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Time-Resolved Resonance Raman Study of Alkaline Isomerization of Ferricytochrome *c*†

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ABSTRACT: A transient intermediate in the course of alkaline isomerization reaction of ferricytochrome *c* has been detected by the use of a stopped-flow resonance Raman spectroscopy, when the pH of the reactant solution was jumped from 6.9 to 12.0. Resonance Raman lines at 1610 and 1640 cm^{-1} have been observed in the transient species. Resonance Raman spectra of totally guanidinated ferricytochrome *c* were also obtained at pH 7.2 and at pH 11.5. At the alkaline pH, the guanidinated derivative has resonance Raman lines at 1478, 1505, 1552, 1588, 1608, and 1637 cm^{-1}. From these frequencies it was suggested that its alkaline form has a structural similarity (around the heme environment) to the alkaline intermediate form of the native cytochrome *c*. This suggestion was supported by the absorption spectrum of the alkaline form of the guanidinated derivative, which has an intense 600-nm band and a blue-shifted Soret band. These Raman frequencies as well as those of the transient species of the native protein are interpreted to consist of high- and low-spin components. They indicate that the distance between the center of the porphyrin core and the pyrrole nitrogen is 2.07 Å for the high-spin component and 2.00 Å for the low-spin component (both of the alkaline form of the guanidinated ferricytochrome *c* and of the alkaline intermediate form of the native cytochrome *c*). Thus, the heme iron is considered to reside in the heme plane regardless of the spin state of the heme iron in these proteins.

Ferricytochrome *c* is known to take several different conformations depending on pH (Theorell & Åkesson, 1941). It undergoes a subtle conformational isomerization near pH 9. At neutral pH, it is in a low-spin state and has Met-80 as the sixth ligand. It has a weak absorption band at 695 nm, which is characteristic of sulfur ligation to the heme iron (Smith & Williams, 1970). As the pH is raised, the alkaline isomerization takes place, and the methionine ligand is replaced by a strong field ligand, which keeps the heme iron in a low-spin state. This new ligand is supposed to be Lys-79 or Lys-72 (Hettinger & Harbury, 1964; Gupta & Koenig, 1971; Davis et al., 1974; Wilgus & Stellwagen, 1974; Kitagawa et al., 1977a,b; Smith & Millett, 1980). By this transition, ferricytochrome *c* loses the reducibility (Greenwood & Palmer, 1965), its 695-nm band disappears, and the methyl signal of

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Met-80 in the NMR spectrum becomes undetectable (Redfield & Gupta, 1971).

The kinetic properties of the alkaline isomerization reaction of horse heart ferricytochrome *c* have been studied by Davis et al. (1974) between pH 8.5 and pH 10.5 by a stopped-flow technique. The reaction has been studied more in detail between pH 8.5 and pH 12.1 (Kihara et al., 1976), and a new transient species has been found above pH 10. This transient intermediate during the reaction has also been detected by Hasumi (1980) and Saigo (1981, 1982). The transient species has an intense absorption band at 600 nm (Saigo, 1981, 1982) and has been suggested to have a high-spin component. However, they could not tell whether the transient species is solely in a high-spin state or in a mixed-spin state. If it is in a mixed-spin state, there are two alternatives: it can be either a thermal mixture of high- and low-spin species or a quantum mechanical admixture (Maltempo et al., 1974).

The resonance Raman spectroscopy has been applied widely to heme proteins and was found to be a sensitive tool to diagnose the oxidation state, spin state, and ligand coordination (Spiro & Strekas, 1972; Yamamoto et al., 1973; Strekas & Spiro, 1974; Rakshit & Spiro, 1974; Spiro & Burke, 1976; Spiro et al., 1979). Normal coordinate analyses of the porphyrin macrocycle have also been reported (Stein et al., 1975; Kitagawa et al., 1976; Abe et al., 1976, 1978; Sunder & Burnstein, 1976). On the basis of these studies, it is expected that the resonance Raman technique can further elucidate a number of important aspects of the heme protein properties.

In this paper, the results of our time-resolved resonance Raman study of alkaline isomerization reaction of ferricytochrome *c* is reported. We utilized a stopped-flow multidetector Raman spectrometer, which we have recently developed (Nishimura & Tsuboi, 1982). With this setup, a resonance Raman spectrum of the transient form of the reaction could be obtained. In many cases of time-resolved resonance Raman studies on heme proteins, reversible reactions such as the photodissociation reaction of (carbon monoxy)-hemoglobin have been subjected to the examination. Here, the reactions were initiated by the laser illumination (Woodruff & Farquharson, 1978; Lyons et al., 1978; Terner et al., 1980; Irwin & Atkinson, 1981). Many biological reactions, however, occur in a time scale of a few millisecond and in irreversible manners. It may therefore be significant to show that resonance Raman spectra of short-lived species can be obtained by the use of a stopped-flow device. It should be mentioned here that Raman spectroscopic studies were also reported of cytochrome *c* reduction process, by the use of a pulse radiolysis (Cartling & Wilbrandt, 1981; Cartling, 1983) and a continuous flow method (Forster et al., 1982).

We have examined, in addition, resonance Raman spectra of guanidinated ferricytochrome *c*, in which all 19 lysine residues are chemically modified to homoarginine. It has been found that this lysine-modified derivative also exhibits a pH-dependent displacement of the methionine ligand with an apparent p*K* value nearly equal to that of a native protein (Stellwagen et al., 1975). The structural similarity between the transient form in the course of the alkaline isomerization reaction of the native protein and the alkaline form of the guanidinated derivative will be discussed here.

Materials and Methods

Horse heart cytochrome *c* (Sigma, type IV) was oxidized with potassium ferricyanide and purified on a CM-Sepharose CL-6B column (Pharmacia). Guanidinated ferricytochrome *c* was prepared by the reaction of the protein with 0.5 M *O*-methylisourea for 11 days at pH 11.0 and at room temperature (Hettinger & Harbury, 1964). This reagent was subsequently removed by dialysis against 40 mM sodium phosphate buffer, pH 7.0, and the resultant dialysate was applied to a column of CM-Sepharose CL-6B. The column was washed with 40 mM sodium phosphate buffer, pH 7.0, and then the guanidinated protein was eluted with 300 mL of the same buffer containing a linear gradient of 0–0.5 M NaCl. The main colored guanidinated ferricytochrome *c* fractions were collected and dialyzed against 40 mM sodium phosphate, pH 7.0, and applied to a column of CM-Sepharose CL-6B. The guanidinated protein was eluted in the same conditions as those for the first chromatography.

The concentration of the native cytochrome *c* was determined optically by using a molar extinction coefficient of 27.6 mM^{-1} cm^{-1} at 550 nm of the reduced protein (Margoliash & Frohwirt, 1959). For the guanidinated protein, the concentration was also optically determined by assuming that its absorption profile is equal to that for the native protein.

Spectrophotometric titrations were performed in 5 mM sodium phosphate–0.1 M glycine buffer at 25 °C. The ionic strength was adjusted to 0.2 by a proper addition of NaCl. Absorption spectra were recorded with a Hitachi Model 100-50 spectrophotometer.

Resonance Raman spectra of the guanidinated ferricytochrome *c* were obtained by using a JASCO R-800 Raman spectrometer with a microprocessor and an NEC Model GLS-3300 argon ion laser.

Experimental arrangement of the stopped-flow multidetector Raman spectrometer system for obtaining time-resolved resonance Raman spectra was reached by a minor change from what was described previously (Nishimura & Tsuboi, 1982). A solution of 120 μM ferricytochrome *c* in 10 mM sodium phosphate buffer, pH 6.9, was mixed with an equal volume of 0.2 M glycine–NaOH buffer in a JASCO stopped-flow device. The pH was 12.0 after mixing. The ionic strength of each of the reactant solutions was adjusted to 0.2 by addition of NaCl. Accordingly, the final salt and buffer concentrations of the solvent are equal to those of the solvent used in the spectrophotometric titration. The ferricytochrome *c* concentration was kept as low as 60 μM so that the effect of self-absorption would not be so critical (Strekas et al., 1974; Nishimura et al., 1983).

The stopped-flow device was controlled by a JASCO Model SFC-5 controller using nitrogen pressure. The mixed solution in the cell was irradiated with an argon ion laser beam of 514.5 nm (NEC Model GLS-3300). The scattered light was collected and examined with a triple monochromator and a Tracor Northern IDARSS detector system, which consists of a TN-1223-4I intensified photodiode array head, a TN-1710-4K analyzer with a TN-1710-21 optical spectrometer module, and an X-Y recorder.

"Delay time" was set at 20 ms on the JASCO SFC-5 controller, which determines the time interval between the trigger of the stopped-flow shot and that of the data acquisition of the detector system. "Exposure time" was set at 200 ms on the TN-1710, which determines the time used for readout of the data in the diode array of the detector. In this combination of settings, therefore, a resonance Raman spectrum should be obtained, accumulated in the period of 20–220 ms after the pH was jumped from 6.9 to 12.0 in the stopped-flow device. Such a spectrum is illustrated in Figure 1D. The spectrum "t = 5" in Figure 1 was obtained, on the other hand, by manually starting the readout of the data in the diode array head, at 5 s after the reaction was initiated. In this case, exposure time was set at 200 ms, the same as for spectrum

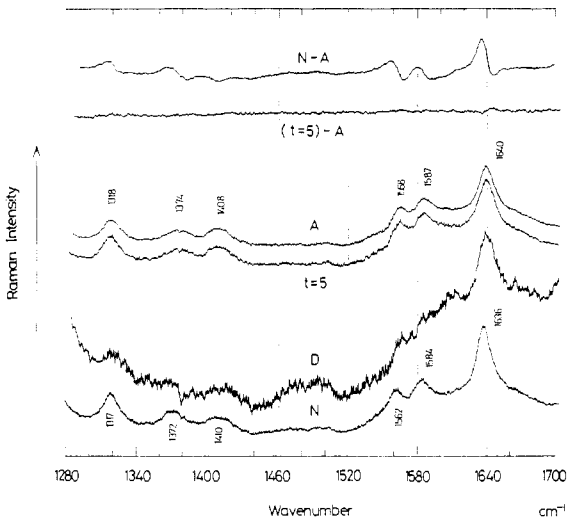

FIGURE 1: Time-resolved resonance Raman spectra of the alkaline isomerization of native ferricytochrome *c*. N: neutral form at pH 6.9. A: alkaline form at 12.0. D: the spectrum obtained from 20 to 220 ms after the initiation of the reaction by setting the time interval and the time to readout of the data as described under Materials and Methods. $t = 5$: the spectrum obtained at 5 s after the reaction was initiated. $(t = 5) - A$: the spectrum obtained by digitally subtracting the alkaline form from that of $t = 5$. $N - A$: the spectrum obtained by digitally subtracting the alkaline form from that of the neutral form. Excitation laser: 514.5 nm at 300 mW. Slit width: 10 cm^{-1}.

D. The temperature of the solution was kept constant at 25 °C throughout the present measurements.

Results

Time-Resolved Resonance Raman Spectra in Alkaline Isomerization of Native Ferricytochrome c. The resonance Raman spectrum of native ferricytochrome *c* at pH 6.9 is shown in Figure 1N and the spectrum of its alkaline form at pH 12.0 in Figure 1A. In the measurements, the sample solutions were placed in the stopped-flow cell (although these were static measurements) to provide for the comparative investigation with the kinetic measurements. These observed spectra are in agreement with those obtained in previous studies (Kitagawa et al., 1977a,b). Some subtle changes are seen in the Raman spectra of these two conformations. These changes, as shown in Figure 1, became more significant in the difference Raman spectrum, N – A, which was obtained by digitally subtracting the spectrum of the alkaline form from that of the neutral one. On the other hand, there are no peaks and no troughs found in the difference Raman spectrum, $(t = 5) - A$, which was obtained by subtracting the spectrum of the alkaline form from the time-resolved spectrum at 5 s after the reaction was initiated (see Figure 1). Thus, it has been confirmed that the alkaline isomerization reaction had been completed at 5 s after the initiation of the reaction and that the whole equipment system was kept stable enough during the stopped-flow performance followed by the 200-ms readout of the Raman spectrum.

Spectrum D, in Figure 1, was obtained from 20 to 220 ms after the reaction was initiated. It was somewhat noisy, probably because a background scattering level was induced by the turbulence of the solution in the stopped-flow cell. Such a background scattering becomes critical when the sample cytochrome *c* concentration is lowered, and we need a compromise here, because the concentration should be kept lower than 100 μM, so that we can neglect the effect of self-absorption (Strekas et al., 1974; Nishimura et al., 1983). It should be noted here that, in spite of its rather poor signal-to-noise ratio, the reproducibility of spectrum D is sufficiently

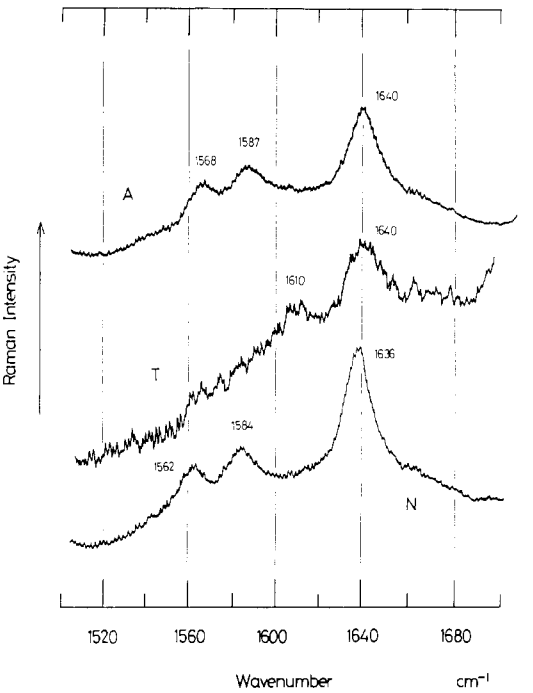

FIGURE 2: Resonance Raman spectrum of the transient form. T: the spectrum of the transient form obtained by subtracting 10% of the neutral and 50% of the alkaline form from spectrum D in Figure 1. N (neutral form at pH 6.9) and A (alkaline form at pH 12.0) are the same as those in Figure 2.

good; a strong peak at 1640 cm^{-1} and a weak band at 1610 cm^{-1} appear every time of our ten trials of the same experiment. It also should be noted that a broad, small peak in the 1480–1500-cm^{-1} range is not a Raman band but it is a false signal caused by a few wrong detector elements of our particular photodiode array.

Spectrum D (Figure 1) is supposed to include not only the Raman scattering of the transient form but also those of the neutral form (start) and alkaline form (final product). On the basis of the rate constants given by Kihara et al. (1976), Davis et al. (1974), Hasumi (1980), and Saigo (1981, 1982), the integral amounts of the neutral and alkaline forms are estimated to be 10% and 50%, respectively, in the 20–220-ms period after the pH 6.9 → 12.0 jump. Therefore, by subtracting 10% of spectrum N and 50% of spectrum A from spectrum D (in Figure 1), the spectrum of the transient form (T) should be obtained. Such a transient spectrum is reproduced in Figure 2 for the spectral region where the spin marker and/or core expansion marker band appear. In this spectrum (T in Figure 2), the strong Raman line observed at 1640 cm^{-1} indicates that the predominant component is in a low-spin state. A weak but appreciable Raman line at 1610 cm^{-1} shows, on the other hand, that a small fraction of a high spin component is involved in the transient species. In other words, the transient intermediate is considered to be a thermally mixed spin species.

pH Titration of Native and Guanidinated Ferricytochrome c. Results of the spectrophotometric titration experiments of native and fully guanidinated ferricytochrome *c* are shown in Figure 3. In each case, as the pH of the solution is raised, alkaline isomerization occurs with an apparent transition point at 9.3. Such a transition was previously observed at pH values in the range from 8.9 to 9.3 depending on the ionic strength of the solution (Greenwood & Wilson, 1971). For guanidinated derivative, also, nearly equal value is obtained for that of the native protein. The transition pH was reported to be 9.4 by Morton (1973), 8.8 by Wilgus & Stellwagen (1974),

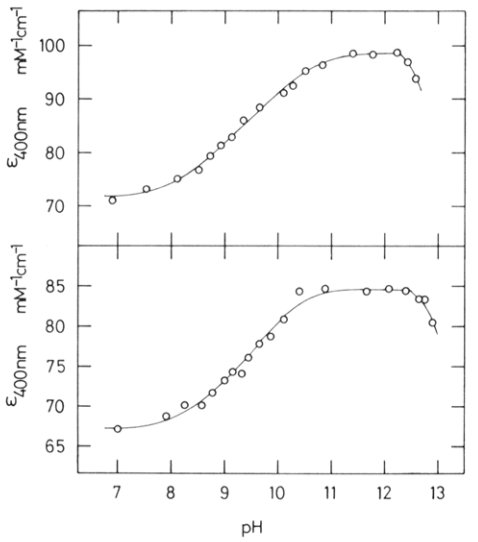

FIGURE 3: Spectrophotometric titrations of ferricytochrome *c*. Upper panel: native protein; 6 μM ferricytochrome *c* in 5 mM sodium phosphate plus 0.1 M glycine. Lower panel: the guanidinated derivative; 7 μM guanidinated ferricytochrome *c* in 5 mM sodium phosphate plus 0.1 M glycine. The ionic strength was adjusted to 0.2 in both cases by an addition of NaCl.

and 8.8 by Stellwagen et al. (1975). The stable conformations at about pH 7 and 11, namely, the neutral and alkaline forms, correspond to state III and state IV, respectively, of Theorell & Åkesson (1941). Another alkaline isomerization process can be seen above pH 12.4 (Figure 3). This process corresponds to the conversion from state IV to state V of the native protein (Theorell & Åkesson, 1941). For guanidinated protein, this process can also be seen in Figure 3.

Absorption spectra of both the native and modified proteins at Soret region, recorded in the course of pH titrations, are shown in Figure 4. They both have isosbestic points, suggesting that only two states are involved in each case, as long as a static measurement is concerned. Its wavelengths of the native and guanidinated ferricytochrome *c* are different (Figure 4).

In Figure 5, longer wavelength absorption spectra of guanidinated ferricytochrome *c* are shown at neutral and alkaline pH. At neutral pH, it has a weak absorption band at 695 nm, indicating that Met-80 is ligating to the heme iron (Smith & Williams, 1970) like that in native protein. On going to the alkaline pH, it losses the 695-nm band and instead shows a gentle rise of absorbance toward near-infrared region. This is not the case of the native protein. This is not considered to be caused by the turbidity of the solution, as it was not reduced by a centrifugation of the sample solution. In addition, the guanidinated derivative has a strong absorption band at 600 nm, and its Soret band has its maximum at 405 nm at alkaline pH. The Soret peak value is somewhat blue shifted to that at neutral pH, 409 nm, which is in contrast to the case of the native protein. In general, high-spin ferric heme proteins are known to be characterized by the charge-transfer band in the range 600–650 nm (Brill & Williams, 1961). Accordingly, it is suggested that the guanidinated derivative has a high-spin component at alkaline pH.

Resonance Raman Spectra of Guanidinated Ferricytochrome c. The resonance Raman spectra of the guanidinated ferricytochrome *c* at pH 7.2 and 11.5 are shown in Figure 6. At neutral pH, it has a very similar profile to that of the neutral form of the native protein (Kitagawa et al., 1977a,b), as seen in Figure 1. This fact suggests that the modification of the protein does not alter the structure around

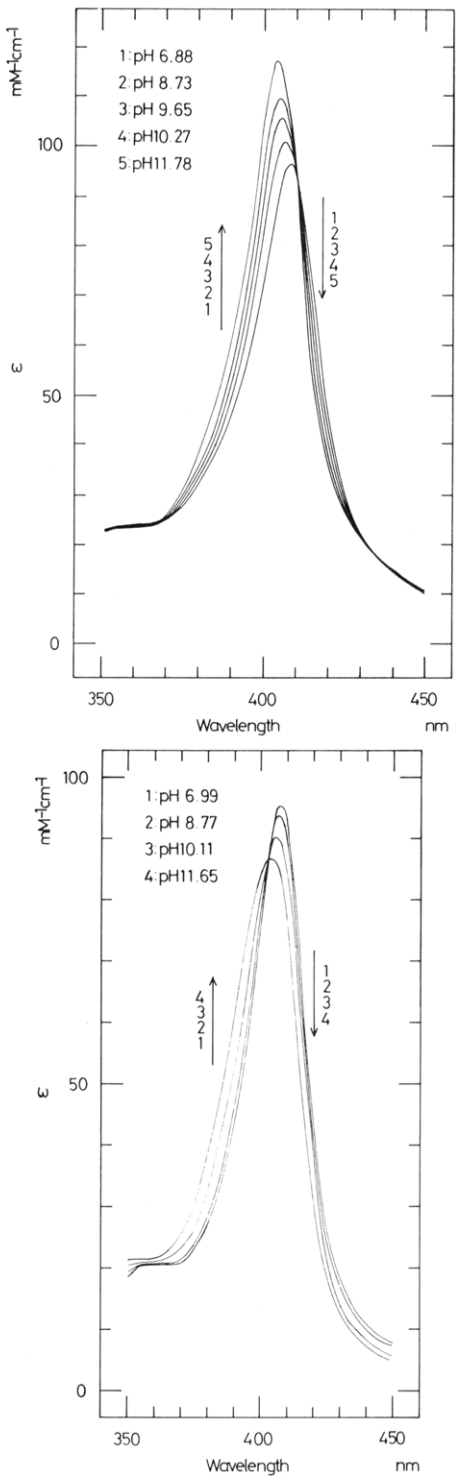

FIGURE 4: Absorption spectra of native (top) and guanidinated (bottom) ferricytochrome *c* in the solutions of different pHs, as indicated. Note that in each group of the spectra, an isosbestic point appears. It is at 410 nm for native cytochrome *c* while at 405 nm for guanidinated one.

the heme environment; the resonance Raman spectrum is in general sensitive to the conformational change of the protein (Lanir et al., 1979).

At alkaline pH, however, it showed a different spectral profile from that of the native protein. It has resonance Raman lines at 1637, 1608, 1588, 1569, 1552, 1505, and 1478 cm^{-1} in a higher frequency region. In a lower frequency region, some spectral changes are also seen on going from native to guanidinated ferricytochrome *c*. The resonance Raman lines at 1246 and 1220 cm^{-1} become weaker and somewhat broader.

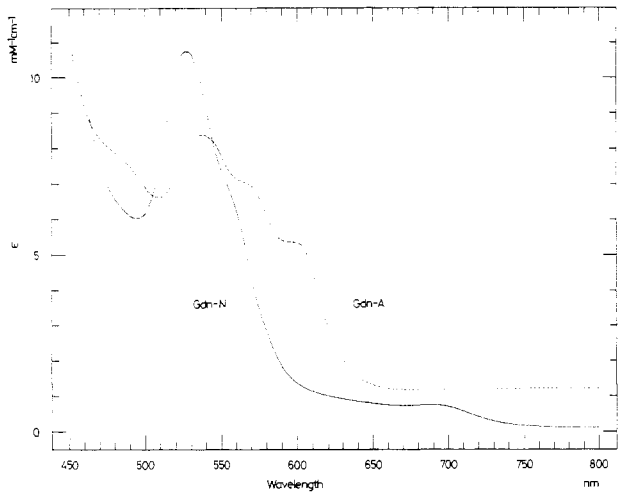

FIGURE 5: Absorption spectra of the guanidinated ferricytochrome *c* at neutral and alkaline pHs. (—) At pH 7.2. (- - -) At pH 11.5. ϵ is the molar extinction coefficient.

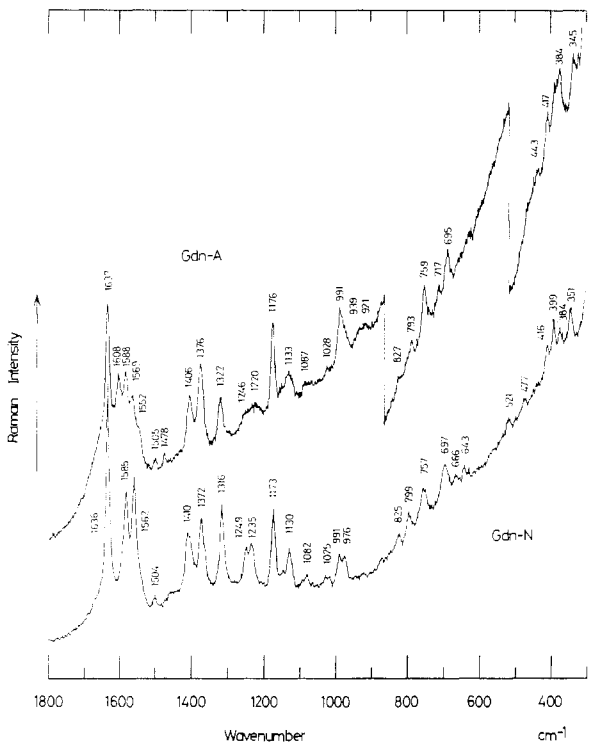

FIGURE 6: Resonance Raman spectra of guanidinated ferricytochrome *c* at neutral and alkaline pHs. (Lower) Gdn-N: neutral form at pH 7.2. (Upper) Gdn-A: alkaline form at pH 11.5. The Raman line at 991 cm^{-1} in the upper spectrum and those at 991 and 976 cm^{-1} in the lower one are those of the phosphate anion which is involved in the solvent. Excitation: 514.5 nm at 300 mW. Slit width: 5 cm^{-1}.

In the spectrum of the alkaline form, some new lines at 717, 921, and 939 cm^{-1} appear and 666, 643, 521, 477, and 399 cm^{-1} disappear. In the spectra of guanidinated cytochrome *c*, now in question, some lines changed their frequencies on raising pH. The stretching mode of the bond between the heme iron and the sixth ligand Met-80 is expected to appear in the frequency region lower than 1000 cm^{-1}. Because of the weak intensity of the Raman lines in this frequency region, however, no interpretation was attempted for it.

Discussion

In a study of a homologous series of model compounds, it was well documented (Spaulding et al., 1975) that the position of some resonance Raman lines of heme proteins and models is correlated to the distance between the center of the porphyrin core and the pyrrole nitrogen, designated as d(Ct–N). The importance of this correlation was reemphasized recently (Huong & Pommier, 1977; Spiro et al., 1979; Scholler & Hoffman, 1979). The relation can be expressed by a linear equation, when the pyrrole tilting dihedral angle is ignored (Spiro et al., 1979), as

$$\nu = K(A - d) \qquad (cm^{-1})$$

Here, ν is the frequencies of the observed resonance Raman lines, d is the Ct–N distance, and the parameters K (cm^{-1}/Å) and A (Å) have the values 555.6 and 4.86 for ν_{19}, 423.7 and 5.87 for ν_{10}, and 375.5 and 6.01 for ν_3 [the numbering of the normal coordinates was given by Abe et al. (1978)]. This formula is considered to be valid with a standard deviation of 0.01 Å for d. On the basis of the equation, the Raman line observed at 1610 cm^{-1} (ν_{10}) in the transient form implies d(Ct–N) = 2.07 Å. This core size is large enough to accommodate the high-spin ferric heme iron to reside in the heme plane, whose d(Fe–N) is 2.07 Å (Koenig, 1965), where d(Fe–N) designates the distance between the heme iron and the pyrrole nitrogen. The Raman line at 1640 cm^{-1} in the transient form corresponds to d(Ct–N) = 2.00 Å. The size here also can accommodate the ferric low-spin heme iron in the heme plane as in the case of neutral and alkaline forms of the native protein, which are low-spin species. Accordingly, it is indicated that the transient form has an in-plane goemetry of the heme iron, regardless of its spin states.

At neutral pH, as stated above, the guanidinated derivative must have the heme environment resembling that of the neutral form of the native protein, and its heme iron resides in the heme plane. At alkaline pH, however, it shows a somewhat complex profile in 1500–1650-cm^{-1} region in contrast to that of unmodified protein. There are at leat seven Raman lines in this region, and these are interpreted to consist of two sets of lines, which are superimposed. The Raman lines at 1478 (ν_3), 1608 (ν_{10}), and 1552 cm^{-1} (ν_{19}) correspond to high-spin species, and following the equation, its d(Ct–N) must be 2.07 Å. This value is equal to that of the high-spin component of the transient form. Other lines, 1505 (ν_3), 1588 (ν_{19}), and 1637 cm^{-1} (ν_{10}), correspond to low-spin species, and its d(Ct–N) must be 2.00 Å. This is equal to d(Ct–N) of the low-spin component of the transient form. The line at 1560 cm^{-1} is one of the so-called oxidation state marker bands and indicates the presence of the ferric heme iron. This line shows a slight high-frequency shift on bringing the pH of the solvent higher from 7, which is considered to correspond to the 1562 → 1568 cm^{-1} shift of native ferricytochrome *c* (as seen in Figure 1). Thus, it remains sufficiently higher than 1540 cm^{-1}, which is considered to be the characteristic frequency of the reduced form, showing that photoreduction did not occur during the measurement and the heme iron was kept at ferric state. Accordingly, the guanidinated derivative has an in-plane geometry in all of its conformations. On the basis of the results so far described, it is suggeted that the transient form in question has a structural similarity around the heme environment to the alkaline form of the guanidinated protein. This suggestion is also supported by the absorption spectra shown in Figure 5. The profile of the alkaline form of the guanidinated derivative has a striking similarity to that of the transient intermediate which appears during the course of alkaline isomerization (Kihara et al., 1976; Saigo, 1981, 1982). Both have a strong absorption band at 600 nm and a Soret band blue shifted (from the neutral form) to about 400 nm. In addition, the guanidinated derivative shows a very broad absorption band in the 650–800-nm range (Figure 5), and a

similar broad band was recently found by Saigo (1982) for the transient intermediate.

In this work, it is shown that the guanidinated derivative has the same pK value as that of the native one (Figure 3). It has been proposed that the alkaline form of the guanidinated derivative is in state V (Theorell & Åkesson, 1941), of the native protein, because of the similarity of its EPR signal at liquid nitrogen temperature (Morton, 1973) to that of native protein at pH 14 (Theorell, 1941). It is seen, however, that an additional conformational change occurs in the guanidinated protein, which begins to occur at pH 12.4, as is in the case of native protein. This fact suggests that the alkaline form of the guanidinated derivative does not correspond to state V of the native protein.

The apparent pK of the first alkaline isomerization process is the same for both proteins, which suggests that the alkaline isomerization is initiated by similar processes in each case. It has been suggested (Davis et al., 1974) that the proton dissociation, which initiates the isomerization, involves the ϵ-NH_3^+ group of the displacing lysine ligand. The possible lysine residue, Lys-72 or Lys-79, is exposed to the solvent (Takano & Dickerson, 1981a,b) so that the intrinsic pK of the residue may be the same as that of free lysine, 10.5. The difference between the apparent pK of native ferricytochrome c (pK = 9.3) and that of the free lysine residue (pK = 10.5) has been explained by considering that a rapid proton dissociation with an intrinsic pK of 11.0 is coupled with a slow, energetically favorable ligand replacement equilibrium (Davis et al., 1974). However, the apparent pK of the protein, which has lysine residues modified to homoarginine (whose pK is 12.5), did not alter at all. Also, trifluoroacetylation of all lysine residues does not prevent the alkaline isomerization (Stellwagen et al., 1975). This protein can also isomerize with an apparent pK nearly equal to that of native protein (Stellwagen et al., 1975). Accordingly, it is concluded that the proton dissociation of the lysine residue is not essential to alkaline isomerization from neutral to alkaline form, though it must be deprotonated in order to become the sixth ligand of the heme iron at an alkaline pH in the native protein.

It has been proposed that the concentration of hydroxide anion reflects the pH dependence of this reaction and this anion displaces the methionine ligand, which is ligating to the heme iron at neutral pH (Stellwagen et al., 1975). From recent X-ray analysis (Takano & Dickerson, 1981a,b), the proximity of the heme environment has been examined in detail. It has been found that three water molecules are buried around here, and one of them is hydrogen bonded with the side chains of Asn-52, Tyr-67, and Thr-78, in the vicinity of the heme group. This water molecule seems to be essential for the alkaline isomerization.

In contrast to ferricytochrome c, an alkaline isomerization of ferrocytochrome c does not occur until the pH is raised to above 12 (Theorell & Åkesson, 1941). This difference between ferri- and ferrocytochrome c can be ascribed to the shift in the position of the water molecule in the heme crevice, which is involved in the hydrogen-bonding network (Takano & Dickerson, 1981b). By their X-ray analysis, the greatest conformational difference has been found around this water molecule. When the pH is raised to alkaline pH, the hydrogen-bonding network may be disturbed. This disturbance would alter the geometry of the Tyr-67, which has hydrogen bonded to the water molecule, and in turn, Met-80, which was coordinated to the heme iron and hydrogen bonded to the Tyr-67, would be affected.

In the course of the isomerization, Met-80 would become unable to strongly ligate to the heme iron, and the transient form would emerge. Following this process, Lys-72 or -79 may be deprotonated at the elevated pH and become able to ligate to the heme iron. This is a strong field ligand so that it can displace the weakly ligating Met-80 residue, if any, and can coordinate as the sixth ligand.

Lastly, the possible significance of finding such a transient form of cytochrome c in its alkaline isomerization is briefly mentioned. The amino acid resides around the 70–80 positions (on the Met-80 side of the heme plane) are highly conserved in many species of cytochrome c. This part of the protein is more flexible than the part on the His-18 side (Northrup et al., 1980) and accessible to chemical modification (McGowan & Stellwagen, 1970). These facts imply that these residues play an important role in the function of cytochrome c. Tyr-67, Lys-79, and Met-80, in question, are located here. It has been indicated that the alkaline form of the native protein loses reducibility (Greenwood & Palmer, 1965). Such a form was once suggested to be used for regulating the electron transfer in vivo, but it is inert in the redox reaction. The transient form, however, involves a high-spin state. In general, the spin state is known to be an important factor affecting the redox potential of cytochrome c (Moore & Williams, 1977), probably because of the change in bond length between the heme iron and the sixth ligand. It was also reported that low-spin ferricytochrome c can be reversibly converted to a high-spin form through association with a gold (I) compound, and it was suggested that this form has a functional role in the electron-transfer mechanism (Otiko & Sadler, 1980). The transient form, now in question, may be similar to this form. If such a transient form is stable enough in the period when cytochrome c is interacting with its redox partners, this could play an important role in vivo.

Registry No. Cytochrome c, 9007-43-6.

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