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Resonance Raman Assignment and Evidence for Noncoupling of Individual 2- and 4-Vinyl Vibrational Modes in a Monomeric Cyanomethemoglobin†

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ABSTRACT: We have investigated the resonance Raman spectra of monomeric insect cyanomethemoglobins (CTT III and CTT IV) reconstituted with (1) protohemes IX selectively deuterated at the 4-vinyl as well as the 2,4-divinyls, (2) monovinyl-truncated hemes such as pemptoheme (2-hydrogen, 4-vinyl) and isopemptoheme (2-vinyl, 4-hydrogen), (3) symmetric hemes such as protoheme III (with 2- and 3-vinyls) and protoheme XIII (with 1- and 4-vinyls), and (4) hemes without 2- and 4-vinyls such as mesoheme IX, deuteroheme IX, 2,4-dimethyldeuteroheme IX, and 2,4-dibromodeuteroheme IX. Evidence is presented that the highly localized vinyl C=C stretching vibrations at the 2- and 4-positions of the heme in these cyanomet CTT hemoglobins are noncoupled and inequivalent; i.e., the 1631- and 1624-cm^{-1} lines have been assigned to 2-vinyl and 4-vinyl, respectively. The elimination of the 2-vinyl (in pemptoheme) or the 4-vinyl (in isopemptoheme) does not affect the C=C stretching frequency of the remaining vinyl. Furthermore, two low-frequency vinyl bending modes at 412 and 591 cm^{-1} exhibit greatly different resonance Raman intensities between 2-vinyl and 4-vinyl. The observed intensity at 412 cm^{-1} is primarily derived from 4-vinyl, whereas the 591-cm^{-1} line results exclusively from the 2-vinyl. Again, there is no significant coupling between 2-vinyl and 4-vinyl for these two bending modes.

The 2- and 4-vinyl groups of iron protoporphyrin IX (protoheme IX) in hemoglobins play an important role in modulating heme reactivity through protein-heme interactions (Gersonde, 1983; Gersonde et al., 1986). The orientation of each vinyl (relative to the heme plane) and the nature of protein-vinyl interactions have been probed by nuclear magnetic resonance (NMR) spectroscopy (La Mar et al., 1978). On the other hand, resonance Raman spectroscopy provides useful information pertaining to the electronic and bonding nature of the vinyl groups in various ligated and unligated hemoglobins (Kerr et al., 1985; Gersonde et al., 1986, 1987; Tanaka et al., 1987).

The vibrational modes associated with the 2- and 4-vinyl groups have been assigned by Spiro and co-workers (Choi et al., 1982a,b; Choi & Spiro, 1983) in their resonance Raman studies on nickel(II) protoporphyrin IX dimethyl ester and its derivatives deuterated at the α-carbon as well as β-carbon atoms of the 2- and 4-vinyl groups. The identification of vinyl vibrational modes has been already made in monomeric insect (Kerr et al., 1985; Gersonde et al., 1986) and tetrameric human (Rousseau et al., 1983) hemoglobins. However, in these studies nonselective deuteration of vinyls in positions 2 and 4 did not allow the assignment of vibrational modes of individual vinyl groups. It is of fundamental interest to know whether or not the two vinyls in hemoglobins are vibrationally coupled.

In this paper, we demonstrate that certain Raman lines in the spectra of cyanomet CTT III can be assigned to 2- and 4-vinyl, respectively. Most importantly, we found that there is no significant coupling between the vibrational modes of 2- and 4-vinyl. The highly localized vinyl C=C stretching vibrations at the 2- and 4-positions are inequivalent. These assignments were made possible by the availability of the following synthetic hemes: 4-vinylprotoheme IX-α,β,β-d_3; monovinyl-truncated hemes, such as pemptoheme (2-vinyl is substituted by hydrogen) and isopemptoheme (4-vinyl is replaced by hydrogen); and symmetric hemes, such as protoheme III (with 2- and 3-vinyl) and protoheme XIII (with 1- and 4-vinyl). In addition, we compare resonance Raman spectra of hemoglobins with identical substituents at postions 2 and 4 (proto-IX, meso-IX, deutero-IX, 2,4-dimethyldeutero-IX, and 2,4-Br_2-deutero-IX CTT IV). We believe that an understanding of the vibrational properties of individual 2- and 4-vinyl is an essential step toward elucidating their role in mediating heme-protein interactions.

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Materials and Methods

Purification of Hemoglobins. The monomeric hemoglobins CTT III and CTT IV were isolated from the lymph of the insect larvae of *Chironomus thummi thummi* as described previously (Gersonde et al., 1972; Sick et al., 1972; La Mar et al., 1983). The chemical homogeneity of these CTT hemoglobins was checked by 500-MHz proton NMR spectra of the cyanomet complex and by polyacrylamide gel (10%) vertical slab electrophoresis at pH 9.5. R_f values were 0.60 and 0.71 for CTT's III and IV, respectively. The purified salt-free hemoglobins were lyophilized and stored at −30 °C.

Reconstitution of Hemoglobins. The globins of CTT's III and IV, respectively, were prepared by employing the modified acetone method (Gersonde et al., 1982). The insertion of iron into the porphyrins was performed as described elsewhere (Overkamp et al., 1976). Deuterohemin IX and mesohemin IX were synthesized by standard methods (Falk, 1964; Baker et al., 1964). Synthesis of protohemin XIII (Smith et al., 1986), pemptohemin (Smith et al., 1983), isopemptohemin (Smith et al., 1983), 2,4-dimethyldeuterohemin IX (Smith & Kehres, 1983), and 2,4-dibromodeuterohemin IX (Minnetian et al., 1989) was performed as indicated in the respective references. Deuteration of protoporphyrin IX resulting in 4-vinylprotoporphyrin IX-α,β,β-d_3 has been described elsewhere (Smith et al., 1983). The reconstitution of CTT's III and IV with deuterohemin IX, mesohemin IX, 2,4-dimethyldeuterohemin IX, 2,4-dibromodeuterohemin IX, 4-vinylprotohemin IX-α,β,β-d_3, pemptohemin, and isopemptohemin, respectively, was performed as described in detail recently (Gersonde et al., 1986).

Sample Preparation. Solutions of hemoglobins (~80 μM) were prepared by dissolving lyophilized oxidized proteins in 0.2 M citrate–phosphate buffer (pH 5.1) and 0.2 M Tris-HCl buffer (pH 9.4), respectively. Very small amounts of solid potassium cyanide were added to the hemoglobin solution to form the cyanomet complex. Nondissolved protein was completely removed by centrifugation. For ligand isotope substitution $K^{13}C^{15}N$ (enrichment, 90% ^{13}C and 92% ^{15}N) was employed.

Resonance Raman Spectroscopy. Resonance Raman spectra were obtained by employing a highly sensitive multichannel Raman system (Yu & Srivastava, 1981) specifically designed for Soret-excited resonance Raman spectroscopy of heme proteins. The system is equipped with a dry ice cooled silicon-intensified target vidicon detector (Model 1254; Princeton Applied Research, Princeton, NJ). The excitation wavelength was the 406.7-nm line of a Kr^+ laser (Model 171; Spectra-Physics, Mountain View, CA). The laser power at the sample was 10–20 mW. The sample was spun in a rotating cell throughout the measurements to avoid the local heating. The Raman spectra were obtained at room temperature by using a data integration time of 303 s (10 000 delay scans). All spectra presented here were not computer-smoothed. The reported wave numbers are accurate to ±1 cm^{-1}. Standard compounds (fenchone, benzene, acetone, etc.) were used for calibration.

Results

Assignment of Vibrational Modes to the 2- and 4-Vinyl in CTT III by Deuteration. The resonance Raman spectra of cyanomet proto-IX CTT III have been described elsewhere in detail and assignments of the Fe–CN stretching (452-cm^{-1}), Fe–C–N bending (412-cm^{-1}), and Fe–N_ϵ (proximal imidazole) stretching (309-cm^{-1}) modes have been made on the basis of iron and ligand isotope replacements (Yu et al., 1984; Gersonde et al., 1987; Kerr et al., 1984). In addition, by reconstitution of CTT III with deuteroheme IX and mesoheme IX, respectively, and by replacement of protoheme IX with protoheme IX deuterated at the vinyl α- and β-carbons, vinyl modes have been identified in the low-frequency region spectra at 591 and 412 cm^{-1} (Gersonde et al., 1987). The 412-cm^{-1} vinyl mode overlaps with the Fe–C–N bending mode. This composite line indicates an additional shoulder at ~423 cm^{-1} of unknown origin. Further examination of the low-frequency region spectra of cyanomet proto-IX CTT III results in the detection of unidentified lines at 520, 504, 479, 381, and 372 cm^{-1} (see spectrum A in Figure 1). The two lines at 381 and 372 cm^{-1} are often not well resolved. The relative intensities of these two lines change with the sample preparation (see three examples in inset I of Figure 1). However, it is certain that the observed change in ratio of intensities does not correlate with a Henderson–Hasselbalch pH titration behavior. Therefore, these spectral changes are not linked to the Bohr effect of CTT III. Both lines are not yet assigned, and their origin is still unknown. In addition, these changes seem to be very localized, as other accompanying changes are not observed in the spectra. We assume that these intensity changes are linked to the ratio of heme rotational components in CTT III (Gersonde et al., 1987). It has been demonstrated for this hemoglobin by proton NMR that the nonsymmetric heme group is inserted by two ways, i.e., rotated by 180° about the α,γ-meso axis, resulting in two heme rotational components A and B (La Mar et al., 1980; Peyton et al., 1987). Hence,

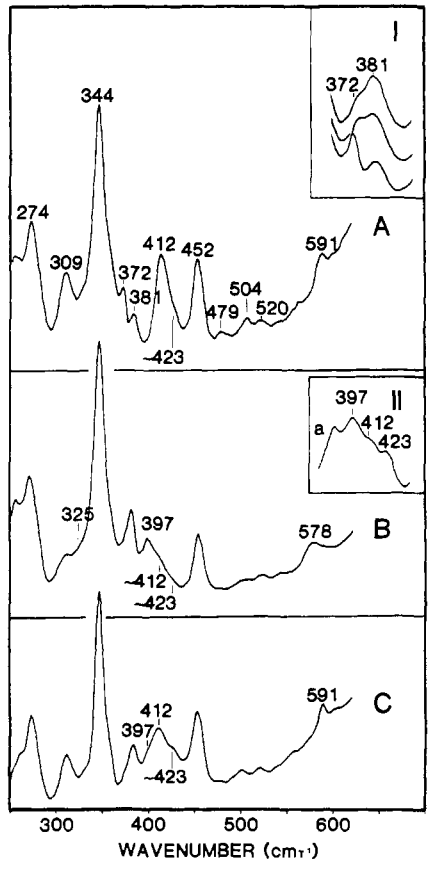

FIGURE 1: Effects of deuteration at the 2,4-divinyls and at the 4-vinyl, respectively, on the low-frequency region (250–650-cm^{-1}) resonance Raman spectra of cyanomet CTT III at pH 9.4. (A) Proto-IX CTT III; (B) 2,4-divinylproto-IX-β,β,β,β-d_4 CTT III; (C) 4-vinylproto-IX-α,β,β-d_3 CTT III. Inset I shows the variations of the relative intensities of the 372- and 381-cm^{-1} lines, corresponding to different ratios of heme rotational components and depending on sample preparation. Inset II shows better resolved peaks at 412 and 423 cm^{-1} in the spectrum of 2,4-divinylproto-IX-β,β,β,β-d_4 CTT III at pH 9.4 (a different sample preparation from that of curve B).

in both forms of this hemoglobin the porphyrin substituents interact with different protein sites (Gersonde et al., 1986). The resonance Raman spectra of cyanomet isopempto and pempto CTT III support the idea that the 372- and 381-cm^{-1} lines are due to heme rotational components. From recent proton NMR studies of the hyperfine-shifted heme protons of these latter complexes it is known that in cyanomet isopempto CTT III one of the two heme rotational components (namely, A) is more stable and hence dominant, whereas pempto CTT III exhibits a spectral behavior that is similar to the natural proto-IX CTT III (Gersonde et al., unpublished data). Hence, the 382-cm^{-1} line in cyanomet isopempto and proto-IX CTT III can be attributed to the major heme rotational component A. The interconnection between the 381- and 372-cm^{-1} lines also supports their possible origin from rotational disorder.

Deuteration of the β-carbons of the vinyl groups at both the 2- and 4-positions results in shifts to lower frequencies by about 15 cm^{-1} for the following lines of the low-frequency region spectra: The 412-cm^{-1} line shifts to 397 cm^{-1}, the 591-cm^{-1} line shifts to 578 cm^{-1}, and a hidden line under the 344-cm^{-1} mode appears as a shoulder at 325 cm^{-1} (Kerr et al., 1985) (see spectrum B in Figure 1). In cyanomet 2,4-divinylproto-IX-β,β,β,β-d_4 CTT III, the formerly intense sharp 412-cm^{-1} line (observed in proto-IX CTT III) splits into three lines occurring now at 423, 412, and 397 cm^{-1}. The 412- and 423-cm^{-1} components may not be ovbious in Figure 1B. However, in a separate sample preparation, we obtained a Raman spectrum that clearly exhibits these two peaks (shown in inset II of Figure 1). Hence, the 412-cm^{-1} line in cyanomet proto-IX CTT III is a composite of at least three modes. The 412-cm^{-1} line, which shifts to 397 cm^{-1} by β-carbon vinyl deuteration, is a vinyl vibrational mode. The 412-cm^{-1} line (a weak shoulder in spectrum B of Figure 1) has to be attributed to the Fe–C–N bending mode, whereas the origin of the 423-cm^{-1} line is still unknown.

In order to identify the vibrational modes from either 2-vinyl or 4-vinyl, we analyzed the spectrum of cyanomet proto-IX CTT III deuterated only at the 4-vinyl group (see spectrum C of Figure 1). The line at 591 cm^{-1} remains unaffected by isotope labeling of the 4-vinyl group. Hence, this line must be assigned to the 2-vinyl group. The 520-, 504-, and 479-cm^{-1} lines (see spectrum A in Figure 1) remain unaffected. However, the intensity of the 412-cm^{-1} line (at pH 9.5) drops markedly, and a new shoulder at 397 cm^{-1} becomes visible, where the spectrum of proto-IX CTT III exhibits a deep trough between the 412- and 381-cm^{-1} lines (compare spectra A and C in Figure 1). This shoulder results from a deuterated 4-vinyl vibrational mode. The same line is observed for cyanomet 2,4-divinylproto-IX-β,β,β,β-d_4 CTT III at pH 9.5 (spectrum B of Figure 1) with somewhat greater intensity, indicating that the 412-cm^{-1} line intensity observed in spectra A and C of Figure 1 has a small contribution from 2-vinyl. Thus, the former 412-cm^{-1} line intensity (spectrum A of Figure 1) has a predominant contribution from the 4-vinyl group. Notice that Figure 1C (only 4-vinyl deuteration) exhibits no shoulder near 325 cm^{-1}, like the one seen in Figure 1B (both 2- and 4-vinyl deuteration). Thus, we conclude that there must be a deuterium-sensitive 2-vinyl mode hidden under the strong 344-cm^{-1} line of Figure 1A.

In the high-frequency region spectra (1300–1700 cm^{-1}) a triplet line (at 1640, 1631, and 1624 cm^{-1}) is observed for cyanomet proto-IX CTT III (see Figure 2). Deuteration at the β-carbon atoms of both vinyls (at positions 2 and 4) leads to a shift of the 1631- and 1624-cm^{-1} lines, appearing as a broad and structureless peak centered at 1591 cm^{-1} (see Figure 2B) (Gersonde et al., 1987). Deuteration only at the 4-vinyl group results in a low-frequency shift of the 1624-cm^{-1} line only. The vinyl mode at 1631 cm^{-1} is not shifted. Hence, the following interpretation becomes valid: The 1624-cm^{-1} mode belongs to the 4-vinyl, whereas the 1631-cm^{-1} mode has to be attributed to the 2-vinyl. The vinyl scissors mode identified at 1431 cm^{-1} (Choi et al., 1982a) shifts underneath the 1399-cm^{-1} mode upon deuteration at the β-carbons of the 2- and 4-vinyl groups (indicated by an arrow in spectrum B of Figure 2). When only the 4-vinyl is deuterated, the 1431-cm^{-1} line loses some intensity, indicating that part of this 1431-cm^{-1} line is also shifted underneath the 1399-cm^{-1} mode (indicated by an arrow in spectrum C of Figure 2). Thus, the observed intensity at 1431 cm^{-1} in Figure 1A is contributed comparably by both 2- and 4-vinyls.

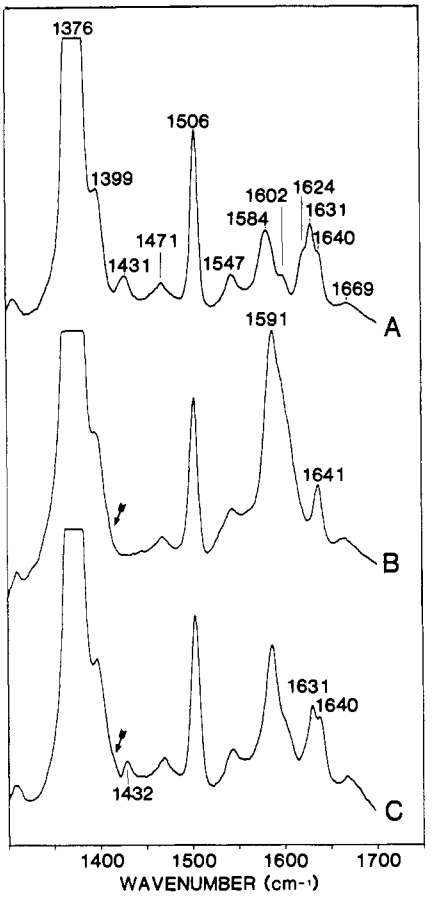

FIGURE 2: Effects of deuteration at the 2,4-divinyls and at the 4-vinyl, respectively, on the high-frequency region (1300–1700-cm^{-1}) resonance Raman spectra of cyanomet CTT III at pH 9.4. (A) Proto-IX CTT III; (B) 2,4-divinylproto-IX-β,β,β,β-d_4 CTT III; (C) 4-vinylproto-IX-α,β,β-d_3 CTT III. Arrow indicates the shift of 1431-cm^{-1} intensity to lower frequency.

Identification of Vinyl Vibrational Modes in Cyanomet Pempto (4-Vinyl) and Isopempto (2-Vinyl) CTT III. In isopempto CTT III the 4-vinyl is replaced by hydrogen, whereas in pempto CTT III the 2-vinyl is substituted by hydrogen. The investigation of the resonance Raman spectra of these cyanomet complexes lends further support to our previous assignment of the vibrational modes to either 2-vinyl or 4-vinyl. There is practically no vibrational coupling between 2-vinyl and 4-vinyl.

In Figure 3 (panel II) we compare the resonance Raman spectra (250–650 cm^{-1}) of both complexes at pH 9.4. One striking difference between cyanomet pempto (spectrum A) and isopempto CTT III (spectrum B) is the vibrational mode

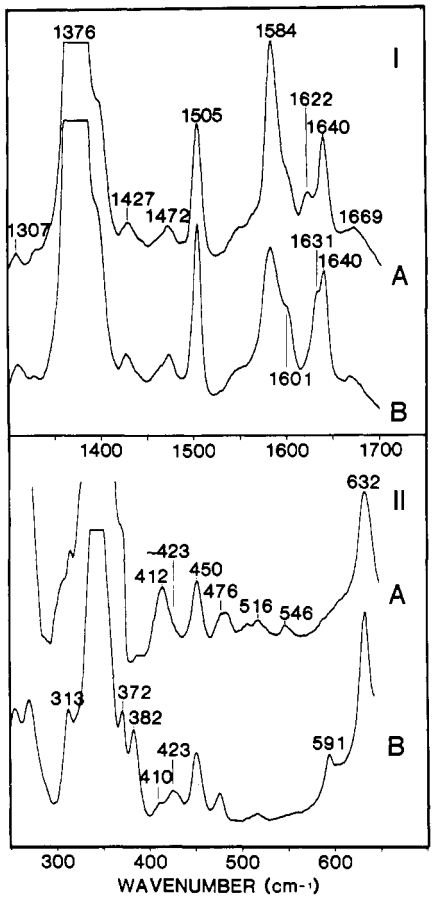

FIGURE 3: Panel I: High-frequency region (1300–1700-cm^{-1}) resonance Raman spectra of cyanomet pempto (A) and cyanomet isopempto (B) CTT III at pH 9.4. Panel II: Low-frequency region (250–650-cm^{-1}) resonance Raman spectra of cyanomet pempto (A) and cyanomet isopempto (B) CTT III at pH 9.4.

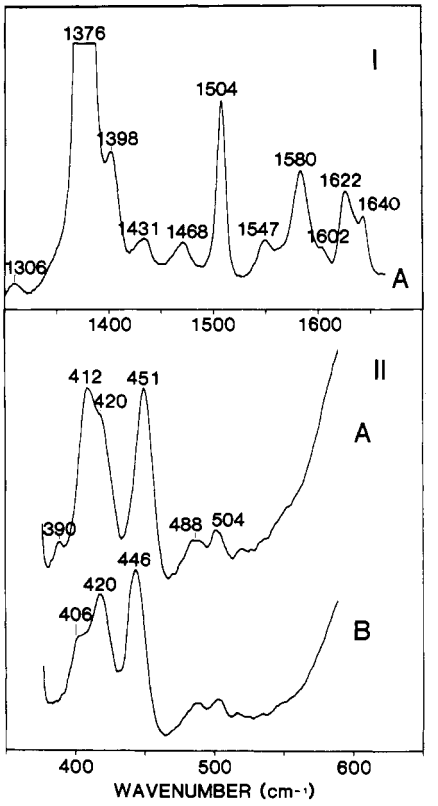

FIGURE 4: Panel I: High-frequency region (1300–1700-cm^{-1}) resonance Raman spectra of cyanomet proto-XIII CTT III at pH 9.4. Panel II: Low-frequency region (350–650-cm^{-1}) resonance Raman spectra of cyanomet proto-XIII CTT III ligated with $^{12}C^{14}N^-$ (A) and $^{13}C^{15}N^-$ (B), respectively, at pH 9.4.

at 591 cm^{-1} which is completely absent in the spectrum of pempto CTT III. Spectral differences are also observed in the 350–450-cm^{-1} region: (i) Pempto CTT III exhibits an intense and broad line at 412 cm^{-1}. This line reflects the triplet structure already described for proto-IX CTT III in Figure 1, indicating the overlap of the Fe–C–N bending mode, the 4-vinyl mode, and the unassigned mode at ~423 cm^{-1} (shoulder). (ii) The spectrum of cyanomet isopempto CTT III clearly exhibits two lines at 410 and 423 cm^{-1}. Comparison of the spectra of pempto and isopempto CTT III allows us to identify the 591-cm^{-1} line as 2-vinyl and the 412-cm^{-1} line as 4-vinyl modes. As the 4-vinyl mode at 412 cm^{-1} is missing in isopempto CTT III, the residual 410-cm^{-1} line must be the δ(Fe–C–N). The intense and sharp 382-cm^{-1} line, which is clearly resolved in the spectrum of isopempto CTT III, is reduced in intensity in the spectrum of pempto CTT III. This line correlates with the change in ratio of the two heme rotational components. This vibrational mode has not yet been assigned.

In Figure 3 (panel I) the high-frequency region (1300–1700-cm^{-1}) spectra of cyanomet pempto and isopempto CTT III at pH 9.4 are shown. Certain Raman lines can also be assigned to individual vinyls. Both pempto (spectrum A) and isopempto CTT III (spectrum B) show the 1640-cm^{-1} line which is also present in the spectrum of proto-IX CTT III (see Figure 2). It is a porphyrin skeleton mode (b_{1g}) (Felton & Yu, 1978; Spiro, 1983). The 1631-cm^{-1} line appears only in isopempto CTT III, but not in pempto CTT III. Hence, the 1631-cm^{-1} line is a 2-vinyl mode. The 1624-cm^{-1} line (observed in proto-IX CTT III) is missing in isopempto CTT III, which does not have a 4-vinyl. But it is present in pempto CTT III occurring at 1622 cm^{-1}, which has a 4-vinyl. Thus, this line has to be attributed to a 4-vinyl mode. The 1431-cm^{-1} mode, formerly assigned to vinyls by deuteration at 2- and 4-vinyl (Gersonde et al., 1987), is affected by these vinyl replacements, showing an intensity drop at 1427 cm^{-1}. Therefore, the 1431-cm^{-1} line is contributed to by both 2- and 4-vinyls.

Vinyl Vibrational Modes in Cyanomet CTT III Reconstituted with Symmetrical Porphyrins. Resonance Raman spectra of cyanomet proto-III CTT III (vinyls in positions 2 and 3) have been reported in detail previously (Gersonde et al., 1987). Here, we present the spectra of cyanomet proto-XIII CTT III (vinyls in positions 1 and 4) (see Figure 4) and compare them with those of cyanomet proto-III CTT III, focusing on the vinyl modes. In the low-frequency region spectrum of proto-XIII CTT III (measured at pH 9.4) the 591-cm^{-1} line, which has been assigned to the 2-vinyl mode, is missing as expected for this porphyrin without a 2-vinyl group. This 591-cm^{-1} line appears in the spectrum of cyanomet proto-III CTT III which possesses the 2- and 3-vinyl groups (symmetric porphyrin) (Gersonde et al., 1987). Furthermore, at pH 9.4 an intense line at 412 cm^{-1} with a shoulder at 420 cm^{-1} was observed in the spectrum of cyanomet proto-XIII CTT III (see spectrum A in panel II of Figure 4). Replacement of $^{12}C^{14}N^-$ by $^{13}C^{15}N^-$ results in a drop in intensity and in a shift in frequency of the 412-cm^{-1} line to 406 cm^{-1}, indicating this line as the Fe–C–N bending mode (see spectrum B in panel II of Figure 4). Hence, the remaining line at 420 cm^{-1} in the $^{13}C^{15}N^-$ complex must be a 4-vinyl vibrational mode (the 1-vinyl may also contribute), equivalent to the 412-cm^{-1} line attributed to 4-vinyl in the $^{12}C^{14}N^-$ complex of proto-IX CTT III. The enhancement of the 412-cm^{-1} intensity is due to the closer proximity to the 420-cm^{-1} line and the

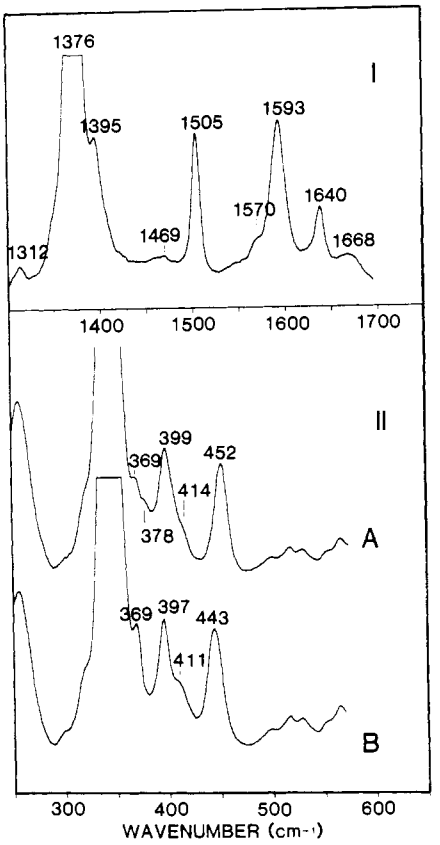

FIGURE 5: Panel I: High-frequency region (1300–1700-cm^{-1}) resonance Raman spectra of cyanomet meso-IX CTT IV showing the absence of two vinyl C=C stretching modes at 1624 and 1631 cm^{-1} at pH 9.4. Panel II: Low-frequency region (250–600-cm^{-1}) resonance Raman spectra of cyanomet meso-IX CTT IV ligated with $^{12}C^{14}N^-$ (A) and $^{13}C^{15}N^-$ (B), respectively, at pH 9.4.

resonance interaction between the Fe–C–N bending and 4-vinyl vibrational mode. In contrast to cyanomet deutero-IX CTT III, the respective proto-XIII and proto-III complexes exhibit only one Fe–C–N bending mode because of the lack of heme rotational disroder in the symmetric heme proteins. The 420-cm^{-1} line is missing in the spectrum of cyanomet proto-III CTT III because of the missing 4-vinyl (Gersonde et al., 1987). The Fe–C–N bending mode in the corresponding proto-III CTT III spectrum occurs at a similar frequency (412 cm^{-1}) (Gersonde et al., 1987).

The high-frequency spectrum of cyanomet proto-XIII CTT III (see panel I of Figure 4) is similar, with respect to the frequencies at 1640 and 1622 cm^{-1}, to that of pempto CTT III. However, the 1622-cm^{-1} line is stronger in proto-XIII CTT III than in pempto CTT III because of the contribution of the equivalent vinyls (1- and 4-vinyl) to the 1622-cm^{-1} intensity. The 1631-cm^{-1} line (assigned to 2-vinyl) is missing in proto-XIII CTT III.

Effect of 2- and 4-Vinyl Replacement on Resonance Raman Spectra of Cyanomet Proto-IX CTT III. It is the purpose of the replacement of vinyls in cyanomet proto-IX CTT III or CTT IV by ethyls (meso-IX CTT IV), methyls (2,4-dimethyldeutero-IX CTT III), bromines (Br_2-deutero-IX CTT III), and hydrogens (deutero-IX CTT IV) to see if those vinyl modes that were previously assigned are missing in the resonance Raman spectra. It also allows us to reveal those modes in the Raman spectra that are hidden under the vinyl modes in natural proto-IX CTT III.

In the low-frequency region spectra of the $^{12}C^{14}N^-$ complex of meso-IX CTT IV (see panel II of Figure 5) the already assigned 2- and 4-vinyl modes are missing. Two Fe–C–N

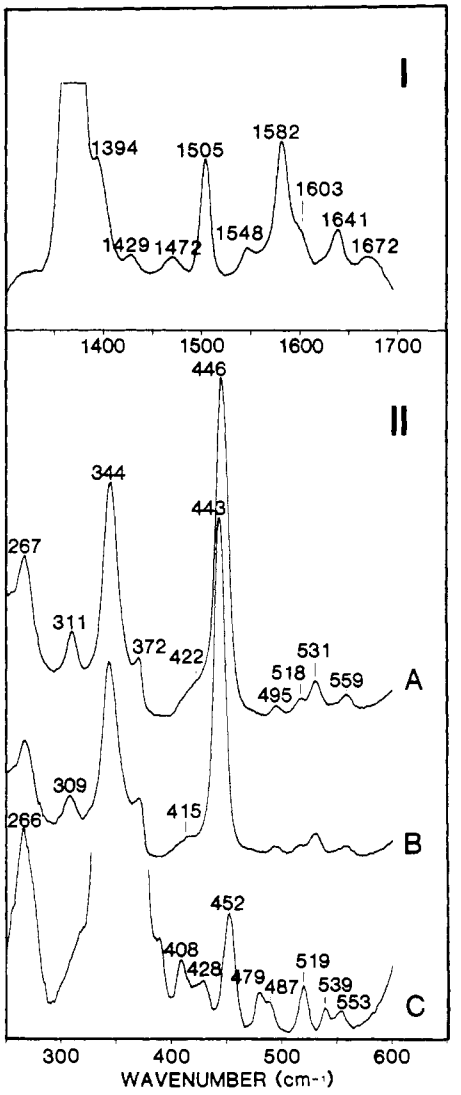

FIGURE 6: Panel I: High-frequency region (1300–1700-cm^{-1}) resonance Raman spectrum of cyanomet 2,4-Br_2-deutero-IX CTT III at pH 9.4. Panel II: Low-frequency region (250–600-cm^{-1}) resonance Raman spectra of cyanomet 2,4-Br_2-deutero-IX CTT III ligated with $^{12}C^{14}N^-$ (A), 2,4-Br_2-deutero-IX CTT III ligated with $^{12}C^{14}N^-$ (A), 2,4-Br_2-deutero-IX CTT III ligated with $^{13}C^{15}N^-$ (B), and deutero-IX CTT III ligated with $^{12}C^{14}N^-$ (C) at pH 9.4.

bending modes which were hidden under the 4-vinyl mode in proto-IX CTT III are now clearly visible. By ligand isotope exchange (replacement of $^{12}C^{14}N^-$ by $^{13}C^{15}N^-$) at pH 9.4 we identify both Fe–C–N bending modes at 399 and ~414 cm^{-1} (for $^{12}C^{14}N^-$) shifted to 397 and ~411 cm^{-1} (for $^{13}C^{15}N^-$). The high-frequency region spectrum of cyanomet meso-IX CTT IV at pH 9.4 (see panel I of Figure 5) lacks the 2- and 4-vinyl modes (observed at 1624 and 1631 cm^{-1} in proto-IX CTT III). The highest porphyrin skeleton fundamental mode (b_{1g}) at 1640 cm^{-1} (Felton & Yu, 1978; Spiro, 1983) is well resolved, but occurs as a shoulder in the spectrum of proto-IX CTT III. Interestingly, this mode is not affected by the vinyl replacement, indicating the lack of coupling between the vinyl stretching and this skeleton vibration. The 1431-cm^{-1} line which has been assigned to both 2- and 4-vinyl in the spectrum of cyanomet proto-IX CTT III (see Figure 2) is missing. This line has been detected in pempto and isopempto CTT III (see panel I of Figure 3).

The low-frequency region spectra of Br_2-deutero-IX CTT III exhibit more sharpened lines (see spectra A and B in panel II of Figure 6). They show differences, compared with that of deutero-IX CTT III (see spectrum C in panel II of Figure

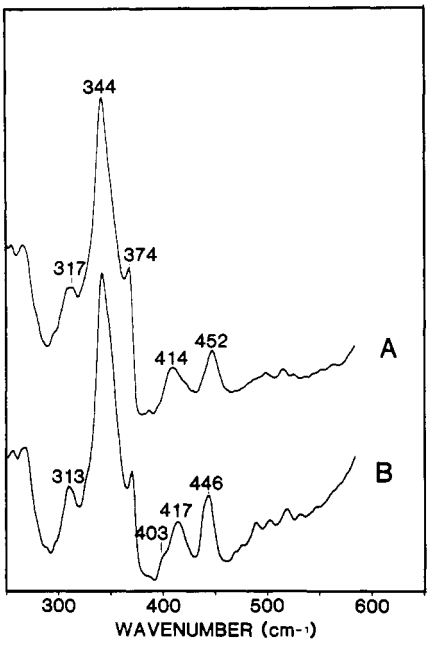

FIGURE 7: Low-frequency region (250–600-cm^{-1}) resonance Raman spectra of cyanomet 2,4-dimethyldeutero-IX CTT III ligated with $^{12}C^{14}N^-$ (A) and $^{13}C^{15}N^-$ (B) at pH 9.4.

6), with respect to the Fe–CN stretching, Fe–C–N bending, and Fe–N_ϵ (proximal histidine). The ν(Fe–CN) is much more enhanced and shifted from 452 cm^{-1} (deutero-IX CTT III) to 446 cm^{-1} (Br_2-deutero-IX CTT III). This line shifts with increasing ligand mass from 446 cm^{-1} ($^{12}C^{14}N^-$) to 443 cm^{-1} ($^{13}C^{15}N^-$). Therefore, the effect of substitution of hydrogen by bromine is to weaken the σ bond between iron and cyanide. The ligand isotope-sensitive line at ~422 cm^{-1} shifts to ~415 cm^{-1} by $^{13}C^{15}N^-$ substitution, indicating that this is the Fe–C–N bending mode. Whereas in deutero-IX CTT III two Fe–C–N bending modes are clearly resolved, in Br_2-deutero-IX CTT III only one Fe–C–N bending mode can be observed. The Fe–N_ϵ (proximal histidine) stretch, which was previously assigned in proto-IX CTT III at 309 cm^{-1} (Kerr et al., 1984), appears at 311 cm^{-1}. This line is well resolved and intense, in contrast to the Fe–N_ϵ stretch in deutero-IX CTT III. It shifts upon $^{13}C^{15}N$ substitution to 309 cm^{-1}.

In the high-frequency region spectra of Br_2-deutero-IX (see panel I of Figure 6), deutero-IX [see Gersonde et al. (1987)], 2,4-dimethyldeutero-IX (spectrum not shown), and meso-IX CTT's (see panel I of Figure 5) the vinyl modes at 1624 and 1631 cm^{-1} (typical for proto-IX CTT III) are missing. This is expected because of the lack of the vinyl in positions 2 and 4. It is interesting that the porphyrin skeleton mode at 1640 cm^{-1} is not affected by the replacement of vinyls.

The low-frequency region spectrum of 2,4-dimethyldeutero-IX CTT III ligated with $^{13}C^{15}N^-$ at pH 9.4 shows a shoulder at 403 cm^{-1} and a 417-cm^{-1} line (see spectrum B in Figure 7). Substitution of $^{13}C^{15}N^-$ by $^{12}C^{14}N^-$ (compare spectrum A in Figure 7) leads to a high-frequency shift of the shoulder at 403 cm^{-1} emerging into the 417-cm^{-1} line and resulting in a 414-cm^{-1} line. Hence, we observe at least one Fe–^{13}C–^{15}N bending mode (at 403 cm^{-1}) in this complex. The Fe–CN stretching frequency of 2,4-dimethyldeutero-IX CTT III is identical with that of proto-IX CTT III.

Discussion

Noncoupling of Vibrational Modes between 2- and 4-Vinyl Groups. The protoheme-IX in cyanomet CTT III contains vinyl groups at positions 2 and 4. These vinyl groups are inequivalent with respect to the α,γ-meso axis. By selective deuteration at the 4-vinyl and comparison with the 2,4-divinyl deuterated complex we are able to assign the C=C stretching mode at 1624 cm^{-1} to 4-vinyl and that at 1631 cm^{-1} to 2-vinyl vibrations. *The elimination of the 2-vinyl (in pemptoheme) or the 4-vinyl (in isopemptoheme) does not affect the C=C stretching frequency of the remaining vinyl.* In the symmetric protoheme-XIII (containing 1- and 4-vinyl) we observe only one vinyl C=C stretching mode at 1622 cm^{-1}. The two equivalent vinyls (symmetric positions at 1 and 4) contribute to the intensity of the 1622-cm^{-1} line. These results indicate that there exists no coupling between the two vinyl C=C stretching vibrations of protoheme-IX in cyanomet CTT III. This is somewhat surprising in view of the close proximity of these vinyls and their possible conjugation with the porphyrin macrocycle. In general, when a molecule possesses two identical functional groups (or oscillators), the vibrational modes from these oscillations may couple to produce two frequencies (in-phase and out-of-phase combination) which are both higher and lower than the unperturbed frequency (Lord & Miller, 1956). Our experimental data clearly indicate that *there is no significant coupling between the vibrational modes of the 2- and 4-vinyl groups.*

The noncoupling of the 2- and 4-vinyl vibrational modes and their inequivalency may be induced by their differential interactions with the protein. The inequivalent C=C stretches at 1624 cm^{-1} (4-vinyl) and 1631 cm^{-1} (2-vinyl) may originate from different orientations of the vinyl groups with respect to the heme plane. The difference in C=C stretching frequencies reflects different degrees of vinyl conjugation with the prophyrin ring in the *ground* state. It is expected that a greater degree of vinyl conjugation should give rise to a lower C=C stretching frequency. In contrast, the 2- and 4-vinyl vibrations are coupled in a protein-free protoporphyrin, as demonstrated for the nickel protoporphyrin IX dimethyl ester by Choi et al. (1982a), who found a 14-cm^{-1} displacement between the in-phase (Raman) and out-of-phase (infrared) combination of the vinyl C=C stretches.

The two Raman lines at 412 and 591 cm^{-1} that are sensitive to vinyl deuteration do not represent the same kind of vibration from two different vinyls because they are too far apart in frequency. As demonstrated in this study, each has unequal intensity contributions from 2- and 4-vinyl groups. The vinyl skeletal bend ($\delta C_bC_\alpha C_\beta$), the in-plane bend of the C_b–C_α bond (δC_b–C_α), the out-of-plane wagging of C_b–C_α (γC_b–C_α), and the torsion about the C_b–C_α axis (τC_b–C_α) are expected to contribute to vibrations in this region (Uchida et al., 1988). However, these coordinates may mix with porphyrin skeletal modes to produce the two different vibrations at 412 and 591 cm^{-1}. Choi and Spiro (1983) assigned the 412-cm^{-1} line of $(ImH)_2$iron(II) protoporphyrin to the $C_bC_\alpha C_\beta$ bending mode. Uchida et al. (1988) found that the 410-cm^{-1} line of oxymyoglobin and (carbonmonoxy)myoglobin significantly loses intensity upon 4-vinyl deuteration and thus ascribed it to a porphyrin in-plane vibration strongly coupled with the skeletal bend ($\delta C_bC_\alpha C_\beta$) of the 4-vinyl, in agreement with our observation that the intensity at 412 cm^{-1} in cyanomet CTT III has a major contribution from 4-vinyl. However, the deuterium-sensitive line at 591 cm^{-1} has not been assigned previously. It may correspond to one of the skeletal E_u modes (Choi & Spiro, 1983) coupled with the above-mentioned vinyl vibrations. Its intensity is derived primarily from 2-vinyl. In contrast, a symmetric vinyl CH_2 scissors mode (coupled with porphyrin skeletal modes) (Choi et al., 1982a) at ~1431 cm^{-1} exhibits significant intensity contributions from both 2- and 4-vinyl groups.

Lack of Heme Rotational Components in Hemoglobins with Symmetric Heme Group. For cyanomet deutero-IX and meso-IX CTT III we observed two Fe–C–N bending modes. These two bending modes reflect the two heme rotational components (Gersonde et al., 1987). The correlation between the Fe–C–N bending mode and the heme rotational component is supported by the data obtained from cyanomet proto-III (Gersonde et al., 1987) and proto-XIII CTT III. In symmetric proto-III and proto-XIII CTT III, only one heme rotational component is possible and the lack of the 4-vinyl mode at $\sim$412 cm^{-1} in cyanomet proto-III CTT III allows the detection of only one Fe–C–N bending mode.

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