Determination by ^1H NMR of the Orientation of Modified Hemes Incorporated into Horseradish Peroxidase. Evidence for Steric Clamping of a Vinyl Group by the Protein

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Abstract: Proton NMR spectroscopy of horseradish peroxidase has been utilized to probe the interaction between the heme side chains of pyrroles I and II and the apo protein. Reconstitution of apo protein with both equivalent 2,4-disubstituted and nonequivalent 2,4-substituted deuterohemins provides a sensitive probe of the heme–protein interactions of pyrroles I and II. Use of specifically deuterated 2,4-modified hemins permits the assignment of heme peaks, which in turn determines the orientation of the heme in the heme pocket. It was determined that 2,4-diacetyldeuterohemin-, 4-acetyldeuterohemin- and 4-vinyldeuterohemin-reconstituted horseradish peroxidase all exist with the heme orientation adopted by native protohemin, while deuterohemin-, 2-acetyldeuterohemin-, and 2-vinyldeuterohemin-reconstituted horseradish peroxidase exist with the heme predominantly in an orientation that is rotated 180° about the heme α–γ axis. From this it was concluded that the heme pocket of the native protein contains more room for the 2,4-vinyl side chains than the 1,3-methyl groups. Furthermore, the preference for the 4-acetyl- and 4-vinyldeuterohemins to exist with the heme in the native orientation, while the 2-acetyl- and 2-vinyldeuterohemins exist predominantly in the 180° flipped form, argues that the 2-vinyl side chain is more sterically constrained than the 4-vinyl side chain, results that are supported by previous investigations of the 2-α-vinyl resonance in HRP compound I. A possible role of the steric constraint of the vinyl groups in stabilizing HRP compound I is discussed.

Although both horseradish peroxidase, HRP, and myoglobin possess protoheme and an axial histidyl imidazole at their active sites and exhibit numerous similarities in physical and spectroscopic properties, it has been proposed that the heme pocket in HRP is more buried, less accessible to solvent, and more constrained than usually found in oxygen-binding hemoproteins.[1-3] The importance and stereospecificity of heme–protein contacts in HRP was emphasized by the recent preliminary report[4] that replacement of the native protohemin group (R_2 = R_4 = vinyl in Figure 1) by deuterohemin (R_2 = R_4 = H) leads to an altered orientation of the prosthetic group within the heme cavity, as depicted in Figure 1. The tighter heme pocket in HRP is also evidenced by the severe restriction on the oscillatory mobility of heme peripheral substituents when compared with metaaquomyoglobin.[1,5]

Since the different functions of HRP and myoglobin must be due wholly to the influence of the polypeptide chain, it is of interest to characterize the nature of the protein constraints on the heme group in HRP. It has already been noted that upon oxidizing HRP to compund I, which is two oxidizing equivalents above the resting enzyme, the porphyrin cation radical in the native enzyme appears to possess a different orbital ground state than the deuterohemin-reconstituted protein.[6,7] Moreover, the stability of compound I for the native enzyme is much greater than that for the deuterohemin-reconstituted enzyme.[8] Since the redox potentials for the hemins are essentially the same outside the protein environment,[9] this suggests that the native compound I may be stabilized by specific interactions between the ubiquitous vinyl groups and the protein.

Proton NMR spectroscopy has been shown to be one of the most versatile and informative probes for delineating structure–function relationships in hemoproteins.[1,10-12] Analses of the hyperfine shifts for both resting enzyme[1] and compound I[12] have provided information on the magnetic and electronic properties of these species. One of the more unique aspects of NMR spectroscopy of paramagnetic hemoproteins is that the pattern of the hyperfine shifts of the individual heme methyls can reveal the orientation of the heme group relative to the axial histidyl imidazole plane.[11,13] This requires unambigous assignment of methyl resonances using specifically deuterium-labeled hemins. Thus, in low-spin ferric systems the axial imidazole π bonding raises the degeneracy of the spin-containing e (π) metal orbitals. The resulting lone-unpaired spin-containing d orbital interacts preferentially with two pyrroles related by inversion through the iron.[11,14,15]

In metMbCN, the imidazole plane is essentially coincident[16] with the N–Fe–N vector through pyrroles II and IV (B in Figure 1), which leads to much larger 1-CH_3 and 5-CH_3 hyperfine shifts[13,17] than for 3-CH_3 and 8-CH_3. On the other hand, cytochrome *c* peroxidase has the 3-CH_3 and 8-CH_3 resonances farthest downfield,[18] consistent with the X-ray findings[19] that the imidazole plane is aligned close to the N–Fe–N vector through pyrroles I and III (A in Figure 1). Thus the correlation between the pair of downfield methyl signals and the imidazole orientation differing

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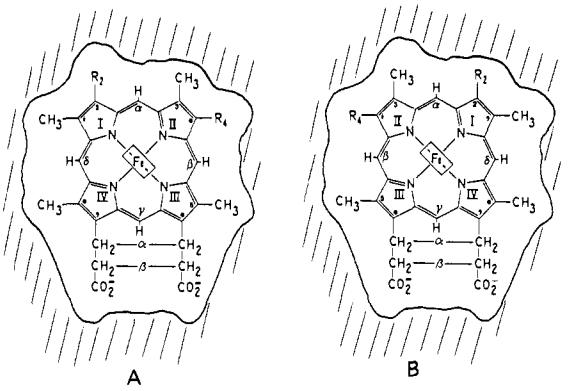

Figure 1. Two possible orientations of the heme relative to the imidazole plane. The arrangement in A is that deduced from NMR for horseradish peroxidase.[4] The heme in B is rotated 180° about the $\alpha-\gamma$ meso axis from that in A and represents the orientation found crystalographically in myoglobin and deduced from the NMR data on deuterohemin-reconstituted horseradish peroxidase.[4] The protein environment is shown schematically so as to allow more room for the larger 2,4-vinyls that the 1,3-methyl for the native orientation of the heme A.

by 90° is established. In view of the fact that the heme methyl peaks must occur in pairs, 1,5-CH_3 or 3,8-CH_3, it is necessary to assign only one of the two downfield methyls in a given low-spin ferric protein in order to establish the orientation of the heme relative to the imidazole plane. In deuterohemin-reconstituted metMbCN, a minor component occurs which has the larger hyperfine shifts for 3-CH_3 and 8-CH_3 than 1-CH_3 and 5-CH_3, establishing[13] that deuterohemin in this component is rotated by 180° about the $\alpha-\gamma$ meso axis (A in Figure 1). Since this initial demonstration[13] of the method for determining the hemin orientation for metMbCN, we have shown that the monomeric *Chironomus* hemoglobins[20] as well as ferricytochrome b_5[21] have the native protohemin group rotationally disordered. The orientation for the major insect hemoglobin component determined by NMR spectroscopy is the same as that found in the most recent X-ray structure.[22]

Preliminary ^1H NNR spectra of HRPCN and the analogous deuterohemin-reconstituted complex, deuteroHRPCN, have already shown[4] that the methyl assignments dictate that in HRPCN the unique heme orientation relative to the imidazole is that given of A of Figure 1, while deuteroHRPCN exists $\simeq$90% with its heme orientation as depicted in B of Figure 1. In order to further characterize the protein–heme interactions at the heme periphery, we report here on the assignments of the ^1H NMR spectra of a variety of hemin derivatives possessing altered 2,4 substituents which allow us to determine if the protein constraints on the heme periphery act similarly on pyrroles I and II or whether they are localized primarily on one of the pyyroles. The modified hemins most appropriate for this study are those possessing a single vinyl or acetyl group at either the 2- or 4-positions. The resulting deduced heme orientations for each case allow us to conclude that a clamping of the heme by the protein occurs primarily at pyrrole I.

Experimental Section

Horseradish peroxidase, type VI, was purchased from Sigma as a lyophilized salt-free powder. The detailed purification, activity assay, and electrophoretic behavior of the proteins used in this study have been published.[1,23] The preparation[24] of apo-HRP and the reconstitution procedure utilized[6,25] have also been described earlier.[1]

Solutions for proton NMR of native or reconstituted HRP were 1–3 mM in protein in 0.2 M NaCl, 99.8% ^2H_2O. The cyanide-coordinated ferric low-spin species was formed by addition of excess KCN to the ferric protein solution. The solution pH, adjusted by using 0.2 M ^2HCl or 0.2 M NaO^2H, was measured with a Beckman 3550 pH meter equipped with an Ingold microcombination electrode. The pH was not corrected for the isotopic effect and is hence referred to as "pH".

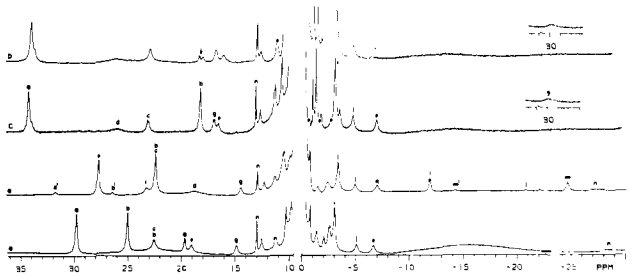

Figure 2. Hyperfine shifted portions of the 360-MHz proton NMR spectra at "pH" 7.0 and 35 °C of (A) native HRPCN, (B) deuteroHRPCN, (C) 2,4-Ac_2-deuteroHRPCN, and (D) [1,3-$(C^2H_3)_2$]-2,4-Ac_2-deuteroHRPCN. The peaks are labeled a–n, with a prime to designate minor component peaks; peaks with clearly reduced intensities due to deuteration are indicated by arrows. Assignments of labeled peaks are given in Table I.

Deuterohemin,[26] 2,4-diacetyldeuterohemin (2,4-Ac_2-deuterohemin),[27] and its 1,3-methyl-deuterated derivative ([1,3-$(C^2H_3)_2$]-2,4-Ac_2-deuterohemin),[28] were prepared by standard methods. 2- and 4-acetyldeuteroporphyrin IX dimethyl esters were prepared by Friedel–Crafts acetylation of copper(II) deuteroporphyrin IX using stannic chloride and acetic anhydride; the copper(II) monoacetyl isomers were separated by using medium-pressure liquid chromatography (150 psi) on Whatman LPS silica gel.[29] Removal of copper was subsequently accomplished by treatment with 10% sulfuric acid in trifluoracetic acid. Reduction of the individual monoacetyldeuteroporphyrins with sodium borohydride, followed by treatment with *p*-toluenesulfonic acid hydrate in *o*-dichlorobenzene, gave[21] the corresponding monovinylporphyrins: pemptoporphyrin dimethyl ester (4-vinyl isomer) from 4-acetyldeuteroporphrin IX, and isopemptoporphyrin dimethyl ester (2-vinyl isomer) from the 2-acetyl derivative. The 1-methyl in 2-acetyl- and the 3-methyl in 4-acetyldeuteroporphyrin IX ester were exchanged by using sodium methoxide in CH_3O^2H, as previously described for the 2,4-diacetyl case,[28] and gave material which was >90% labeled in the appropriate 1- or 3-methyl. Deuterium label which had been incorporated into the acetyl methyl groups of these compounds was simply removed by brief treatment with aqueous acid, a process which does not affect the deuterium in the nuclear methyl (1,3) groups. The labeled pemptoporphyrin and isopemptoporphyrins were prepared from the deuterated monoacetyldeuteroporphyrins by reduction with sodium borodeuteride, followed by dehydration, as previously, in *p*-toluenesulfonic acid/*o*-dichlorobenzene. Iron was inserted into the porphyrins, and the methyl esters were hydrolyzed as previously described.[30] Proton NMR showed that the pemptohemin or 4-vinyldeuterohemin (4-V-deuterohemin) was labeled to an extent of 70% in the 3-methyl, >90% in the α-vinyl, and 70% at the β-vinyl methylene. Likewise, the isopemptohemin or 2-vinyldeuterohemin (2-V-deuterohemin) was labeled 80% with ^2H in the 1-methyl, >90% in the 2-α-vinyl, and 80% in the 2-β-vinyl positions. The loss of deuterium at the β-vinyls is probably due to exchange processes taking place during the *p*-toluene-sulfonic acid dehydrations. The proteins reconstituted with modified hemins are prefixed by 2-R-and/or 4-R-deutero- to indicate the substituents.

The 360-MHz proton NMR spectra were recorded on a Nicolet NT-360 Fourier transform NMR spectrometer operating with quadrature detection. Typical spectra consisted of 5000 to 10000 pulses using 8000 data points over a 30000 Hz bandwidth (10 μs 90° pulse). The residual water peak was suppressed by a 25-ms presaturation pulse; signal-to-noise was improved by exponential apodization which introduced 2–5 Hz line broadening. Peak shifts were referenced to the residual water line which in turn was calibrated against internal DSS.

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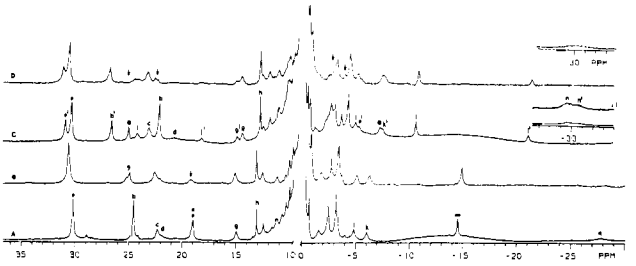

Figure 3. Hyperfine shifted portions of the 360-MHz proton NMR spectra at "pH" 7.0 and 35 °C of (A) 4-V-deuteroHRPCN, (B) [3-(C^2H_3),4-($C^2HC^2H_2$)]-4-V-deuteroHRPCN, (C) 2-V-deuteroHRPCN, and (D) [1-(C^2H_3),2-($C^2HC^2H_2$)]-2-V-deuteroHRPCN. Peak labeling follows the pattern established in Figure 2; peaks with clearly reduced intensities due to deuteration are indicated by arrows. Assignments of labeled peaks are given in Table II.

Chemical shifts are reported in parts per million referenced to DSS, with downfield shifts positive.

Results

The hyperfine-shifted region of the 360-MHz ^1H NMR spectra for HRPCN, deuteroHRPCN, 2,4-Ac_2-deuteroHRPCN, and [1,3-(C^2H_3)$_2$]-2,4-Ac_2-deuteroHRPCN are illustrated in Figure 2A–D, respectively. The methyl assignments for the first two complexes have been reported previously,[4] as have those for the vinyl protons,[31] a propionate[31] 6-H_β and 7-H_α and tentative assignments of axial imidazole $C_\delta H$ and $C_\epsilon H$.[32] The heme 2- and/or 4-H appear in a characteristic upfield window.[32] 2,4-Ac_2-deuteroHRPCN yields a single set of resonances indicative of a unique heme orientation. The degree of deuteration is 50% at 1-methyl and 70% at 3-methyl, so that the methyl resonance with diminished intensity in D of Figure 2 can be assigned to 3-CH_3. Since the pair of hyperfine shifted methyls[4,13,20,21] must be either 3,8 or 1,5, the one assignment establishes that the heme orientation in 2,4-Ac_2-deuteroHRPCN is the same as for the native enzyme (B in Figure 1).

The 360-MHz ^1H NMR spectra in the region below 10 ppm and above 0 ppm from DSS for 4-V-deuteroHRPCN, [3-(C^2H_3),4-($C^2HC^2H_2$)]-4-V-deuteroHRPCN, 2-V-deuteroHRPCN, and [1-(C^2H_3),2-($C^2HC^2H_2$)]-2-V-deuteroHRPCN are displayed in A–D of Figure 3. While the spectrum of 4-V-deuteroHRPCN exhibits the same number of downfield resonances as HRPCN and hence is consistent with a unique protein structure, the spectrum of 2-V-deuteroHRPCN exhibits twice the number of resonances below 12 ppm as found in HRPCN. Moreover, the relative intensities do not follow the simple 3:1 or 1:1 ratio found in HRPCN. These peaks, however, are consistent with two protein forms with the components present in the ratio ~2:1. The major component peaks are labeled by the letters a–h, j–n while the minor component peaks are labeled a′–h′, i′–n′.

Comparison of the spectrum of 4-V-deuteroHRPCN (A) with that of [3-C^2H_3,4-$C^2HC^2H_2$]-4-V-deuteroHRPCN (B) in Figure 3 yields the unique assignment of 3-CH_3 and 4-vinyl H_α peaks. The decreased intensity of the peaks at −2.5 ppm in B (and D) of Figure 2 is due to simultaneous deuteration of the vinyl β-position. The same resonances arise from 3-CH_3 and 4-H_α as in native HRPCN, establishing that the unique heme orientation is that found for HRPCN (A in Figure 1). A similar comparison of the spectra of 2-V-deuteroHRPCN (C) and [1-C^2H_3,2-$C^2HC^2H_2$]-2-V-deuteroHRPCN (*d*) in Figure 3 reveals that one of the methyls for the major component, b, is 1-CH_3 and the vinyl H_α peak, d, is 2-H_α; the other methyl must then be 5-CH_3 and hence the heme orientation for the component is the same as for deuteroHRPCN, i.e., B in Figure 1. The minor component, on the other hand, does *not* have 1-CH_3 downfield, and thus must have 3-CH_3 and 8-CH_3 downfield, which dictates the native orientation (A in Figure 1).

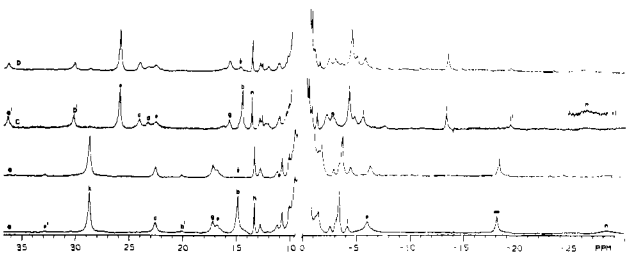

Figure 4. Hyperfine shifted portions of the 360-MHz proton NMR spectrum at "pH" 7.0 and 35 °C of (A) 4-Ac-deuteroHRPCN, (B) [3-(C^2H_3)]-4-Ac-deuteroHRPCN, (C) 2-Ac-deuteroHRPCN; and (D) [1-(C^2H_3)]-2-Ac-deuteroHRPCN. Peak labeling follows the pattern established in Figure 2; peaks with clearly reduced intensities due to deuteration are indicated by arrows. Assignments of labeled peaks are given in Table II.

Table I. Chemical Shifts of Cyanide Complexes of Horseradish Peroxidase Containing 2,4-Substituted Deuterohemins[a]

peak[c]	HRPCN orient A[b]	deuteroHRPCN orient B[b]	deuteroHRPCN orient A[b]	2,4-Ac_2deuteroHRPCN orient A[b]
a, a′	29.76 (8)	27.64 (5)	31.63 (8)	34.17 (8)
b, b′	24.97 (3)	22.32 (1)	26.31 (3)	18.13 (3)
c, c′	22.50	22.32		23.06
d, d′	22.50	18.68		25.87
e, e′	19.61 (4)	NP	NP	NP
f, f′	18.99 (7)	23.16 (6)		16.46 (7)
g, g′	14.82	14.35		16.89
h, h′	12.97	12.81		12.99
i, i′	−3.06	−3.30		−2.99
j, j′	−5.13	−4.90		−4.62
k, k′	−6.71	−6.92		−6.80
l, l′	NP	−11.81 (4)	−20.75 (4)	NP
m, m′	NP	−24.59 (2)	−14.17 (2)	NP
n, n′	−28.4	−27.2		−30.1

[a] Peak shifts ±0.02 ppm, recorded at 360 MHz at 35 °C and "pH" 7.0. [b] Peak shifts are reported for both major and minor (when determinable) heme orientation as shown in Figure 1. [c] The lettering scheme used is that for HRPCN (see Figure 2). The assignments are indicated where possible; the number in parentheses after the peak shift indicates the assignment as numbered in Figure 1. NP is used to indicate protons that are not present in these HRPCNs.

The ^1H NMR spectra of the analogous acetyl derivatives 4-Ac-deuteroHRPCN and 2-Ac-deuteroHRPCN are illustrated in A and C of Figure 4. In the former compound we again have a single set of resonances indicative of a unique heme orientation, and the methyl deuteration (B in Figure 4) reveals that one of the downfield peaks is 3-CH_3, which requires the same heme orientation as HRPCN (A in Figure 1). Similarly, the spectrum of 2-Ac-deuteroHRPCN displays two sets of resonances (C in Figure 4), and deuteration (D in Figure 4) reveals that only the major component has 1-CH_3 (b) downfield. Hence, the major component has the orientation as in B of Figure 1, while the minor isomer has the orientation as in A of Figure 1.

The various resonances assigned by unambiguous isotope labeling, by the characteristic pH dependent shifts and line widths exhibited by the presumed propionate H_α next to the methyl with the larger hyperfine shift,[31] and by the unique window in which 2-H and 4-H resonate[33] are listed in Table I, along with the resonance positions of, as yet, unassigned resonances. The methyl assignments indicate that replacing both vinyls with acetyl groups leaves the heme orientations unchanged; removal of both vinyls reverses the heme orientation. The presence of a single vinyl or acetyl group at the 4-position leads to a unique heme orientation which is the same as for the native protein. The presence of a

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Table II. Chemical Shifts of Cyanide Complexes of Horseradish Peroxidase Containing Monosubstituted Deuterohemins[a]

peak[c]	4-V-deuteroHRPCN orient A[b]	2-V-deuteroHRPCN orient A[b]	2-V-deuteroHRPCN orient B[b]	4-Ac-deuteroHRPCN orient A[b]	2-Ac-deuteroHRPCN orient A[b]	2-Ac-deuteroHRPCN orient B[b]
a, a′	30.40 (8)	30.93 (8, 3)	30.38 (5)	28.69 (8)	36.37 (8, 3)	26.00 (5)
b, b′	24.66 (3)	26.64 (3, 8)	22.22 (1)	14.84 (3)	30.25 (3, 8)	14.57 (1)
c, c′	22.42		23.14	22.52		24.17
d, d′	22.01	19.00 (?)	20.78	27.27		23.40
e, e′	19.01 (4)		25.05 (2)	NP	NP	NP
f, f′	19.01 (7)	18.27 (7)	24.25 (6)	16.73 (7)		22.65 (6)
g, g′	14.99	14.94	14.49	17.14		15.76
h, h′	13.08	12.58	12.87	13.30		13.69
i, i′	−3.26		−4.35	−3.47		−4.39
j, j′	−4.98	−5.42	−5.09	−4.27		−5.66
k, k′	−6.18	−7.57	−7.39	−6.10		
l, l′	NP	−21.14 (4)	−10.70 (4)	NP	−19.37 (4)	−13.43 (4)
m, m′	−14.74 (2)	NP	NP	−18.14 (2)	NP	NP
n, n′	−28.1	−30.5	−29.5	−28.3		−26.4

[a] Peak shifts ±0.02 ppm, recorded at 360 MHz at 35 °C and "pH" 7.0. [b] Peak shifts are reported for both major and minor (when determinable) heme orientation as shown in Figure 1. [c] The lettering scheme used is that for HRPCN (see Figure 2). The assignments are indicated where possible; the number in parentheses after the peak shift indicates the assignment as numbered in Figure 1. NP is used to indicate protons that are not present in these HRPCNs.

single vinyl or acetyl group on the 2-position, however, leads to heme disorder with the favored orientation of the heme rotated 180° about the α–γ meso axis from that in the native protein.

Discussion

The severe restriction on the oscillatory mobility of vinyl and propionate side chains in HRP[1,31] relative to that found in myoglobin[5] is suggestive of heme–protein interactions at the heme periphery which are basically repulsive in nature. The unique orientation of native protohemin is thus probably determined by the fact that the packing of amino acid side chains crowd both the 1,3-methyls and 2,4-vinyls but leave slightly more room for the larger vinyl groups, as illustrated schematically in Figure 1. On this premise, the 180° rotation about the α–γ meso axis of the heme upon converting from protohemin (R_2 = R_4 = vinyl) to deuterohemin (R_2 = R_4 = H) can be rationalized by the fact that the reversed orientation places the 1,3-methyles in the larger space provided for the 2,4-vinyls and the 2,4-protons in the smaller space originally occupied by 1,3-methyls, thereby reducing steric interaction with the protein for all heme substituent groups on pyrroles I and II.

If the degree of steric interactions with the protein is equal for pyrrole I (2-vinyl) and pyrrole II (4-vinyl), then both monovinyl deuterohemins might be expected to yield heme orientational preference intermediate between that observed for protohemin (A in Figure 1) and deuterohemin (B in Figure 1), leading to partial populations of both orienations. However, in the case that the steric interactions are localized primarily at one of the pyrroles, it may be anticipated that one of the monosubstituted deuterohemins should maintain the native orientation (A in Figure 1), and the other isomer should adopt primarily the reversed (B in Figure 1) orientation. The fact that 4-V-deuteroHRPCN exists in the native orientation indicates that the 4-vinyl can be accommodated in the allocated space within the heme pocket in the orientation A in Figure 1. On the other hand, 2-V-deuteroHRPCN is present largely with the reversed heme orientation (B in Figure 1), indicating that the 2-vinyl is not as easily accommodated in the heme pocket in the native orientation. Thus, we conclude that the heme–protein interactions which dominate the heme orientation are primarily steric or repulsive interactions between the 2-vinyl group and the amino acids near pyrrole I in the native protein.

Direct information on the nature of this steric interaction between the vinyl group and protein is not obtainable from the ^{1}H NMR spectrum of HRPCN because the spin distribution results in too small a hyperfine shift for the 2-vinyl resonances to be resolved from the intense diamagnetic envelope.[31] However, if we assume that the heme–protein interactions at the heme periphery are similar in HRPCN and compound I, the previously reported "splitting" of uniquely the 2-vinyl H_α peak into two components[12] provides strong support for a severe steric interaction localized at pyrrole I. The differential paramagnetic line broadening of the two 2-vinyl H_α component peaks was shown[12] to be consistent with a vinyl group which is clamped into two slowly exchanging orientations each of which has the vinyl and heme planes close to parallel. These two orientations, in the limit of coplanarity, are depicted in Figure 5. Thus, both the 2-vinyl H_α resonance of HRP compound I, as well as the dependence of the heme orientation on the location of vinyl groups in low-spin cyanide complexes of the resting enzyme, argues for severe steric interactions between the protein side chains and pyrrole I, leading to the clamping of the 2-vinyl group into a more in-plane orientation than present in models or proteins with less constrained heme pockets.

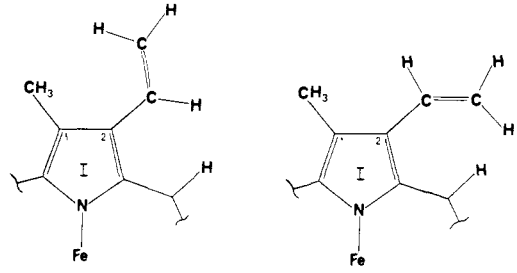

Figure 5. The two limiting coplanar orientations of the vinyl group of pyrrole I in which the vinyl group lies in the plane of the pyrrole, as deduced from the line width of the split 2-vinyl H_α peak in compound I.[12]

While it is not clear that this clamping of the 2-vinyl group has any direct functional significance, it is noted that for model compounds and most other hemoproteins, the vinyls are usually not significantly coplanar[22,34–36] with the heme, due to the steric interaction with the adjacent methyl and meso-H. Thus, there is limited conjugation between vinyl and heme π systems. The forced near coplanarity of 2-vinyl in HRP could serve as a stabilizing influence on the porphyrin cation radical[36] formed in compound I of the native enzyme by expanding the π system over which the charge can be delocalized.

The unique heme orientation identical with that for the native protein found for 2,4-Ac_2-deuteroHRPCN is consistent with the fact that acetyl groups are similar to vinyl groups in both size and geometry. The acetyl π planes are similarly deterred from being coplanar with the heme due to interference from the adjacent

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methyl and meso-H. Thus, it is not surprising that 4-Ac-deuterohemin has the same orientation as found for 4-V-deuterohemin, while 2-Ac-deuterohemin displays heme disorder similar to that of 2-V-deuterohemin, with the major compound possessing the same reversed orientation (B in Figure 1). Acetyl resonances are not resolved in any low-spin ferric complex. Unfortunately, the significantly reduced stability of compound I for acetyl derivatives of HRP precludes detection of their ^1H NMR spectra.

Thus, we conclude that determination of the influence peripheral substituents have on the porphyrin orientation in b-type low-spin ferric hemoproteins using our NMR method[13] may provide a general technique for detailed characterization of heme-protein interactions. We further conclude that the heme–protein steric interactions which tend to force the 2 substituent into the heme plane are primarily responsible for determining the orientation of the heme in the heme pocket of HRP. A possible functional role of the steric clamping is suggested to be stabilization of the porphyrin cation radical of the compound by extension of the π system to the vinyl group.

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Registry No. HRP, 9003-99-0; heme, 14875-96-8; 2-Ac-deuterohemin, 68866-16-0; 2-V-deuterohemin, 78694-18-5; 4-Ac-deuterohemin, 68949-21-3; 4-V-deuterohemin, 78694-17-4; 2,4-Ac_2-deuterohemin, 14977-95-8; deuterohemin, 21007-21-6.

Chemistry, Spectroscopy, and Isotope-Selective Infrared Photochemistry of a Volatile Uranium Compound Tailored for 10-μm Absorption: $U(OCH_3)_6$

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Abstract: This contribution reports on the chemical, vibrational spectroscopic, and infrared multiphoton photochemical properties of uranium hexamethoxide, $U(OCH_3)_6$, a prototype molecule for laser-induced uranium isotope separation with a carbon dioxide laser. Uranium hexamethoxide can be prepared from UCl_4 by conversion to $Li_2U(OCH_3)_6$, followed by oxidation with lead tetraacetate. Vapor pressure studies on $U(OCH_3)_6$ indicate that $\Delta H_{sub}° = 23 \pm 3$ kcal/mol and $\Delta S_{sub}° = 76 \pm 4$ eu; at 33 °C, the vapor pressure is 17 mtorr. The vibrational spectra of $U(^{16}OCH_3)_6$ and $U(^{18}OCH_3)_6$ have been assigned by using infrared and laser Raman data. Under idealized O_h symmetry, the $U(^{16}OCH_3)_6$ U–O stretching fundamentals are assigned at 505.0 (A_{1g}), 464.8 (T_{1u}), and 414.0 cm^{-1} (E_g). Tentative assignments are also made for several of the overtone and combination transitions evidence for possible lowering of the symmetry is presented. In gas-phase infrared photochemical experiments, the predominant $U(OCH_3)_6$ photoproducts isolated are $U(OCH_3)_5$, methanol, and formaldehyde. These are suggestive of multiphoton U–O bond homolysis to produce uranium pentamethoxide and methoxy radicals. The enrichment of unreacted $U(OCH_3)_6$ in ^{235}U is maximum at ca. 927 cm^{-1} (near what may be a U–O stretching overtone transition) and exhibits both a low fluence threshold and diminution at high fluence.

Introduction

The efficient separation of isotopes by selective laser-induced multiphoton infrared excitation of polyatomic molecules is now an experimental reality as well as a subject of considerable current theoretical interest.[2] A wide variety of systems have been studied, and laboratory-scale isotope separation has been achieved for a host of elements including hydrogen,[2,3] boron,[4] carbon,[4b,5] oxygen,[6] silicon,[4b] sulfur[2,7] chlorine,[8] molybdenum,[9a] selenium,[9b] and osmium.[10] The key molecular requirements for any efficient laser-induced isotope separation process employing polyatomic molecules include (i) volatility, preferably approaching ca. 1 torr at ambient temperatures (operating pressures appreciably in excess of this are likely a disadvantage), (ii) the existence of an infrared-active normal vibrational mode (fundamental, overtone, or combination) that exhibits a nonzero isotope shift and absorbs in the spectral region corresponding to the output of an efficient laser system, (iii) the absence of interfering ligand absorptions, (iv) the ready synthesis of large quantities of the target molecule, (v) the facile separation and recycling of enriched and depleted material, and (vi) the absence of undesirable photochemical side reactions such as nonselective photodecomposition.

In practice, the isotope-sensitive transition of the target molecule is brought into resonance or near resonance in the gas phase with a high fluence infrared laser source. The result is isotope-selective

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