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# Determinants of the Vinyl Stretching Frequency in Protoporphyrins. Implications for Cofactor−Protein Interactions in Heme Proteins

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**Abstract:** Soret-excitation resonance Raman (RR) spectra are reported for the bis-imidazole complexes of a series of mono- and divinylhemins. The complexes include 2-vinyldeuterohemin IX, 4-vinyldeuterohemin IX, protohemin IX, and protohemin III. For all four hemins, two polarized RR bands are observed at ~1620 and ~1631 cm$^{-1}$. Both of these bands are absent from the spectrum of the deuterohemin IX, which contains no vinyl substitutents. The relative intensities of the 1620- and 1631-cm$^{-1}$ bands are ~60:40 for all of the vinylhemins studied. However, the intensity of each band of both monovinyl complexes is approximately one-half that of the analogous bands of both divinyl complexes. The appearance of the 1620- and 1631-cm$^{-1}$ bands is independent of solvent although the 1631-cm$^{-1}$ band is difficult to identify in aqueous solutions wherein the hemins are aggregated. Temperature-dependent RR studies indicate that the intensity of the 1630-cm$^{-1}$ band monotonically decreases relative to that of the 1620-cm$^{-1}$ feature as the temperature is lowered. The 1620-cm$^{-1}$ feature has generally been assigned as the characteristic vinyl stretching mode ($\nu_{C=C}$) of vinylhemins. The 1631-cm$^{-1}$ band has not been previously identified in the RR spectra of vinylhemins in solution but has been observed in the spectra of heme proteins which contain protohemin IX. For the proteins, the 1631-cm$^{-1}$ band has been assigned as a second $\nu_{C=C}$ mode. The appearance of two $\nu_{C=C}$ modes has generally been attributed to site-specific vinyl group−protein interactions which render the 2- and 4-vinyl substituents of the protohemin IX cofactor inequivalent. In the case of the vinylhemins in solution, we also assign the 1631-cm$^{-1}$ band to a second $\nu_{C=C}$ mode. However, the simultaneous appearance of two $\nu_{C=C}$ modes is attributed to the existence of two nearly equal-energy vinyl torsional conformers which are intrinsic to a single vinyl group. In the divinyl complexes, both conformers occur for each vinyl group; however, the 2- versus 4-vinyl substituents cannot be distinguished due to the absence of vibrational coupling. Local density functional calculations on a vinylporphyrin model and several vinyl-substituted small molecules confirm that two vinyl torsional conformers should exist and that these conformers are close in energy (within 450 cm$^{-1}$ or less). In the porphyrin, the vinyl group of the lower energy form (Conformer I) is nearly in plane and points toward the $\beta$-pyrrole methyl group. The vinyl group of the higher energy form (Conformer II) is out of plane by ~40° and points toward the *meso*-hydrogen. Explicit second-derivative calculations on the small molecules indicate that the frequencies of the $\nu_{C=C}$ modes of the two vinyl torsional conformers differ by 10−20 cm$^{-1}$. The calculations further suggest that the 1620- and 1631-cm$^{-1}$ $\nu_{C=C}$ modes observed for the vinylhemins in solution are associated with Conformers I and II, respectively. The fact that vinylhemins can occupy two nearly-equal energy torsional conformers has significant implications for the interpretation of the RR spectra of proteins that contain protohemin IX. In particular, the appearance of two $\nu_{C=C}$ modes does not necessarily justify an interpretation which invokes site-specific vinyl group−protein interactions.

## I. Introduction

Many biologically important tetrapyrroles contain peripheral substituent groups that can conjugate with the $\pi$-electron system of the macrocycle.[1] Protoheme IX with 2,4-divinyl substituents is the most commonly occurring cofactor. This prosthetic group is found in oxygen carriers, peroxidases, and *b*-type cytochromes.[2] Variants of protoheme are also found in nature, for example, heme *a* and spirographis heme, which contain 4-vinyl-8-formyl and 2-formyl-4-vinyl substituents, respectively. The former tetrapyrrole is a redox cofactor in cytochrome *c* oxidase;[3] the latter serves as the binding site for molecular oxygen in the oxygen carrying proteins of certain invertebrates.[1] Conjugating substituents are also found in the photosynthetic pigments chlorophyll *a* and bacteriochlorophyll *a*.[1,4] These cofactors contain 2-vinyl and 2-acetyl groups, respectively. Conjugating substituents provide a conduit for electronic communication between the porphyrin macrocycle and the protein matrix. Such interactions have been proposed for the vinyl groups of a protoheme IX in a number of proteins,[5−9] for formyl group of heme *a* in cytochrome *c* oxidase,[10] and the acetyl group of bacteriochlorophyll *a* in photosynthetic reaction centers.[11]

Resonance Raman (RR) spectroscopy is a sensitive probe of the $\pi$-electronic structure of tetrapyrroles.[12] Over the past 15 years, there have been a number RR studies aimed at character-

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izing the vibrational properties of the conjugating substituents on these macrocycles.[10d,12b,13−15] Protoheme IX is arguably the most extensively studied naturally occurring tetrapyrrole. Spiro and co-workers were the first to conduct detailed vibrational studies on this cofactor and related metalloprotoporphyrins.[13a,b] These workers assigned two bands in Ni(II) protoporphyrin IX (RR band at ~1634 cm$^{-1}$; IR band at ~1620 cm$^{-1}$) to the vinyl stretching vibrations ($\nu_{C=C}$). The splitting of these bands was attributed to vibrational coupling. Kitagawa and co-workers assigned two RR bands in nickel(II) protoporphyrin (1610 and 1633 cm$^{-1}$) as the $\nu_{C=C}$ modes.[13c] Later, several groups provided evidence that the $\nu_{C=C}$ modes in protoporphyrins are not in fact vibrationally coupled. Asher and co-workers performed RR studies on both mono- and divinylhemins and showed that the $\nu_{C=C}$ vibrations of both occur at ~1620 cm$^{-1}$.[15] Champion and co-workers examined single crystal RR spectra

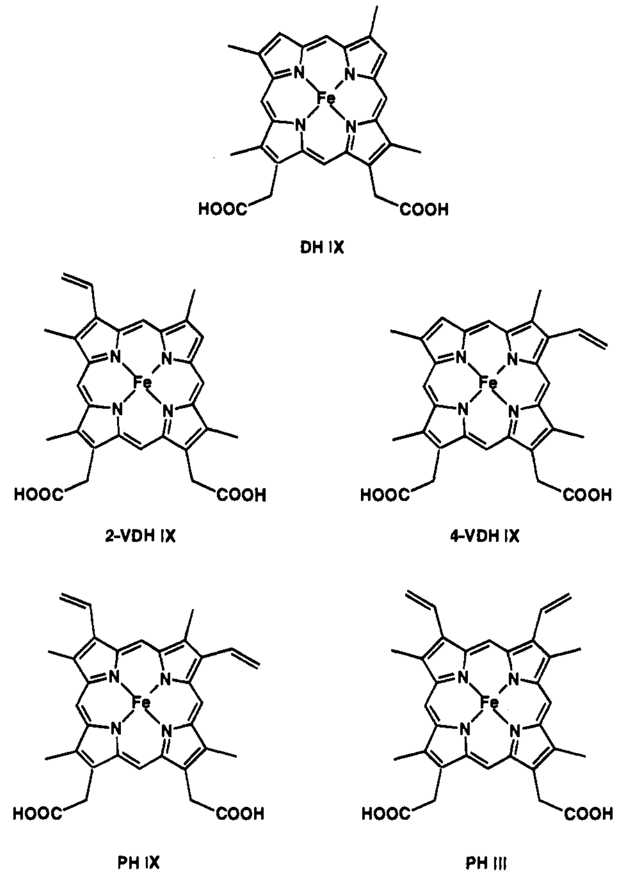

**Figure 1.** Structures of the various hemins examined in this study. The axial imidazole ligands are omitted for clarity.

of myoglobin and found that the two $\nu_{C=C}$ modes of the protoheme IX cofactor exhibit the same frequency and give rise to a single band at ~1620 cm$^{-1}$.[16] The absence of interaction between the two vinyl groups of protoheme is also consistent with the results of INDO/CI calculations which indicate that the torsional potential of a given vinyl group is independent of the torsional angle of the other.[17] In certain protoheme IX containing proteins, such as insect hemoglobins,[5i] horse radish peroxidase (HRP),[9c,d] and cytochromes $P\text{-}450_{LM2}$ and $P\text{-}450_{SSC}$,[8] two $\nu_{C=C}$ modes are observed in the RR spectra (typically near 1620 and 1630 cm$^{-1}$). The appearance of two $\nu_{C=C}$ modes has generally been attributed to a protein-induced inequivalence of the two independent vinyl groups rather than a manifestation of vibrational coupling.

Despite the substantial number of vibrational studies reported for protoporphyrins, significant unanswered questions remain concerning the vibrational properties of the vinyl groups in these molecules both in solution and in heme proteins: (1) What is the origin of the second vibrational band assigned as a $\nu_{C=C}$ mode? If this band is in fact due to a $\nu_{C=C}$ vibration, what structural properties dictate its frequency? (2) In heme proteins which exhibit multiple $\nu_{C=C}$ bands, why are there only two bands and why are their frequencies always near 1620 and 1630 cm$^{-1}$? (3) What structural features of protoheme IX in the protein matrix are associated with the appearance of multiple $\nu_{C=C}$ modes? In order to address these issues, we undertook a detailed RR study of the series of vinylhemins shown in Figure 1. These include deuterohemin IX (DH IX), 2-vinyldeuterohemin IX (2-VDH IX), 4-vinyldeuterohemin IX (4-VDH IX),

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protohemin IX (PH IX), and protohemin III (PH III). The RR studies were accompanied by theoretical calculations which map out the minima and transition states along the vinyl torsional coordinate and examine the frequencies of the $\nu_{C=C}$ vibrations of different conformers. Collectively, the experimental and computational studies delineate for the first time the factors which govern the vibrational characteristics of the $\nu_{C=C}$ vibration. The results dictate a reevaluation of the interpretations previously advanced for the behavior of this mode in both model complexes and heme proteins. The studies also have significant implications for the interpretation of the vibrational characteristics of other conjugating substituent groups on tetrapyrroles, in particular carbonyl stretching modes ($\nu_{C=O}$).

## II. Methods

**A. Experimental Procedures.** PH IX chloride and free base deuteroporphyrin IX were purchased from Porphyrin Products (Logan, UT) and used as received. Iron insertion into the free base and isolation and purification of the chloride salt were performed by standard procedures.[18] The chloride salts of 2-VDH IX, 4-VDH IX, and PH III were synthesized by literature methods.[18,19] The organic solvents used for the RR studies (acetone, methanol (MeOH), ethanol (EtOH), and dimethyl sulfoxide (DMSO)) were all spectroscopic grade. These solvents and the detergent Triton X-100 (Aldrich) were used as received. Aqueous solutions were prepared in deionized water at pH 10.5. Imidazole (Aldrich) was recrystallized three times from benzene. The low-spin, bis-imidazole hemins (Fe(III) complexes) were prepared by adding an excess (~1000-fold) of the ligand to the solution containing the hemin chloride. High-spin hemes (Fe(II) complexes) were prepared by adding an excess of $Na_2S_2O_4$ to the hemin chloride in DMSO. The identity of the complexes was confirmed by UV–vis spectroscopy.

Ambient-temperature RR spectra were obtained from samples contained in either a spinning cell or a quartz capillary tube. Low-temperature spectra were obtained from samples mounted on the cold tip of a closed cycle refrigeration system (ADP Cryogenics, DE-202 Displex). The sample concentrations used for the RR experiments were 100–150 $\mu$M. The RR spectra were acquired with a triple spectrograph (Spex 1877) equipped with a holographically etched 2400 groove/mm grating in the final stage. The scattering was collected at 90° to the incident laser beam by use of a camera lens (Canon 50 mm f/1.2). The excitation wavelengths were provided by the outputs of a Kr ion laser (Coherent Innova 200-K3). A 1152 × 298 pixel, front-illuminated, UV-enhanced charge-coupled device was used as the detector (Princeton Instruments, LN/CCD equipped with a EEV 1152-UV chip). The laser powers were typically 5 mW. The frequencies were calibrated using the known frequencies of indene. The frequencies are accurate to ±1 cm$^{-1}$ for strong and/or isolated bands. The slit widths were set to provide ~2 cm$^{-1}$ resolution.

**B. Theoretical Calculations.** The potential energy surface along the vinyl torsional coordinate was investigated using density functional theoretical (DFT) calculations. Although both DFT and *ab initio* Hartree–Fock (HF) calculations have been successfully applied to molecules the size of porphyrins,[20] DFT was chosen for the following reasons: (1) DFT can implicitly account for electron correlation and often gives results that excel those obtained at the *ab initio* HF level.[21–23] (2) Compared to conventional calculations, DFT calculations are far more computationally facile. (3) HF theory exhibits qualitatively incorrect and pathological behavior in describing the potential energy surfaces of porphyrinic molecules, favoring frozen resonance forms over realistic, delocalized structures.[20a,c] This problem is overcome by simple schemes of electron correlation, such as second-order Møller–Plesset perturbation theory (MP2) or DFT.[20a,c] DFT, in particular the local density functional (LDF) calculations employed herein, have the advantage of being vastly less CPU-time and memory intensive than MP2 calculations.

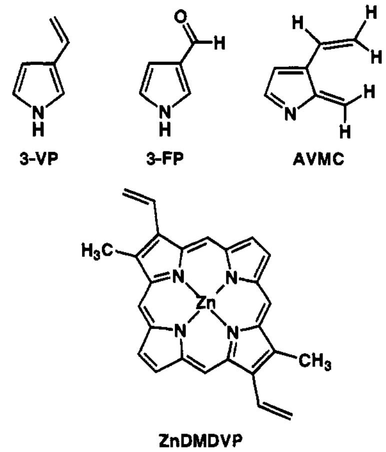

**Figure 2.** Structures of the molecules examined with LDF calculations.

The LDF calculations were carried out on the Zn(II) complex of 1,5-dimethyl-2,6-divinylporphine (ZnDMDVP) and several small molecules containing vinyl or formyl substituents. These latter molecules include 3-vinylpyrrole (3-VP), 3-formylpyrrole (3-FP), and 1-aza-4-vinyl-5-methylidenecyclopenta-1,3-diene (AVMC). The structures of all these molecules are shown in Figure 2. AVMC was examined because the exocyclic methylene group simulates the steric character of the *meso* position of the porphyrin macrocycle. ZnDMDVP was chosen to model the vinylhemin complexes because the disposition of the substituent groups imposes a symmetry constraint that minimizes the CPU time. This molecule also features methyl groups vicinal to the vinyl groups and, therefore, presumably closely mimics the steric environment of protoporphyrins. In the calculations both vinyl groups were simultaneously rotated to maintain $C_2$ symmetry. The LDF calculations were performed with the DMol[24] program system using the local exchange-correlation functional of von Barth and Hedin.[25] The basis sets used were numerically tabulated on a grid and were of double-$\zeta$ plus polarization quality.[24] The performance of this level of theory is well documented.[20a,d,e,23] A very fine mesh (XFINE[24b]) was used for numerical integrations to minimize numerical noise that could adversely affect the precision of the small energy differences between total energies of conformers of large molecules. A fine mesh also helped to reduce the roughness of the potential energy surface which enhanced the accuracy of calculated force constants. Geometry optimizations were carried out till the maximum Cartesian component of the nuclear gradient was lower than 0.0005 atomic units.

Vibrational frequencies were calculated only for the small molecules shown in Figure 2. ZnDMDVP was not included owing to the extensive

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CPU time required to construct the force constant matrix for the large molecule. All calculations on the small molecules were performed at the optimized geometries. Cartesian first derivatives of the total energy were calculated analytically. Second derivatives were calculated by two-point finite differencing. Using these second derivatives (force constants), the vibrational analysis was carried out in a standard manner.[26] The vibrational calculations confirm that the structures analyzed represent minima on the potential energy surface. These calculations revealed no imaginary frequencies for these structures, with the lowest frequency corresponding to the torsional mode ($\tau_{CCCX}$; X = C or O) of the conjugating substituent as expected.

## III. Results

**A. RR Spectra.** In the results presented below, we focus on the bis-imidazole complexes of the various hemins. In these low-spin Fe(III) systems, the $\nu_{10}$ band of the porphyrin macrocycle occurs near 1640 cm$^{-1}$ and does not interfere with the observation of the $\nu_{C=C}$ modes which occur in the 1620–1630-cm$^{-1}$ region.[12b,13b,15] The high-spin Fe(III) and low-spin Fe(II) complexes are problematic in this regard because $\nu_{10}$ downshifts into the latter spectral region. In the case of high-spin Fe(II) complexes, the 1620–1630 cm$^{-1}$ is also free from interference because $\nu_{10}$ occurs at much lower frequency. Therefore, RR studies were conducted on selected high-spin heme complexes. The features observed in the region of the $\nu_{C=C}$ modes of these latter complexes were found to be identical to those observed for the low-spin hemins. This result strongly suggests that the results obtained for the latter complexes are general rather than specific to particular oxidation and/or spin state of the metal ion. In the sections below, we first described the ambient-temperature RR spectra of the various hemins. We then describe the effects of solvent and temperature on vibrational characteristics of the $\nu_{C=C}$ modes.

The high-frequency regions of the ambient-temperature Soret-excitation RR spectra of the bis-imidazole complexes of the various hemins are shown in Figure 3. The spectra shown were acquired in DMSO solutions. The RR spectra are typical of low-spin Fe(III) porphyrins and are characterized by strong polarized RR bands assigned to $\nu_2$ (~1580 cm$^{-1}$), $\nu_3$ (~1503 cm$^{-1}$), and $\nu_4$ (~1375 cm$^{-1}$).[12] Weaker depolarized RR bands are also observed, most importantly the $\nu_{10}$ mode (~1639 cm$^{-1}$). In all four of the vinylhemins, a polarized RR band attributable to a $\nu_{C=C}$ mode is observed at ~1620 cm$^{-1}$. The intensities of the 1620-cm$^{-1}$ bands of 2-VDH IX and 4-VDH IX are approximately equal and approximately one-half those of the analogous bands of PH III and PH IX. This result is consistent with the contribution of both vinyl substituents of the latter complexes to the 1620-cm$^{-1}$ band indicative of the absence of interaction between these groups.[5i,15–17] The relative intensities, which are based on comparison with the Raman bands of the solvent, are only approximate because the Soret absorption maxima of the different vinylhemins are slightly different (within a 5-nm range). Hence the absolute RR intensities are somewhat different at a given excitation wavelength.

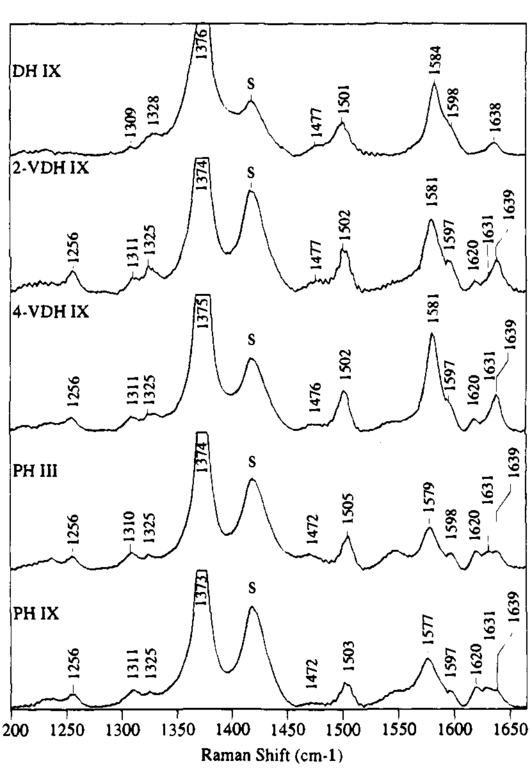

**Figure 3.** High-frequency region of the ambient-temperature (295 K) Soret-excitation RR spectra of the bis-imidazole complexes of the various hemins in DMSO. All spectra were acquired with $\lambda_{ex}$ = 413.1 nm except for that of DH IX where $\lambda_{ex}$ = 406.7 nm. The band marked by S is due to the solvent.

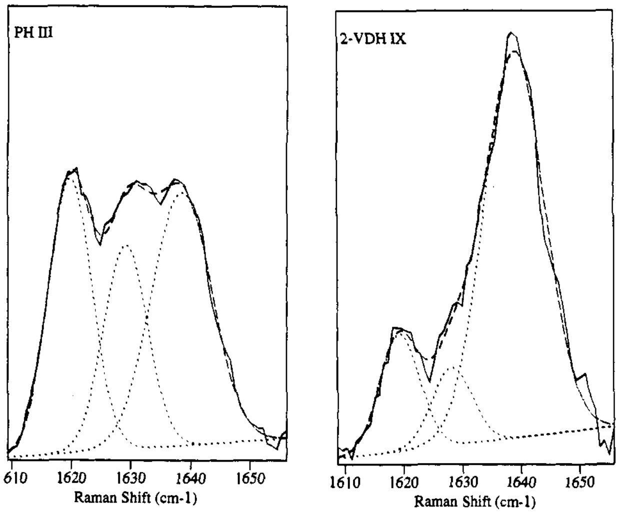

**Figure 4.** Expanded view of the RR spectra of PH III (left panel) and 2-VDH IX (right panel) in the region of the $\nu_{C=C}$ modes. The observed spectra (solid lines) are the same as those shown in Figure 2. The other traces are simulations (dashed line) based on three Gaussian peaks (dotted lines) (see text). The relative intensities of the RR bands in the left and right panels are scaled to the solvent band (see Figure 2).

The above-noted features of the RR spectra of the various vinylhemins are in all respects typical of those previously reported for these complexes.[12b,13b,15] However, closer inspection of the RR spectra reveals that both the mono- and divinylhemins exhibit an additional RR band near 1631 cm$^{-1}$. This band as well as the 1620-cm$^{-1}$ band is absent form the spectrum of DH IX. The 1631-cm$^{-1}$ band is clearly evident in the spectra of both PH IX and PH III where it fills in the gap between the $\nu_{C=C}$ and $\nu_{10}$ bands at 1620 and 1639 cm$^{-1}$, respectively. In the case of 2- and 4-VDH IX, the 1631-cm$^{-1}$ feature is weaker; however, its presence is clearly discernible as a shoulder on the $\nu_{10}$ band. The presence of the 1631-cm$^{-1}$ band is more clearly illustrated in the expanded views of the RR spectra of PH III and 2-VDH IX shown in Figure 4. Simulations of the band contour are also included in the figure. In the simulations, the width of the $\nu_{10}$ band was constrained to be the same as that observed for DH IX (Figure 3, top trace) and the widths of the 1620- and 1631-cm$^{-1}$ bands were constrained to be equal. Although the fits are not unique, the simulations indicate that a minimum of three bands are required

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[26] Wilson, E. B., Jr.; Decius, J. C.; Cross, P. C. *Molecular Vibrations: The Theory of Infrared and Raman Vibrational Spectra*; McGraw-Hill: New York, 1955.

to fit the spectra of both complexes regardless of constraints. With the above noted constraints, the intensity ratio of the 1620- and 1631-cm$^{-1}$ bands is ~60:40 for both PH III and 2-VDH IX. The intensity of each band of PH III and 2-VDH IX is approximately one-half that of the former. Identical results were found for PH IX and 4-VDH IX (not shown). The ~60:40 intensity ratio satisfactorily accounts for the spectral data acquired at both of the excitation wavelengths used in this study ($\lambda_{ex}$ = 406.7 and 413.1 nm).

The spectral characteristics observed for the various vinylhemins strongly suggest that the 1631-cm$^{-1}$ band is a second $\nu_{C=C}$ mode. The fact that the monovinyl hemins exhibit two $\nu_{C=C}$ modes indicates that the structural properties which result in multiple $\nu_{C=C}$ modes are intrinsic to a single vinyl substituent. In the case of the divinyl hemins, the presence of the second vinyl group is manifested only in that the intensity of each $\nu_{C=C}$ mode is approximately twice that of the monovinyl complexes. This provides independent confirmation that the two vinyl groups exhibit nearly identical vibrational characteristics.[5i,15–17] This view is further reinforced by the fact that both PH IX (inequivalent vinyls) and PH III (equivalent vinyls) exhibit identical spectra.

In order to explore further the vibrational characteristics of the 1631-cm$^{-1}$ RR band, additional experiments were performed. The results of these experiments are described in more detail below.

(1) Polarized RR spectra were obtained for the various hemins (not shown). In all cases, the 1631-cm$^{-1}$ band was found to be strongly polarized. This observation is consistent with the assignment of the band to a $\nu_{C=C}$ mode and precludes the possibility that it is a $\nu_{10}$ mode of some minor species. On the other hand, the polarization data do not rule out the possibility that the 1631-cm$^{-1}$ band is due to an overtone or totally symmetric combination mode. These possibilities are somewhat unlikely, however, because polarized features are completely absent from this region of the RR spectrum of DH IX.

(2) The RR spectra of the various hemins were examined in a variety of solvents. Representative RR spectra obtained for bis-imidazole complexes of PH IX in aqueous and nonaqueous solvents are shown in Figure 5. The trends observed in these spectra reflect those observed for all the other vinylhemins. As can be seen, the spectral features observed in the region of the $\nu_{C=C}$ modes are identical in all the nonaqueous solvents (DMSO, MeOH, acetone). In contrast, the 1631-cm$^{-1}$ band is much less prominent in neat water solutions. The only hint of this band is the filling in observed between the 1620- and 1639-cm$^{-1}$ bands. The RR features observed in neat water are different from those observed in the nonaqueous solvents because the hemins strongly aggregate in water. [Aggregate formation is indicated by the appearance of a second strongly red-shifted Soret band.[12b]] This is demonstrated by the fact that dispersion of the aggregate by the addition of a small amount (0.1%) of detergent (Triton X-100) results in an RR spectrum which is essentially identical to those observed in the nonaqueous solvents.

(3) The temperature dependence of the RR spectra of the various hemins was examined over the range 30–295 K. Representative RR spectra of DH IX, 4-VDH IX, and PH IX obtained at several temperatures are shown in Figures 6, 7, and 8, respectively. Inspection of the data obtained for both 4-VDH IX and PH IX reveals that the 1631-cm$^{-1}$ band decreases in intensity relative to the 1620-cm$^{-1}$ band as the temperature is lowered. At 30 K, the 1631-cm$^{-1}$ feature is nearly absent. The relative intensity change is approximately monotonic; no abrupt change is observed upon freezing of the solvent. Concomitantly, the 1620-cm$^{-1}$ band appears to gain intensity as the temperature is lowered. The intensity increase of the 1620-cm$^{-1}$ band is difficult to quantitate, however, because the band shapes (heights and widths) of both the porphyrin and solvent bands also change as the temperature is lowered. The temperature dependence of the relative intensities of the 1620- and 1631-cm$^{-1}$ bands suggests that these modes are associated with two species in thermal equilibrium. Assuming a two-state model, the observed RR intensities predict that the free-energy difference between the species is 30–70 cm$^{-1}$ and that the form associated with the 1620-cm$^{-1}$ band is lower in energy. It should be noted, however, that the assessment of the populations via RR intensity measurements is subject to appreciable error. The accuracy of this measurement is predicted on the knowledge of the absorption characteristics ($\lambda_{max}$ and $\epsilon$) of the different species and the Franck–Condon factors for the mode in question.[27] In a multidimensional oscillator, the frequencies and Frank–Condon factors for all the other modes must also be known to develop an accurate picture of the RR intensities. Any or all of these properties could be different for the two conformers giving rise to the 1620- and 1631-cm$^{-1}$ RR bands.

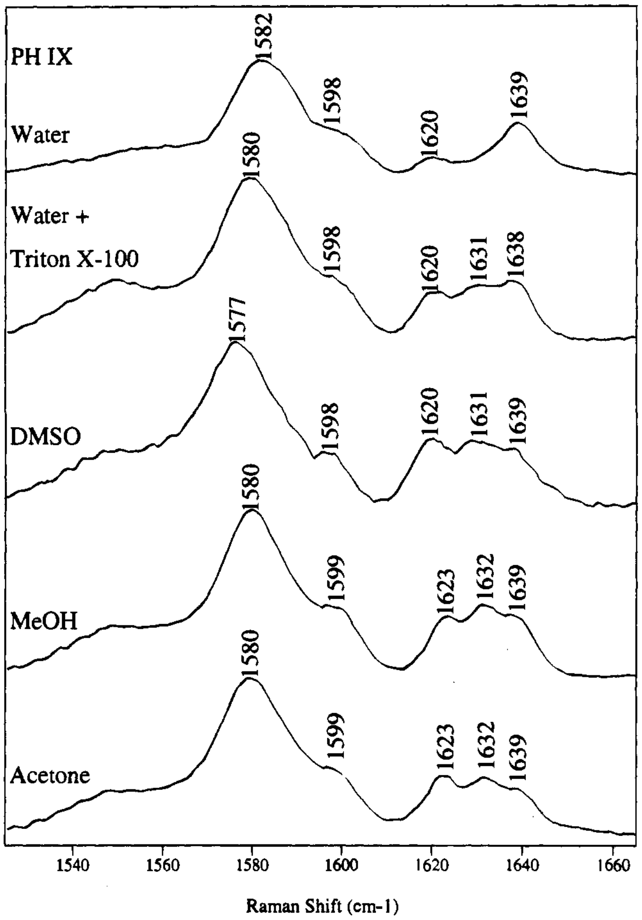

**Figure 5.** Solvent dependence of the Soret-excitation RR spectra of PH IX. All other conditions are the same as those given in Figure 2.

Other noteworthy features are observed in the temperature dependence of the RR spectra of the various hemins. In particular, the $\nu_2$ bands (~1580 cm$^{-1}$) of all the vinylhemins upshift and narrow considerably as the temperature is lowered (Figures 7 and 8). The $\nu_2$ band of DH IX also upshifts; however, the shift is smaller than that observed for the vinylhemins. The line width of the $\nu_2$ band of DH IX is also independent of temperature (Figure 6). The behavior of the $\nu_2$

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(27) Myers, A. B.; Mathies, R. A. In *Biological Applications of Raman Spectroscopy*; Spiro, T. G., Ed.; Wiley: New York, 1987; Vol. 2, pp 1–58.

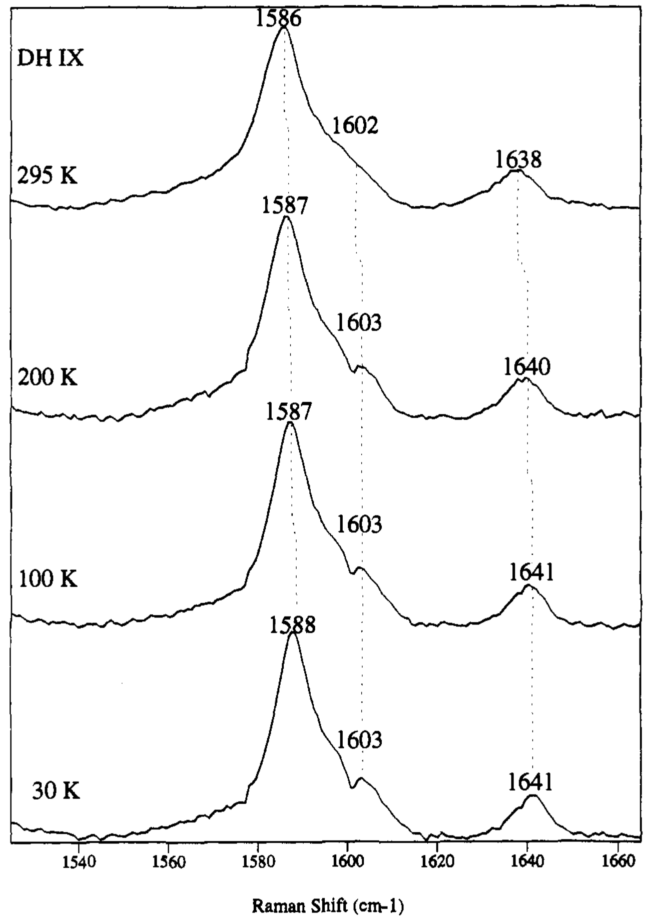

**Figure 6.** Temperature dependence of the Soret-excitation ($\lambda_{ex}$ = 406.7 nm) RR spectrum of DH IX in EtOH.

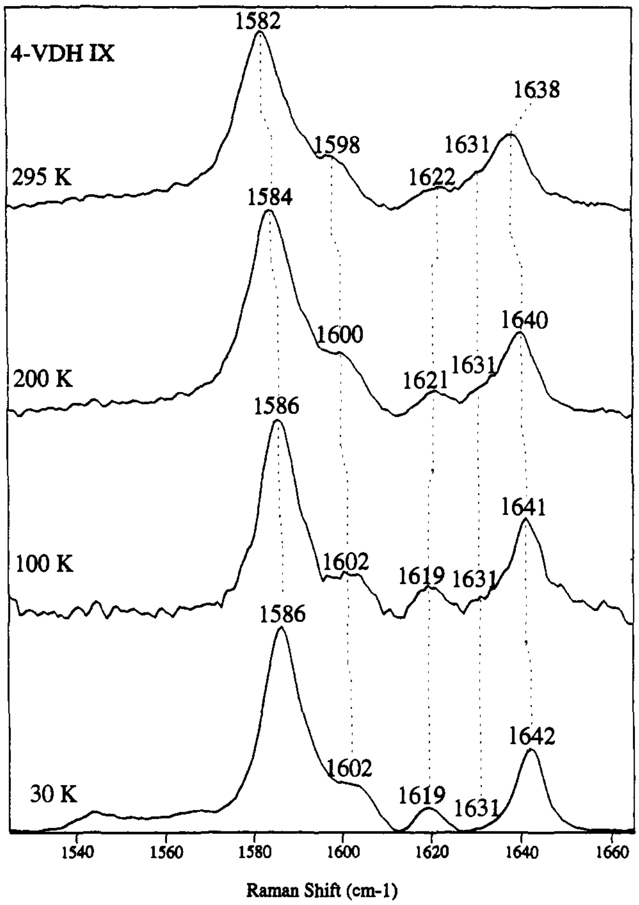

**Figure 7.** Temperature dependence of the Soret-excitation ($\lambda_{ex}$ = 413.1 nm) RR spectrum of 4-VDH IX in EtOH.

**Table 1.** Torsional Angles and Relative Energies (cm$^{-1}$) of the Conformers

| | conformer I | Conformer II | | transition state(s) | |
|---|---|---|---|---|---|
| | $\tau$ | $\tau$ | $\Delta E^a$ | $\tau$ | $\Delta E^a$ |
| 2-VP | 0° | 180° | 171 | ~90° | *b* |
| 3-FP | 0° | 180° | 126 | ~90° | *b* |
| AVMC | 0° | 165° | 232 | 91° | 1668$^c$ |
| ZnDMDVP | <5° | 140° | 897$^d$ | 90° | 3352$^d$ |
| | | | | 180° | 1127$^d$ |

[a] Energies relative to those of Conformer I. [b] These energies were not calculated. [c] The exact energy of the second transition state ($\tau \sim$ 180) was not calculated; however, it is ~100–200 cm$^{-1}$ above that of Conformer II. [d] These energies are for two vinyl substituents; accordingly, the energies for a single vinyl group should be approximately one-half these values (see text).

bands of the vinylhemins suggests that multiple unresolved bands occur in this region and that the lower-frequency component(s) loses intensity as the temperature is lowered. This behavior parallels that observed for the 1620- and 1631-cm$^{-1}$ bands. In this picture, the lower frequency $\nu_2$ mode would be associated with the higher frequency $\nu_{C=C}$ mode and vice versa. It also appears that certain other RR bands of the vinylhemins undergo temperature-dependent shifts; however, these shifts are difficult to quantitate due to the weakness of these bands.

**B. LDF Calculations.** The RR spectral results presented above suggest that vinylhemins exist as at least two species in thermal equilibrium at ambient temperature and that the frequencies of the $\nu_{C=C}$ modes are different for the two species. This prompted us to seek a more detailed characterization of the portion of the molecules' potential surface that are associated with the vinyl torsion. In this regard, previous INDO/CI calculations located distinct isomers (conformers) of 2,4-divinylporphyrin which differ with respect to the dihedral angle between the porphyrin and vinyl planes.[17] The INDO/CI surface is exceedingly flat, with both conformers and the transition state connecting them lying within and energy range of 100–500 cm$^{-1}$ (<1.5 kcal/mol). Although predictions of the INDO/CI calculations are in qualitative agreement with the experimental results indicating two thermally populated conformers, this level of theory is not accurate or reliable for a detailed description of such a flat potential surface. Accordingly, we employed LDF theory to calculate the energetics of the stationary points on the vinyl torsional surface.

The results of the LDF calculations on the three small molecules (3-VP, 3-FP, AVMC) and the porphyrin model complex (ZnDMDVP) are summarized in Figure 9 and Table 1. Figure 9 presents a schematic representation of the potential surface of ZnDMDVP showing the relative energies of the extremum conformers. Table 1 lists the relative energies of the various conformers of all the molecules. For all four molecules, two unique vinyl torsional conformers (hereafter referred to as I and II) were found to be local minima on the potential surface. In Conformer I, the vinyl (formyl) group points approximately toward the $\beta$-hydrogen/substituent. In Conformer II, the vinyl (formyl) group points approximately toward the α- or *meso*-hydrogen/substituent. The two conformers are close in energy, with Conformer I being more stable by 250 cm$^{-1}$ or less for the small molecules and 897 cm$^{-1}$ for ZnDMDVP. Because ZnDMDVP has two vinyl groups which were simultaneously rotated in the calculation to preserve $C_2$ symmetry, the energy difference between the two conformers of a given vinyl substituent is ~450 cm$^{-1}$. For both conformers of 3-VP and 3-FP, the conjugating substituents and the ring are

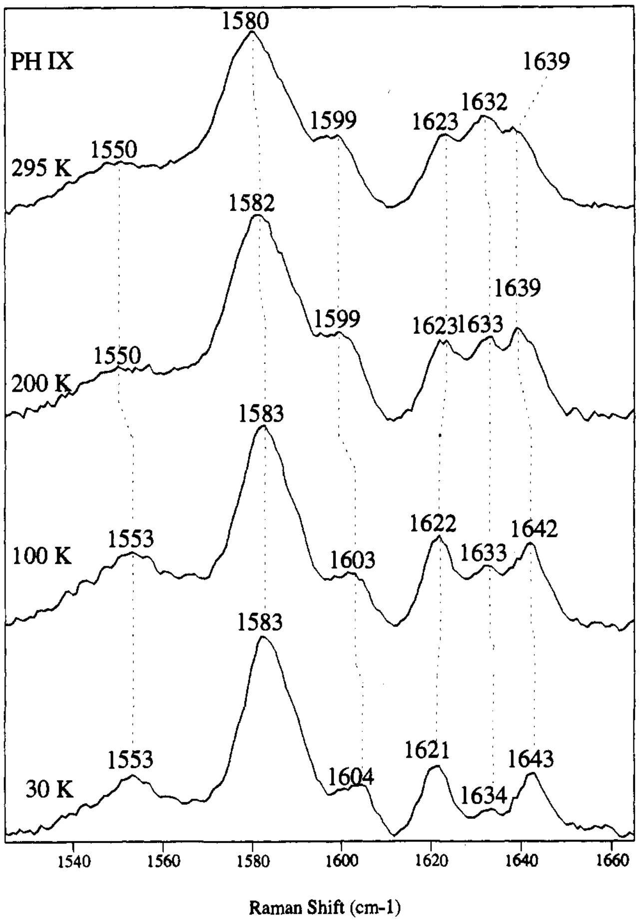

**Figure 8.** Temperature dependence of the Soret-excitation ($\lambda_{ex}$ = 413.1 nm) RR spectrum of PH IX in EtOH.

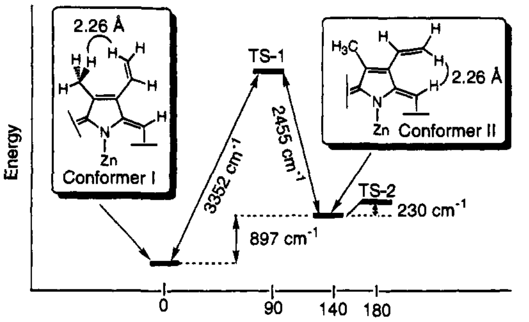

**Figure 9.** Schematic representation of the calculated vinyl torsional potential energy surface for ZnDMDVP. The structures of Conformers I and II are shown in the inserts. The distances are the closest contacts between the vinyl hydrogens and the $\beta$-pyrrole/*meso* hydrogens.

coplanar (Conformer I, $\tau$ = 0°; Conformer II, $\tau$ = 180°). Conformer I of AVMC is also planar; however, the vinyl group of Conformer II is rotated out of plane ($\tau \sim$ 165°). The transition state between these conformers ($\tau$ = 90°) is ~1600 cm$^{-1}$ higher in energy. This value is similar to that of the torsional barrier in 1,3-butadiene (1600 cm$^{-1}$ above the *cis* conformer)[28] but substantially greater than that in vinyl-substituted aromatic compounds such as styrene (623 cm$^{-1}$).[29] This is qualitatively understandable because AVMC may be regarded as a modified fulvene, which is not quite aromatic. Conformer I of ZnDMDVP is also essentially planar ($\tau$ < 5°); the vinyl group of Conformer II is out of plane ($\tau \sim$ 140°). However, the planar transition state (TS-2) of ZnDMDVP is only 230 cm$^{-1}$ above Conformer II (Figure 9). The transition state connecting conformers I and II (TS-1) is ~3352 cm$^{-1}$ (9.55 kcal/mol) above Conformer I or 2455 cm$^{-1}$ (7.00 kcal/mol) above Conformer II. Accordingly, the barrier for a single vinyl group in ZnDMDVP should be ~1676 or ~1228 cm$^{-1}$ relative to these two conformers. The presence of a barrier at $\tau$ = 90° implies that conjugative interaction occurs between the $\pi$ system of the porphyrin and vinyl moieties. This prediction is qualitatively consistent with the results of the INDO/CI calculations. Further work is in progress to elucidate which porphyrin $\pi$ molecular orbitals participate in the conjugative interaction.

Although both the LDF and INDO/CI calculations predict the existence of two distinct conformers, the detailed features of the vinyl torsional potential surfaces calculated at the two levels of theory are different. The most striking difference between the two surfaces is that the LDF calculations predict a significantly higher transition state (~1228–1676 cm$^{-1}$ per vinyl group) connecting Conformers I and II than do the INDO/CI calculations (100–500 cm$^{-1}$ per vinyl group). In addition, the latter calculations predict a very high barrier of ~5000 cm$^{-1}$ at the planar form ($\tau$ = 180°) which is essentially absent on the LDF surface. [Note that the torsional angles of the planar forms described in ref 17 are opposite those used here.] At the LDF level, the planar form of ZnDMDVP ($\tau$ = 180°) is only 230 cm$^{-1}$ above Conformer II. The present LDF calculations also predict that Conformer I is essentially planar ($\tau \sim$ 0°), whereas the INDO/CI calculations predict a distinctly nonplanar form ($\tau \sim$ 50°). Both calculations predict that Conformer II is out of plane ($\tau \sim$ 140°).

The low energies of the planar forms of ZnDMDVP and the smaller model system AVMC predicted by the LDF method are explained by the optimized geometries. These geometries indicate that no severe steric interactions occur between the vinyl hydrogens and groups at either the $\beta$-pyrrole or $\alpha$/*meso* positions. In the case of ZnDMDVP, the closest contacts between the vinyl hydrogens and the $\beta$-methyl (Conformer I) or *meso* hydrogens (Conformer II) are 2.26 Å for both conformers (Figure 9). The large interactions between the vinyl group and *meso*-hydrogen predicted by the INDO/CI method probably reflect errors introduced by using unoptimized geometries. They may also reflect inadequacies in the semiempirical method. Finally, it should be noted that the equilibrium vinyl torsional angles ($\tau$ < 5 and ~40°) calculated via the LDF method are smaller than the angle determined via NMR measurements ($\tau$ ~51°).[30] However, this latter value represents a thermal average over all populated conformers and torsional vibrational states. In an extremely anharmonic well which is relatively flat near the minimum (which is expected for the $\tau_{CCCC}$ mode[17,29]), the average torsional angle can be very different from the angle at the potential minimum. This can be seen by inspecting data presented in Table 2 in ref 17.

The vibrational frequencies calculated for the $\nu_{C=X}$, $\tau_{CCCX}$ (X = C or O), and selected ring-skeletal modes of 3-VP, 3-FP, and AVMC are shown in Figure 10. For all three molecules, the frequencies of the $\nu_{C=X}$ modes of Conformer I and Conformer II differ by 10–20 cm$^{-1}$. These differences are comparable to those observed for the two $\nu_{C=C}$ modes of the vinylhemins (~10 cm$^{-1}$). In the case of AVMC the lower frequency $\nu_{C=C}$ mode is associated with Conformer I; the opposite is the case for 3-VP (and for the $\nu_{C=O}$ modes of 3-FP). These results, in conjunction with the RR studies of the vinylhemin complexes, suggest that AVMC is more representa-

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[28] Carreira, L. A. *J. Chem. Phys.* **1975**, *62*, 3851–3854.

[29] Carreira, L. A.; Townes, T. G. *J. Chem. Phys.* **1975**, *63*, 5283–5286.

[30] Bothner-By, A. A.; Gayathri, C.; van Zijl, P. C. M.; Maclean, C.; Lai, J. J.; Smith, K. M. *Magn. Reson. Chem.* **1985**, *23*, 935–938.

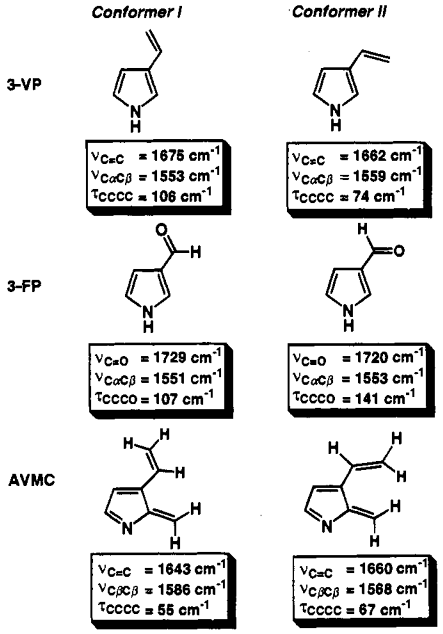

**Figure 10.** Calculated vibrational frequencies of selected modes of 3-VP, 3-FP, and AVMC.

tive of the porphyrin system than 3-VP (or 3-FP). In particular, the lower frequency $\nu_{C=C}$ mode of the vinylhemins (1620 cm$^{-1}$) appears to be associated with the lower energy form (Conformer I). The LDF calculations also indicate that the $\nu_{C=C}$ modes of 3-VP and AVMC are mixed with certain other vibrations of the molecule. In 3-VP, the most significant mixing is with the $C_\alpha C_\beta$ stretching vibration of the ring. In 3-AVMC, the extent of mixing is different in Conformers I and II. However, for both conformers, the ring-skeletal vibration most strongly mixed with the $\nu_{C=C}$ mode involves the $C_\beta C_\beta$ (rather than $C_\alpha C_\beta$) bond. Regardless of which ring-skeletal mode is more strongly mixed with the $\nu_{C=C}$ mode, a lower value for the highest skeletal-mode frequency is associated with a higher $\nu_{C=C}$ frequency and vice versa. This calculated trend parallels the experimentally observed behavior for the $\nu_2$ and $\nu_{C=C}$ modes of Conformers I and II of the vinylhemins (vide supra).

## IV. Discussion

**A. General Characteristics of the Vinyl Conformers.** The experimental and theoretical studies reported herein provide a self-consistent picture of the factors that contribute to the appearance of multiple $\nu_{C=C}$ bands in the vibrational spectra of vinyl-substituted porphyrins. In particular, the studies indicate that the multiple bands are due to the presence of at least two nearly equal-energy vinyl torsional conformers. These conformers are intrinsic to a single vinyl substituent. In this respect, the porphyrins are similar to small molecules containing vinyl groups, both the cyclic species examined here and acyclic species investigated elsewhere.[31] All of these small molecules exist in two conformers in thermal equilibrium at ambient temperature. The calculations further suggest that multiple conformers are to be expected for other tetrapyrroles which contain conjugating substituents such as vinyl and carbonyl groups. These include chlorophyll *a* (vinyl group), bacteriochlorophyll *a* (acetyl group), and heme *a* and spirograpis heme (formyl groups).

One question which remains concerns the reasons why the 1631-cm$^{-1}$ $\nu_{C=C}$ mode has not been previously identified in the RR spectra heme model complexes. Several factors probably contribute to this oversight: (1) Due to interference from $\nu_{10}$, the 1630-cm$^{-1}$ band can only be clearly observed in Soret-excitation RR spectra of low-spin Fe(III) and high-spin Fe(II) complexes. Even in these complexes, the excitation wavelength must be chosen to minimize the intensity of $\nu_{10}$. This requires excitation near the Soret maximum rather than on the red side of the band contour. RR studies performed with excitation in this latter region are common because an Ar ion laser emission line occurs at 457.9 nm. (2) The 1630-cm$^{-1}$ band is only clearly identifiable in nonaggregated samples. This requires the use of nonaqueous solvents. RR studies of the free acids of PH IX and related compounds have typically been performed in aqueous solutions.[12b,13b,15,32] On the other hand, RR studies have been performed on the dimethyl ester of PH IX and related systems in nonaqueous solutions.[12b,13b,32] The 1631-cm$^{-1}$ band is very difficult to identify in these complexes because the intensity of the $\nu_{10}$ mode is larger in the dimethyl ester than in the free acid.[12b,32,33] It also appears that the $\nu_{C=C}$ bands are somewhat broader in the dimethyl ester (Kalsbeck, W. A.; Bocian, D. F., unpublished results).

In the preceding discussion, the vinyl group conformation has been described in terms of two well-defined conformers. This view is an oversimplification of the actual situation for the following reasons. The frequency of the torsional mode is very low (100 cm$^{-1}$ or less); consequently, a number of torsional vibrational levels are populated at ambient temperatures. In addition, the surface for the torsional oscillator is predicted to be very anharmonic.[17,29] The large anharmonicity leads to significantly different equilibrium torsional angles in the various populated vibrational levels. Accordingly, the conjugation of the vinyl group with the porphyrin ring will be different in each torsional level. The amount of coupling between the $\nu_{C=C}$ mode and the ring-skeletal vibrations, such as $\nu_2$, will also be different. As a consequence, the frequencies of the $\nu_{C=C}$ mode will be different in each torsional state. The RR bands due to the different $\nu_{C=C}$ modes in the thermally populated torsional states are not resolved; however, they contribute to the breadth and asymmetry of the band envelope assigned to the $\nu_{C=C}$ modes of Conformers I and II. Depending on the exact shape of the torsional surface, the band envelope could assume very different shapes. It is also likely that the Franck–Condon factors for the $\nu_{C=C}$ vibration are different in the various torsional levels. This possibility severely complicates the analysis of the RR band intensities. These factors may contribute to the observation that the $\nu_{C=C}$ modes of the PH IX dimethyl ester are broader than those of free acid (although it is somewhat difficult to imagine why the torsional surface for the vinyl modes would be different for the two species). Regardless of the complications associated with extracting the relative populations of Conformers I and II from the RR intensities, the free-energy differences determined from these measurements (30–70 cm$^{-1}$) are qualitatively similar to those predicted for the four molecules studied computationally (450 cm$^{-1}$ or less). Any differences between the observed and calculated relative energies of the two conformers should be regarded as small because factors such as solvent effects can easily modify these energies by several hundreds of wavenumbers.

**B. Implications for Cofactor–Protein Interactions.** The fact that PH IX exists in two conformers in solution has significant impact on the interpretation of the RR spectra of proteins that contain this cofactor. As was previously noted,

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[31] (a) Durig, J. R.; Tang, Q.; Little, T. S. *J. Raman Spectrosc.* **1992**, *23*, 653–666. (b) Durig, J. R.; Tang, Q.; Little, T. S. *J. Mol. Struct.* **1992**, *269*, 257–283.

[32] Spaulding, L. D.; Chang, C. C.; Yu, N.-T.; Felton, R. H. *J. Am. Chem. Soc.* **1975**, *97*, 2517–2524.

[33] Spiro, T. G.; Strong, J. D.; Stein, P. *J. Am. Chem. Soc.* **1979**, *101*, 2648–2655.

certain heme proteins exhibit a single $\nu_{C=C}$ mode near 1620 cm$^{-1}$ whereas others exhibit two bands near 1620 and 1630 cm$^{-1}$. Myoglobins,[12b,13b,16] tetrameric hemoglobins,[12b,13b,34] and cytochrome *b*[35] fall in the first group; insect hemoglobin,[5i] HRP,[9c,d] and cytochrome P-450$_{LM2}$ and P-450$_{SSC}$[8] fall in the second. The appearance of a single $\nu_{C=C}$ mode near 1620 cm$^{-1}$ has generally been attributed to the superposition of the unresolved bands of both vinyl groups of the PH IX cofactor. The implication is that either both vinyl groups have similar orientations with respect to the porphyrin plane or if the orientations are different the frequency of the $\nu_{C=C}$ mode is not sensitive to this difference. The appearance of two $\nu_{C=C}$ modes has been assumed to indicate the existence of site-specific vinyl group–protein interactions which differentially perturb one vinyl group versus the other. The vinyl group which exhibits the 1630-cm$^{-1}$ $\nu_{C=C}$ mode would be identified as the more perturbed substituent because of this mode is different from that which has been thought to be characteristic of PH IX in solution (~1620 cm$^{-1}$). The interpretation of the structural factors which give rise to one versus two $\nu_{C=C}$ modes must be reevaluated in light of the results reported herein.

If the protein matrix exerts no constraints on one or both vinyl groups, then two $\nu_{C=C}$ modes should be observed in the RR spectrum. Accordingly, the appearance of a single $\nu_{C=C}$ mode implies that the protein preferentially stabilizes one of the two conformers observed in solution. In addition, a similar conformation is stabilized for both vinyl groups. The fact that the $\nu_{C=C}$ mode is typically observed near 1620 cm$^{-1}$ implies that this form is qualitatively more like Conformer I of ZnDMDVP ($\tau < 5°$) than Conformer II ($\tau \sim 140°$). This view is generally supported by the high-resolution X-ray crystal structures available for a number of heme proteins that exhibit a single $\nu_{C=C}$ mode.[36] In all cases, the torsional angles for both vinyl groups fall in the range 0–90°. In most cases, both groups are out of plane and exhibit torsional angles in the range 30–60°. Given that the torsional angles of the 2- and 4-vinyl groups are generally different, it would be expected that the frequencies of the two $\nu_{C=C}$ modes would also be somewhat different. This may be the case; however, differences of as much as 5 cm$^{-1}$ would be difficult to resolve owing to the line width of the $\nu_{C=C}$ RR band (~12 cm$^{-1}$). Finally, it should be noted that the extent of protein-induced stabilization of Conformer I versus Conformer II need not be very large (~1 kcal/mol) to render the latter unobservable in the RR spectrum.

The studies reported herein have more serious implications for the characterization of heme proteins which exhibit two $\nu_{C=C}$ modes. The results indicate that the appearance of two modes is the norm rather than the exception. Accordingly, this phenomenon cannot be taken as necessarily indicative of differential vinyl group–protein interactions at the 2-versus 4-position of the porphyrin macrocycle. Indeed, the simplest interpretation for the RR data would be that both vinyl groups are unconstrained by the protein matrix and free to assume the conformations preferred in solution. More complicated interpretations are also possible; for example, one vinyl group could be constrained and the other could be free from interactions. In this case, the relative intensities of the RR bands of the two $\nu_{C=C}$ modes should be different from those observed in solution. However, such differences would be extremely difficult to quantitate owing to the overlap between the $\nu_{C=C}$ modes and $\nu_{10}$.

If one or both vinyl groups of the PH IX cofactor were unconstrained by the protein matrix, then relatively large uncertainties would be expected in the crystallographically determined orientation of the group. At present, no high-resolution X-ray structural data are available for heme proteins in which two $\nu_{C=C}$ modes have been clearly identified in the RR spectrum. However, in the case of cytochrome P-450$_{LM2}$ and P-450$_{SSC}$, structural data are available for related variants, in particular cytochrome P-450$_{cam}$.[37] The structural data obtained for the latter protein do not indicate unusually large uncertainties in the position of either vinyl group on the PH IX cofactor. The crystallographic data do, however, show that the two vinyl groups have very different torsional angles (2-vinyl, $\tau = 18 \pm 10°$; 4-vinyl, $\tau = 89 \pm 9°$). The difference between these torsional angles (~70°) is not as large as that calculated for the two conformers of ZnDMDVP (~135°). However, it is larger than those reported for proteins which exhibit a single $\nu_{C=C}$ mode.[36] Together, these observations suggest that appearance of two $\nu_{C=C}$ modes in the RR spectra of cytochrome P-450$_{LM2}$ and P-450$_{SSC}$ is in fact due to site-specific interactions with the protein matrix which preferentially stabilize different vinyl torsional conformers at the 2- versus 4-positions of the porphyrin ring.

HRP is another heme protein which clearly exhibits two $\nu_{C=C}$ modes in the RR spectrum.[9c,d] Although no X-ray structure is available for this protein, the features observed in the NMR spectrum provide insight into the conformational characteristics of the vinyl substituents.[9a,b] The NMR signature observed for the 4-vinyl group of the PH IX cofactor is unremarkable. In contrast, the 2-vinyl group exhibits unusual spectral features which indicate that this substituent is trapped into two conformers that slowly interconvert on the NMR time scale. The exact structure of these two conformers is not known; however, the vinyl torsional angle for both is ~20–30°.[9b] It has been suggested that the two conformers are similar to those identified here as Conformers I and II and that the protein matrix forces the vinyl group into these unusual forms.[9a] The results reported herein suggest an alternative explanation. In particular, the 2-vinyl group occupies more or less normal conformations indicating minimal interactions with the protein at torsional angles corresponding to the equilibrium forms. However, strong vinyl group–protein interactions occur at torsional angles near the transition state. These interactions significantly raise the energy of this latter state. This view is supported by the fact that the conformational interconversion is slow on the NMR time scale. Interconversion would be fast on this time scale if the transition state energy were comparable to that calculated for ZnDMDVP (7–10 kcal/mol).

In the absence of independent structural data, it is not possible to distinguish whether the appearance of two $\nu_{C=C}$ modes is indicative of site-specific vinyl group–protein interactions or the absence of such interactions. Another way to address this issue is to examine the RR spectra of proteins reconstituted with either divinylhemins specifically labeled at the 2- versus 4-positions or with monovinyl hemins such as 2-VDH IX versus 4-DH IX. This approach has been taken to investigate the vibrational characteristic of the $\nu_{C=C}$ modes of insect hemoglobins whose native PH IX cofactor also exhibits two $\nu_{C=C}$ modes near 1620 and 1630 cm$^{-1}$.[5i] The studies using the

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[34] Rouseau, D. L.; Ondrias, M. R.; La Mar, G. N.; Kong, S. B.; Smith, K. M. *J. Biol. Chem.* **1983**, *258*, 1740–1746.

[35] (a) Babcock, G. T.; Widger, W. R.; Cramer, W. A.; Oertling, W. A.; Metz, J. G. *Biochemistry* **1985**, *24*, 3638–3645. (b) Hurst, J. K.; Loehr, T. M.; Curnutte, J. T.; Rosen, H. *J. Biol. Chem.* **1991**, *266*, 1627–1634.

[36] For a tabulation of the vinyl torsional angles of the PH IX cofactor in a variety of heme proteins, see ref 6b.

[37] (a) Poulos, T. L.; Finzel, B. C.; Howard, A. J. *Biochemistry* **1986**, *25*, 5314–5322. (b) Raag, R.; Poulos, T. L. *Biochemistry* **1989**, *28*, 7586–7592.

monovinyl DH-IX derivatives indicate that the former band is exclusively due to the 4-vinyl group whereas the latter is exclusively due to the 2-vinyl substituent. These results unambiguously indicate that site-specific interactions occur in this protein which preferentially stabilize different vinyl torsional conformers for the 2 versus 4 substituents.

## V. Summary and Conclusions

The studies reported herein present a new view of the factors which influence the vibrational characteristics of the $\nu_{C=C}$ modes of protoporphyrins. Both the experimental and theoretical studies indicate the existence of two nearly equal-energy vinyl torsional conformers. The relatively soft potential for the vinyl torsion explains the plethora of vinyl torsional angles observed for the substituent groups of the PH IX cofactor in heme proteins. The theoretical studies further suggest that other types of conjugating substituents, such as formyl and acetyl groups, will exhibit the similar torsional properties. The fact that two conformers of the vinyl (and other) substituents exist in thermal equilibrium necessitates a reevaluation of the RR and other spectroscopic data obtained for heme and other proteins. The observation of a single $\nu_{C=C}$ mode in the RR spectrum indicates that both vinyl groups on the PH IX cofactor interact with the protein matrix and that these interactions result in the preferential stabilization of indistinguishable vinyl torsional conformers. The observation of two $\nu_{C=C}$ modes has several possible explanations. The widely invoked explanation is that site-specific vinyl group–protein interactions render the 2- and 4-vinyl groups inequivalent. This interpretation appears to be appropriate for certain heme proteins (insect hemoglobin and cytochrome P-450) but only partially correct for others (HRP). In the worst case, the appearance of two $\nu_{C=C}$ modes could indicate that both vinyl groups are free from interactions with the protein and assume the torsional conformers normally found in solution. These cases cannot be distinguished by RR spectroscopy alone in lieu of additional studies on cofactors which have been specifically labeled or modified at the 2- versus 4-positions of the porphyrin macrocycle. In the absence of these data, care must be exercised in drawing conclusions regarding the nature of the cofactor–protein interactions.

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