Such effects are not observed for binding of Lys79 (IVb) presumably owing to the closer proximity of this residue to the coordination site.

For a few modes, these tendencies are also reflected in the low-frequency region. For instance, the bands at 349.2 and 361.0 cm^{-1} are shifted by −4.6 and +2.9 cm^{-1}, respectively, in IVa compared to III, while in IVb, they remain at essentially the same positions. Both modes include Fe−N(pyrrole) stretching vibrations,[16] which should in fact respond to changes of the core size. Aside from these two modes, the spectroscopic similarities of IVa and IVb are readily apparent. Moreover, the spectroscopic differences of these two species compared to III are much more pronounced than in the marker band region. The most significant changes are noted between 370 and 400 cm^{-1}, where the RR spectrum of III displays three bands at 375.1, 381.9, and 398.2 cm^{-1} and the spectra of IVa and IVb possess four bands.

To gain greater insight into the nature of these bands, we measured the RR spectra of the wild-type ferricytochrome and both the Lys79Ala and Lys73Ala variants in D_2O as a function of pH. Component analysis of the resulting spectra of the variants conducted as described above permitted resolution of the spectra of the individual deuterated conformers. At pH 7.0, there was no indication of a contribution from the alkaline state IVa (IVb). For state III of both variants, minimal spectroscopic changes were observed in D_2O in agreement with previous findings for the wild-type protein.[25] Comparably small effects are noted for the high alkaline species Va and Vb (see, Table 3).

In the spectra of conformers IVa and IVb derived from the RR spectra of the Lys79Ala and Lys73Ala variants, respectively, significant frequency shifts and intensity changes were observed upon H/D exchange that are evident upon visual inspection of the spectra (Figure 6). These changes are most pronounced in the region between 330 and 430 cm^{-1}. The most remarkable

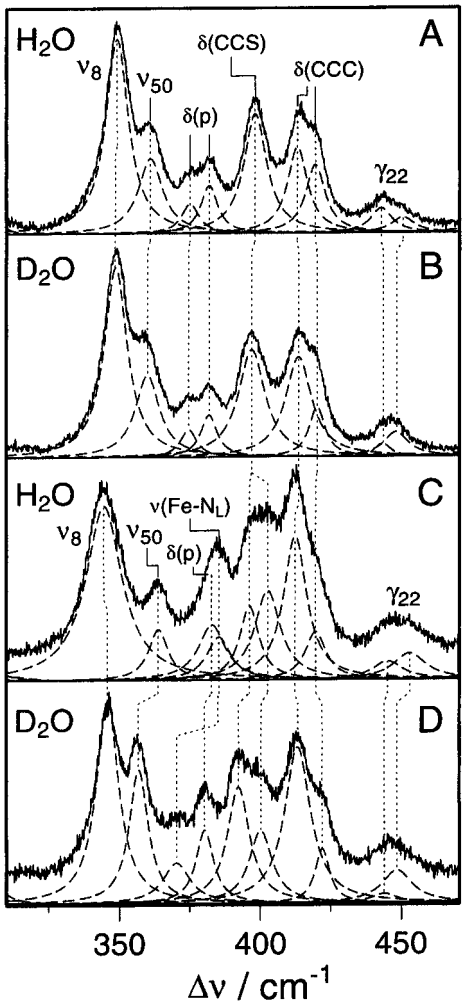

Figure 6. RR spectra of Lys79Ala variant conformers in the low-frequency region (310−470 cm^{-1}) obtained from H_2O and D_2O solutions with 413-nm excitation: (A) III, H_2O; (B) III, D_2O; (C) IVa, H_2O; (D) IVa, D_2O. Spectra A and B were measured directly at pH 7.0. Spectra C and D were obtained from the RR spectra measured in the pH range between 7.8 and 9.5 after subtracting the contribution of the neutral form as determined by the component analysis. The dashed lines represent the Lorentzian band shapes fitted in a conventional band analysis.

effect of H/D exchange is the appearance of a new band at ∼370 cm^{-1} in the spectra of conformers IVa and IVb (the latter spectrum is not shown).

Despite the uncertainty in determining the frequencies of closely spaced bands (e.g., the composite peak at ∼382 cm^{-1}), isotopic shifts of 3−10 cm^{-1} in the spectra of states IVa and IVb compare with shifts of <1 cm^{-1} in state III. For the latter species, these subtle spectroscopic changes resulting from H/D exchange have been attributed to structural changes of the hydrogen-bonding network in the heme pocket that lead to small modifications of the porphyrin geometry.[25] However, such an explanation cannot account for the unique and much greater effects of H/D exchange on the RR spectra of conformers IVa and IVb. On the other hand, the Lys and His ligands in both species give rise to iron−ligand vibrations that should exhibit a distinct isotopic mass effect upon H/D exchange. The corresponding modes with predominant Fe−N(His) and Fe−N(Lys) stretching character are expected to occur between 200 and 250 cm^{-1} and between 350 and 400 cm^{-1}, respectively.[18] In the RR spectra of the deuterated state IVa (IVb), the most likely candidate for the latter mode is the band at 370 (369)

cm^{-1}, which apparently corresponds to the bands at ~385 cm^{-1} observed in H_2O. This assignment implies that there is a 15 cm^{-1} downshift upon H/D exchange and suggests that the effective mass of the Lys ligand only includes that of the NH_2 group. This shift in frequency as well as the frequencies themselves can be reproduced readily with a simple three-body oscillator model[26] using force constants and geometric parameters similar to those employed for calculations of bis(imidazolyl)hemes.[27]

Moreover, since isotopic shifts are also observed for the bands in the immediate vicinity of the 385 cm^{-1} band, we conclude that the Fe−ligand(Lys) vibration or internal ligand vibrations are coupled to nearby porphyrin modes. A similar coupling has, in fact, been observed for bis(imidazolyl)porphyrins by Mitchell et al.[27] Such a coupling implies that the composition of the normal modes between 340 and 400 cm^{-1} is somewhat different in states IVa and IVb than in state III and also varies in the alkaline species upon H/D exchange. These effects can readily account for the changes of the relative band intensities that accompany the frequency shifts (see, Table 3).

The identification of the mode primarily involving the Fe−N(His) stretching between 200 and 250 cm^{-1} is more difficult. The only new band in IVa (IVb) compared to III is a weak band at 244 cm^{-1}, which is close to the position calculated by the three-body oscillator model (cf., ref 26). The low intensity of this band and interference from the more intense bands at 230 cm^{-1} precluded unambiguous detection of an isotopic shift of this band in D_2O, which, in addition, is expected to be much smaller than for the Fe−N(Lys) stretching.

Interestingly, the observation of the Fe−N(Lys) stretch in the alkaline species is not necessarily expected. Previous attempts to identify the Fe−ligand stretching modes in state III [i.e., Fe−S(Met), Fe−N(His)] failed.[16] Presumably, in that study, appropriate resonance conditions were not met with Soret band excitation so that the RR intensities of these modes remained too weak for detection among the various porphyrin modes. On the other hand, hemes with two axial nitrogen ligands have been shown to exhibit relatively strong iron−ligand stretching modes that are resonance enhanced upon Soret band excitation.[27] This behavior is apparently also the case for the IVa and IVb conformers.

Structural Characterization of the Conformers Va and Vb. In these conformational states, the axial ligand exchange is reflected by substantial changes in the RR spectra. Distinct frequency shifts and alterations of the relative band intensities in the marker band region are observed that support the view that the new ligands differ from those of the neutral and the alkaline conformers. The pK_as for formation of Va and Vb suggest that at pH 10.5−11.0 strong ligands are formed that can displace the ϵ-amino groups of Lys73 and Lys79 from the sixth coordination site of the heme iron. Hydroxide or alkoxide ions are strong field ligands and are the most likely candidates for the sixth ligand in Va and Vb. Such ligands may be provided by water or amino acid side chains (Tyr, Thr) in this pH range. In fact, several candidates of this type that are in close proximity to the coordination site can be identified in the three-dimensional structure of the wild-type cytochrome, i.e., WAT166 (5.0 Å) and the hydroxy groups of Thr78 (7.0 Å) and Tyr67 (5.0 Å).[4] The latter group can be ruled out as a ligand in Va and Vb because, according to the RR spectra, these conformers are also formed by the Tyr67Phe variant without any significant structural alterations relative to the wild-type protein. This is also true for Thr78 because the RR spectra of the Thr78Ala variant at pH > 11 are very similar to the weighted sum of the spectra of states Va and Vb (spectra not shown).[28] Coordination by a hydroxide would lead to H/D-sensitive iron−ligand stretching vibrations; however, our attempts to identify these modes in the region between 400 and 550 cm^{-1} on the basis of the expected frequency downshift (≤10 cm^{-1}) failed.[29] This negative result, however, does not rule out coordination by hydroxide if resonance enhancement is not sufficient at the excitation wavelengths used (413, 530, and 568 nm). Thus, a deprotonated water (e.g., WAT166) remains the most probable ligand to the heme iron in states Va and Vb.

The striking spectroscopic differences between conformational states Va and Vb in the marker band region reflect structural differences of the porphyrin despite the (most likely) identical ligation pattern. Such differences evidently include the interactions of the heme propionates and the thioether bridges with the immediate protein environment since there are distinct alterations of the corresponding bending modes of these substituents in states Va and Vb, i.e., between 380 and 420 cm^{-1} (Figure 5 and Table 3).

Comparison with the Conformational State of Cytochrome *c* Bound in Electrostatically Stabilized Complexes. It has been suggested that alkaline conformers of ferricytochrome *c* might be structurally similar or identical to the conformational state of the ferricytochrome formed upon reaction or complex formation with cytochrome *c* oxidase (C*c*O).[7] This conformer is also observed to form when ferricytochrome *c* binds to model agents or surfaces that mimic the binding domain of C*c*O to some extent (polyanions, phospholipid vesicles, electrodes).[11a,12,17] Such models permit more detailed spectroscopic characterization of the cytochrome than can be achieved with the cytochrome *c*−C*c*O complex itself due to the lack of spectroscopic interference from the heme groups of C*c*O. A RR spectrum of this conformational state, denoted as state B2,[30] can be obtained readily upon binding cytochrome *c* to polytungstates[31] (Figure 7). In this case, significant changes in the RR spectrum of the protein are immediately evident both in the marker band and in the low frequency region that distinguish the resulting form of the cytochrome from the native conformation and from conformational states IVa and IVb (Figure 5). This observation is no longer surprising because lysine residues including Lys79 and Lys73 have been implicated as participants in electrostatic interactions with the negatively charged binding domain of C*c*O or related model systems.[17,32] Thus, when cytochrome *c* binds to electrostatically complementary species, Lys73 and Lys79 are not available for coordination to the heme iron and formation of IVa and IVb should be inhibited.

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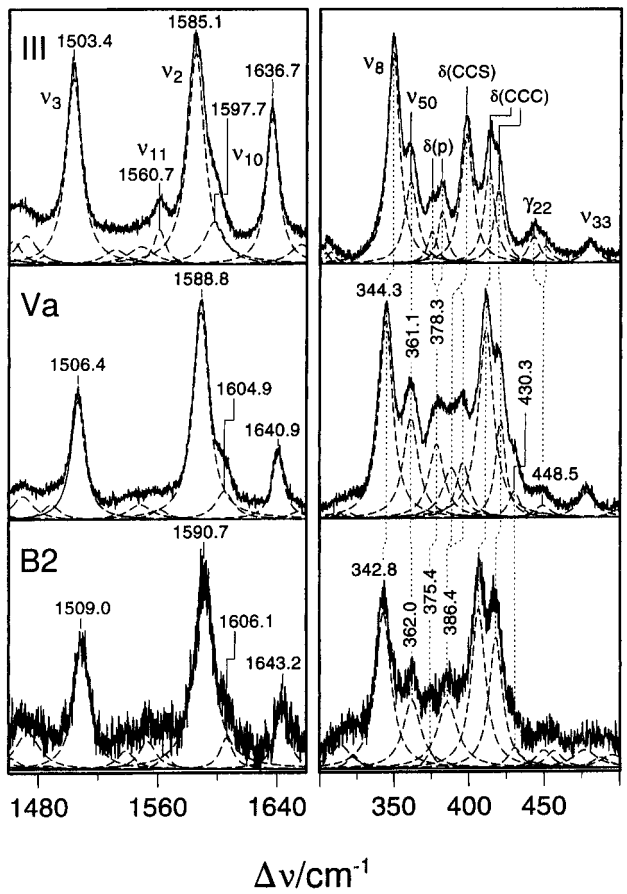

Figure 7. RR spectra of various conformers of wild-type ferricytochrome *c* in the marker band (1460−1660 cm^{-1}) and low-frequency (200−500 cm^{-1}) regions obtained with 413-nm excitation: (A) state III, measured at pH 7.0; (B) state Va, obtained from the RR spectra measured in the pH range between 10.0 and 12.0 after subtracting the contributions of the other conformers determined by the component analysis; (C) state B2, obtained from the RR spectra of wild-type ferricytochrome *c* bound to polyanions as described in the text. The dashed lines represent the Lorentzian band shapes fitted in a conventional band analysis.

On the other hand, there are far-reaching spectroscopic similarities between the state B2 and the conformer Va (Figure 7), including (i) the significant upshift of the marker bands, (ii) the increased intensity of ν_2, (iii) the downshift in frequency of ν_8, and (iv) the decreased intensity of δ(CCS) and γ_{22} in the low-frequency region. This close resemblance of the spectra of states B2 and Va indicates a similar structure of the heme (pocket) of both species. In particular, it implies that the Met80 ligand is replaced by a hydroxide in state B2 despite the formation of this cytochrome conformer even at neutral pH. In fact, a recent study of the Met80Ala variant has demonstrated that hydroxide binding may well occur at pH 7 once the native methionine sulfur ligand is not available.[33] One implication of these observations is that at least some of the biological electron-transfer reactions of cytochrome *c* may be coupled to a conformational transition that involves exchange of an axial ligand to the heme iron.

Factors Controlling the Ligand Exchange. The apparent identity of coordination environments of conformational states Va and B2 combined with the possible decrease in pK_a for the formation of Va and Vb in the Lys73Ala/Lys79Ala double variant may contribute to better understanding of the ligand exchange mechanism. In state B2 and the double-variant, lysyl residues 73 and 79 cannot coordinate to the heme iron and hydroxide replaces Met80 at pH < 8 and pH < 10, respectively. In both cases, the positive charges at positions 73 and 79 are neutralized by either intermolecular electrostatic interactions with negatively charged entities (state B2) or replacement by alanines (Lys73Ala/Lys79Ala). It is tempting to assume that, in the wild-type protein, these positive charges are crucial for stabilizing the structure of the heme pocket.

Kinetics studies have been interpreted as suggesting that the formation of the alkaline forms IV occurs via the deprotonation of a yet unidentified ionizable ("trigger") group (p$K_a \sim 11$), followed by a rapid conformational change (p$K_a \sim -2$) in which the Met80 is replaced as the axial ligand to the heme iron.[34] At present, however, the identity of the so-called trigger group remains unresolved. Inasmuch as solvent exposed lysines are expected to exhibit pK_a values slightly below 11, it is plausible to assign these ionizable groups to Lys73 and Lys79. Other candidates for this conformation state-linked titratable group include His18[35] and one of the heme propionate groups.[36]

The present results indicate that, once chemical modification of the ϵ-amino groups of Lys73 and Lys79 [corresponding to the neutralization of the positive charge(s) (cf. ref 1, and references therein)] prevent binding to the heme iron, hydroxide may replace the Met80 ligand with a relatively low pK_a. This possibility might permit reinterpretation of Bosshard's study,[37] in which no change in the rate of acetylation of the individual surface lysines, including Lys73 and Lys79, was observed between pH 7 and pH 11.

Conclusions

1. Wild-type ferricytochrome *c* exhibits complex conformational equilibria between pH 7 and pH 12 that involve the neutral or native form III, two alkaline forms IV, i.e., IVa and IVb, and two high alkaline species V, i.e., Va and Vb.

2. In the forms IVa and IVb, Lys73 and Lys79 replace the Met80 ligand with similar pK_as (8.7 and 8.9), while a hydroxide, probably provided by WAT166, binds to the heme iron in Va and Vb with pK_as of 10.5 and 11.0, respectively.

3. The pK_as of formation of Va (and Vb) are dramatically lowered if the positive charges at the positions 73 and 79 are neutralized either by substitution with alanines (p$K_a \leq 9.5$) or by intermolecular interactions with negatively charged surfaces (pK_a < 8), suggesting that the charge distribution on the protein surface in the lysine-rich domain around the heme crevice controls the heme structure.

4. The RR spectrum of the conformational state B2 of cytochrome *c* that is formed in electrostatically stabilized complexes, e.g., with polyanions[31] or the physiological reaction partner cytochrome *c* oxidase,[11a,12] is closely related to state Va, suggesting that biological electron-transfer reactions of cytochrome *c* can be coupled with ligand exchange at the active site of the protein.

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