Proton Nuclear Magnetic Resonance Spectroscopy of Horseradish Peroxidase Isoenzymes: Correlation of Distinctive Spectra with Isoenzyme Specific Activities†

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ABSTRACT: High-resolution proton NMR spectra are reported for the paramagnetic ferric native and cyano complexes of the five major horseradish root peroxidase (HRP) isoenzymes (A_1, A_2, A_3, B, and C). Axial imidazole resonances are observed in the native and cyano-complex spectra of all the isoenzymes, thus indicating the presence of a common axial histidine ligand. Proton NMR spectra outside the usual diamagnetic region are identical for sets of A_1 and A_2 isoenzymes and for the B and C isoenzyme set. Variation in heme residue chemical shift positions may be controlled in part by porphyrin vinyl side chain–protein interactions. Diverse upfield spectra among the isoenzymes reflect amino acid substitutions and/or conformational differences near the prosthetic group, as signals in this region must result from amino acid residues in proximity to the heme center. Acid–base dependence studies reveal an "alkaline" transition that converts the native high-spin iron(III) porphyrin to the low-spin state. The transition occurs at pH 9.3, 9.4, 9.8, and 10.9 for respective HRP A_1, A_2, A_3, and C isoenzymes, respectively. Significantly, this ordering also reflects specific activities for the isoenzymes in the order $A_1 = A_2 > A_3 > B = C$. Identical proton NMR spectra for A_1/A_2 and B/C isoenzyme sets parallel equivalent specific activities for members of a particular set. Proton NMR spectra thus appear to be highly sensitive to protein modifications that affect catalytic activity.

Horseradish peroxidase (HRP) has been the subject of numerous physiochemical studies as a consequence of the favorable stability and commercial availability of the enzyme (Dunford & Stillman, 1976; Dunford, 1982). Up to 12 isoenzymes have been identified, including both acidic (A_1, A_2, and A_3) and basic (B, C, D, and E_1–E_6) forms (Shannon et al., 1966; Kay et al., 1967; Aibara et al., 1981). The majority of physical measurements have been performed with mixtures of the major C isoenzyme and the minor B isoenzyme as supplied by commercial vendors. Ready availability of the B/C isoenzyme mixture has dictated that these proteins should serve as the benchmark for reactivity and spectroscopic properties among peroxidases. However, further detailed characterization of the acidic isoenzymes of HRP is clearly warranted in view of the fact that specific activities of A isoenzymes are up to 20 times higher than those of the B/C isoenzymes (Kay et al., 1967).

Nuclear magnetic resonance (NMR) spectroscopy provides one of the most powerful techniques for elucidation of hemoprotein solution dynamics and electronic and molecular structure. Spectra of hemoproteins in their paramagnetic states are particularly rich in information content, as signals for nuclei in proximity to the iron center are likely to be shifted well outside the crowded 0–10 ppm "diamagnetic" spectral region. For example, proton NMR spectroscopy of native HRP has been useful in demonstrating that the proximal histidyl imidazole moiety remains protonated upon coordination to the iron(III) center (La Mar & de Ropp, 1979; La Mar et al., 1982a,b). Characterization of various ligated (Williams et al., 1975; Morishima et al., 1977, 1978; La Mar et al., 1980b) and oxidized forms of HRP (Morishima & Ogawa, 1978; La Mar et al., 1981, 1983a; La Mar & de Ropp, 1980) has also benefited from NMR measurements. All previous high-resolution proton NMR studies have dealt with only the basic B/C isoenzymes. Findings described here will serve to demonstrate that significant spectroscopic differences are seen for the acidic A isoenzymes. It is shown that a direct correspondence exists between NMR chemical shift patterns and the specific activity of a given HRP isoenzyme. Furthermore, specific activities of the isoenzyme are correlated with pK_a values for an "alkaline" high-spin to low-spin conversion as measured by proton NMR spectral changes.

Experimental Procedures

Horseradish peroxidase with $RZ = 0.8$ (ratio of Soret to 280-nm absorbance) was purchased from Sigma Chemical Co. (type I) as an essentially salt-free lyophilized powder. One gram of the crude peroxidase was dissolved in 8 mL of 100 mM acetate buffer at pH 4.4. The dark red viscous solution was filtered through a Whatman GF/D glass microfiber filter to remove any undissolved material. At 4 °C, the solution was loaded onto a 2.5 × 100 cm column of Sephadex G-100 previously equilibrated with 100 mM acetate buffer at pH 4.4. The column was run at a rate of 10 mL/h, and 1.5-mL fractions were collected. After the fractions rich in HRP were pooled, the *RZ* value had increased to 1.67. The pooled material was then applied to a 10-mL microcolumn (Bio-Rad Econo-column) containing Whatman CM-52 equilibrated in the same buffer. The peroxidase isoenzymes A, B, and C were not retained by this column and were eluted free of tightly bound impurities by using the 100 mM acetate buffer. Some 230 mg of HRP with $RZ = 1.95$ was recovered after this procedure. The material thus obtained was dialyzed overnight against 60 volumes of 5.0 mM acetate buffer at pH 4.4 and applied to a 2.5 × 20 cm CM-52 column previously equilibrated with the acetate buffer. Isoenzymes B and C are retained by the column while isoenzymes A (A_1, A_2, and A_3) are readily eluted with 60 mL of the same buffer. Isoenzymes B and C practically coeluted when a gradient of 5.0–75 mM

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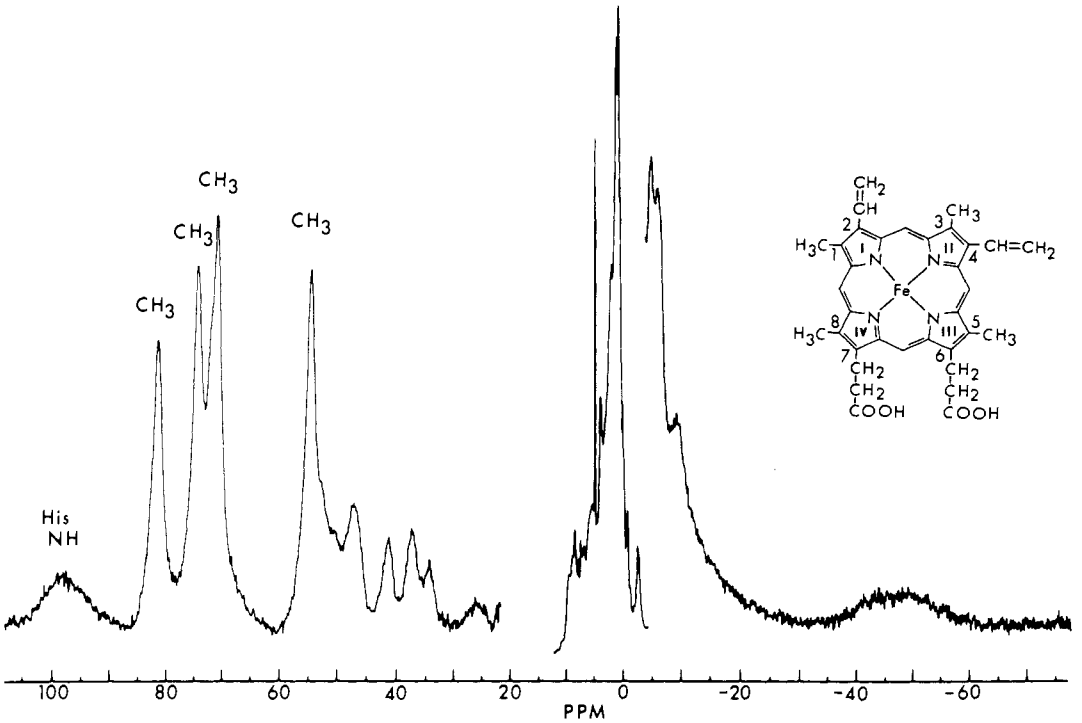

FIGURE 1: Proton NMR spectrum of ferric native HRP isoenzyme C. Conditions: 25 °C, D_2O solution, 0.10 M phosphate buffer, pD 6.40, 2.0 mM enzyme, DSS reference.

acetate buffer as described by Shannon et al. (1966) was used. Partial separation was effected through elution with a double gradient of 1 L of 5.0 mM, 1 L of 50 mM, and 1 L of 100 mM acetate buffer at pH 4.4 in a 4 × 40 cm column of CM-52. Even under these conditions complete separation of HRP B and HRP C was not complete, but sufficient material for NMR spectroscopy was obtained by pooling the edges of the two overlapping bands. Separation of isoenzymes A_1, A_2, and A_3 was carried out by modification of the procedure of Shannon et al. (1966) using a 1.5 × 20 cm Whatman DE-53 column equilibrated with 5.0 mM tris(hydroxymethyl)-aminomethane (Tris) buffer at pH 8.4 and a 0.0–60 mM NaCl gradient. The corresponding *RZ* values for the isoenzymes A_1, A_2, A_3, B, and C at pH 4.4 were 4.25, 3.97, 3.26, 3.56, and 3.30, respectively. This modification affords the isolation of the three acidic isoenzymes in only one step. The purity is comparable to the four-column purification described earlier (Shannon et al., 1966).

Samples were prepared for NMR spectral measurements through concentration by ultrafiltration and repeated exchange with D_2O containing 100 mM phosphate buffer at pD 6.40, or 50 mM Tris buffer at pD 6.9. Uncorrected solution "pD" values were measured in the NMR tube using an Aldrich micro-NMR tube electrode calibrated with standard aqueous (H_2O) buffers. Proton NMR spectra were recorded at 360 MHz with a Bruker WM-360 spectrometer. Sweep widths of 71 kHz were routinely employed, and from 3000 to 50 000 transients were recorded. The residual water signal and adjacent protein signals were suppressed by prepulse irradiation. Spectra were recorded at 25 °C unless otherwise indicated. Chemical shift values are referenced to internal 4,4-dimethyl-4-silapentanesulfonate (DSS); the 4.75 ppm residual HDO signal provided a convenient secondary reference. Uncertainties in chemical shift values range from ±0.2 ppm for broad signals to ±0.03 ppm for relatively sharp resonances.

Results and Discussion

Proton NMR Spectroscopy of Native HRP Isoenzymes. The proton NMR spectrum of HRP isoenzyme C is provided in Figure 1. Far-downfield and near-upfield features are essentially identical with those previously reported at 360 MHz (La Mar et al., 1980b). The far-upfield region has not been described in earlier NMR studies of HRP, but a resonance with a line width on the order of 5 kHz is readily apparent at −47 ppm. Although this signal approaches in breadth the base-line "roll" artifacts from our spectrometer, the upfield feature is totally reproducible for relatively concentrated HRP solutions using a variety of spectral widths and offsets. The broad feature was absent in spectra of cyano-HRP and in those for metmyoglobin. The signal remains to be unambiguously assigned.

The far-downfield proton NMR spectral region is recorded in Figure 2 for native preparations of the five major HRP isoenzymes. Chemical shift values are presented in Table I. A number of common features and significant spectral differences are apparent. Spectra for sets of enzymes A_1/A_2 and

Table I: Downfield Proton NMR Resonances for Ferric Native HRP Isoenzymes[a]

signal	isoenzyme				
	A_1	A_2	A_3	B	C
His N–H	94.8	95.2	95.8	97.8	98.8
CH_3	79.4	79.6	79.9	81.4	81.5
CH_3	69.4	69.5	69.5	74.3	74.3
CH_3	65.8	65.9	67.5	70.6	70.8
CH_3	53.3	53.4	54.4	54.4	54.4
a	65.8	66.3	68.7	71.7	72.0
b	51.1	51.1		50.1	50.0
c	45.4	45.4	46.6	46.9	47.0
d	43.5	43.6	42.2	41.2	41.2
e	34.7	34.7	36.8	37.2	37.3
f	32.6	32.5	32.9	34.2	34.2
g	25.3	25.3	27.8		
h			63.5[b]		
i			59.5[b]		
j				52.6	52.5

[a] Conditions: 25 °C, D_2O solution, 0.10 M phosphate buffer, pD 6.40, enzyme concentrations 0.5–2.0 mM, DSS reference, signal labels match those in Figure 2. [b] Minor signals from denatured enzyme or unidentified isoenzyme.

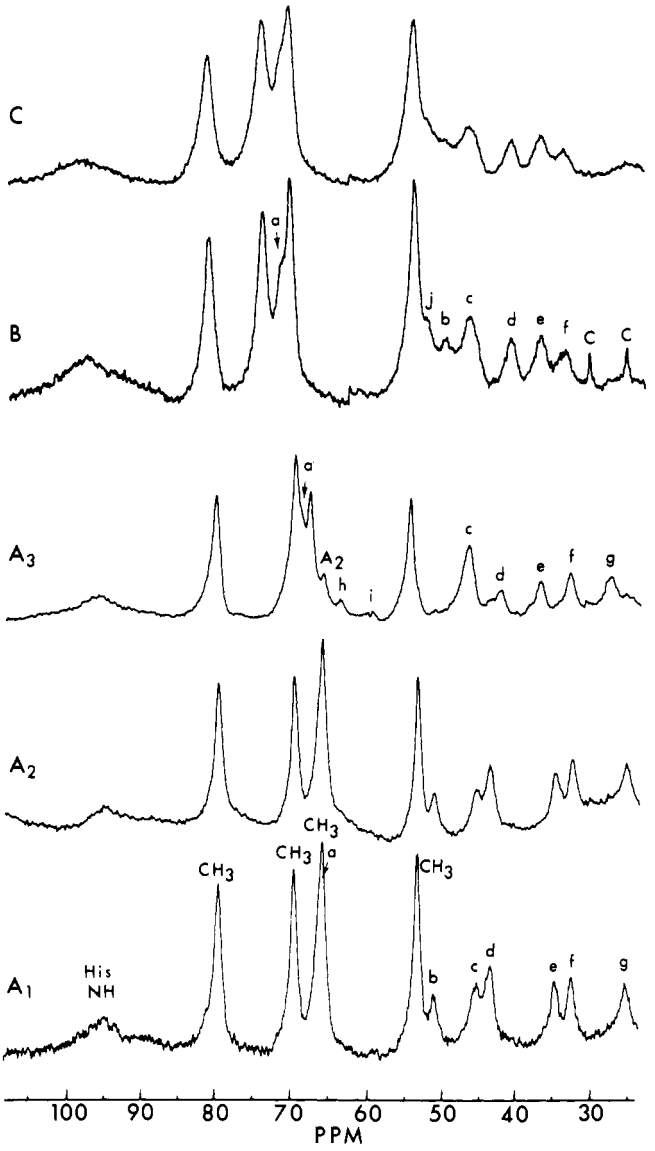

FIGURE 2: Downfield proton NMR spectra of ferric native HRP isoenzymes. Conditions: 25 °C, D_2O solution, 0.10 M phosphate buffer, pD 6.40, 0.5–2.0 mM enzyme, DSS reference. Signals labeled C in the HRP B spectrum are from ring methyl resonances of cyano-HRP as a consequence of trace cyanide contamination.

for B/C are essentially identical, and this accounts for the absence of signal splitting in earlier NMR studies of the B/C isoenzyme mixture. Spurious signals labeled C are noted in the spectrum of isoenzyme B due to traces of contaminating cyanide ion. The isoenzyme A_3 preparation contained minor amounts of the A_2 form as is evident in appearance of the signal labeled A_2 at 65.8 ppm. Other minor signals (reproducible for two separate preparations) labeled h and i are likely due to denatured enzyme or to a previously unidentified minor isoenzyme that coelutes with HRP A_3 under the conditions employed. Line widths are clearly smaller for the A_1/A_2 isoenzymes as compared to the B/C forms. Line-width differences between the B and C isoenzyme spectra are apparently a consequence of the higher isoenzyme C concentration.

La Mar and co-workers (La Mar et al., 1980b) have unambiguously assigned the specific porphyrin ring methyl signals for HRP B/C. In accordance with the numbering scheme shown in Figure 1, ring methyl assignments are 5, 1, 8, 3 (downfield to upfield ordering). Assuming that this ordering is applicable to the HRP A isoenzymes, it is clear that ring methyls at positions 5 and 3 (pyrroles III and II) are perturbed little by the amino acid substitutions in different isoenzymes. However, the 1,8-ring methyl residues on the opposite side of the porphyrin (pyrroles I and IV) must be in appreciably different environments in HRP A as compared to the HRP B/C polypeptide. "Steric clamping" of the 2-vinyl residue in HRP B/C has previously been detected by high-resolution NMR methods (La Mar et al., 1983b). Modulation of this protein–heme interaction among the various isoenzymes provides a possible explanation for the large chemical shift differences of isoenzyme 1-methyl signals and apparent differences in the 2-vinyl C–H signals. In this regard, signals labeled a in the B/C isoenzyme spectra have been tentatively assigned to the 2-vinyl C–H residue (La Mar et al., 1980b). Corresponding single-proton signals lie under heme methyl peaks in the A_1/A_2 spectra. An upfield shift of some 6 ppm for these signals (as compared with the B/C isoenzymes) parallels the pattern of ring methyl shifts and is fully consistent with location of the residue on pyrrole I as a 2-vinyl C–H group. The 4-vinyl C-H signal has been tentatively assigned to one of the components of signal c in the B/C enzymes (La Mar et al., 1980b). On the basis of relatively small differences observed for the 3-methyl (pyrrole II) signal of the five isoenzymes, the 4-vinyl signal position is expected to be relatively invariant. Although the assignment is not a conclusive one, the signal(s) labeled c is (are) indeed observed in the 45.4–47.0 ppm region for all five proteins.

Six additional single-proton signals in the 55–25 ppm region for HRP B/C have been grouped as representative of proximal histidyl CH_2 and porphyrin ring CH_2 propionic acid moieties (La Mar et al., 1980b) (common labels placed on these signals in Figure 2 are not meant to imply corresponding assignment for the various isoenzymes). These six signals are evident for A_1/A_2 isoenzymes (signal c is not included in this count, and signal d integrates with a two-proton intensity). The 55–25 ppm spectral region for the A_3 isoenzyme appears to have the same number of signals, but overlap of three signals at c, possible overlap with a methyl group signal at 54.4 ppm, and contributions from minor amounts of the A_2 isoenzyme leave the spectrum rather complex. Little can be offered with regard to specific assignment of signals in the 55–25 ppm region. However, it should be noted that the signals for HRP B/C exhibit a relative downfield bias much as is the case for ring methyl resonances in these isoenzymes.

The broad, far-downfield proximal histidyl imidazole N–H signal is seen in all the isoenzymes (signal intensity and line width are distorted by our base-line smoothing procedure). A downfield bias is seen for this signal as one compares the HRP A vs. HRP B/C isoenzymes—the range of respective chemical shift values is 95–99 ppm. This relatively small difference is particularly important, as no gross change in proximal imidazole hydrogen bonding or perturbation of axial ligand–iron interactions is apparent. Furthermore, the downfield chemical shift bias for both the axial residue and heme ring residues of HRP B/C (as compared with HRP A) indicates that a dipolar shift mechanism is not totally responsible for isoenzyme spectral differences, as axial and equatorial dipolar shifts would be in opposite directions.

The upfield spectral region for the five major HRP isoenzymes is shown in Figure 3, and chemical shift values are listed in Table II. Spectra for the B/C forms are essentially identical with those previously reported at 35 °C by La Mar and co-workers (La Mar et al., 1980b). Significant spectral differences are observed among A isoenzymes, but as was the case for the downfield region, spectra are identical for the A_1/A_2 forms and for the B/C forms. In this spectral region,

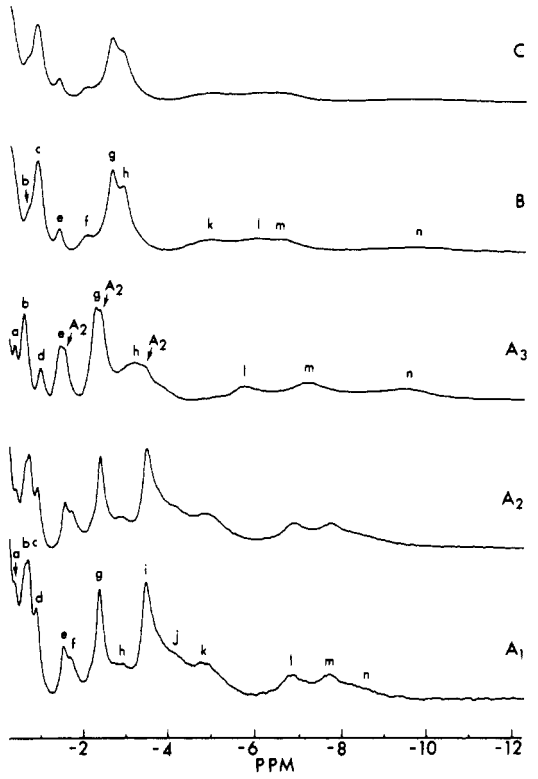

FIGURE 3: Upfield proton NMR spectra of ferric native HRP isoenzymes. Conditions: 25 °C, D_2O solution, 0.10 M phosphate buffer, pD 6.40, 0.5–2.0 mM enzyme, DSS reference.

Table II: Upfield Proton NMR Resonances for Ferric Native HRP Isoenzymes[a]

	isoenzyme				
signal	A_1	A_2	A_3	B	C
a	−0.41	−0.41	−0.41		
b	−0.62	−0.62	−0.60	−0.70	−0.67
c	−0.70	−0.72		−0.89	−0.89
d	−0.89	−0.89	−0.96		
e	−1.50	−1.52	−1.42	−1.40	−1.40
f	−1.69	−1.69		−2.05	−2.05
g	−2.37	−2.36	−2.27	−2.66	−2.66
h	−2.9	−2.9	−3.2	−2.93	−2.90
i	−3.46	−3.48			
j	−4.1	−4.2			
k	−4.8	−4.8		−4.9	−4.9
l	−6.8	−6.8	−5.7	−6.0	−6.1
m	−7.7	−7.7	−7.2	−6.6	−6.6
n	−8.2	−8.3	−9.4	−9.8	−9.8

[a] Conditions: 25 °C, D_2O solution, 0.10 M phosphate buffer, pD 6.40, enzyme concentrations 0.5–2.0 mM, DSS reference, signal labels match those in Figure 3.

only resonances l and m have been specifically assigned as representative of two of the four vinyl CH_2 signals of HRP B/C. The corresponding vinyl signals are seen at −8.3 and −10.8 ppm in the native ferric cytochrome *c* peroxidase spectrum (Satterlee et al., 1983a). Other upfield signals are expected to result from ring current and dipolar shift contributions to the resonance positions of alkyl amino acid residues in proximity to the heme prosthetic group. Among the HRP isoenzymes, only HRP C has been sequenced (Welinder & Mazza, 1977), and thus, detailed comparisons among isoenzymes are not possible. However, amino acid substitutions and/or conformational differences near the heme center must be responsible for spectral differences in the upfield region (vide infra).

Native HRP isoenzyme C has been shown to bind a single calcium ion with high affinity. Removal of the ion brings

Table III: Downfield Proton NMR Resonances for Ferric Cyano-HRP Isoenzymes[a]

	isoenzyme				
signal	A_1	A_2	A_3	B	C
CH_3	30.12	30.07	30.79	30.93	30.95
CH_3	27.64	27.62	28.12	25.98	25.96
a	23.27	23.27	23.3	23.73	23.72
b	22.4	22.4	22.33		
c	21.56	21.51	21.44		
d	19.17	19.17	20.06	20.07	20.06
e				19.5	19.5
f	16.92	16.90	17.46		
g				15.50	15.53
h	12.74	12.74	12.33	13.28	13.27
i				12.91	12.90
j	11.26	11.26	10.53	11.1	11.1
k	10.36	10.34	10.31	10.39	10.40
l	9.79	9.76	10.07	10.15	10.15
m			26.60[b]		

[a] Conditions: 25 °C, D_2O solution, 0.10 M phosphate buffer, pD 6.6, enzyme concentrations 0.5–2.0 mM, KCN concentrations typically 10 mM, DSS reference, signal labels match those in Figure 4. [b] Minor signal from denatured enzyme or unidentified isoenzyme.

Table IV: Upfield Proton NMR Resonances for Ferric Cyano-HRP Isoenzymes[a]

	isoenzyme				
signal	A_1	A_2	A_3	B	C
a	−0.72	−0.77	−0.91	−1.01	−1.00
b			−1.52	−1.55	−1.55
c	−1.90	−1.89	−1.85	−1.89	−1.82
d				−2.55	−2.54
e	−2.83	−2.79	−3.07	−3.12	−3.09
f	−3.34	−3.33	−3.48	−3.39	−3.36
g				−3.58	−3.60
h	−4.15	−4.15			
i	−4.64	−4.63	−4.97		
j				−5.66	−5.64
k	−7.48	−7.47		−7.13	−7.20
l			−9.35		
His 2-H	−27.7	−27.7	−30.4	−30.1	−30.0

[a] Conditions: 25 °C, D_2O solution, 0.10 M phosphate buffer, pD 6.6, enzyme concentrations 0.5–2.0 mM, KCN concentrations typically 10 mM, DSS reference, signal labels match those in Figure 5.

significant proton NMR spectral changes for the high-spin ferric native form (Ogawa et al., 1979). Involvement of calcium ion with HRP A isoenzymes has not been demonstrated, but presumably, the ion would not have been removed by the separation procedures employed in this study (spectra reported here for HRP B/C match those for the calcium-bound form). Identical proton spectra were obtained for the HRP A_3 isoenzyme before and after addition of 5 mM calcium chloride to a pD 6.9 0.05 M Tris buffer and 0.3 mM enzyme solution. This experiment served to demonstrate either that calcium ion was bound in the initial enzyme preparation or that the isoenzyme has little affinity for the metal ion. It should also be noted that use of Tris vs. phosphate buffer in this instance yielded identical proton NMR spectra, thus indicating no critical dependence on the nature of buffers used in this work.

Cyano Complexes of HRP Isoenzymes. A detailed proton NMR spectroscopic study of cyano and alkaline low-spin forms of HRP B/C has recently appeared (de Ropp et al., 1984). Downfield and upfield spectra of B/C isoenzymes shown in Figures 4 and 5 are equivalent to those reported in the earlier study at 25 °C. Tables III and IV provide listings of resonances shifted well outside the diamagnetic region. As is the case for the native high-spin states, cyano forms of sets of isoenzymes B/C and A_1/A_2 exhibit identical proton NMR

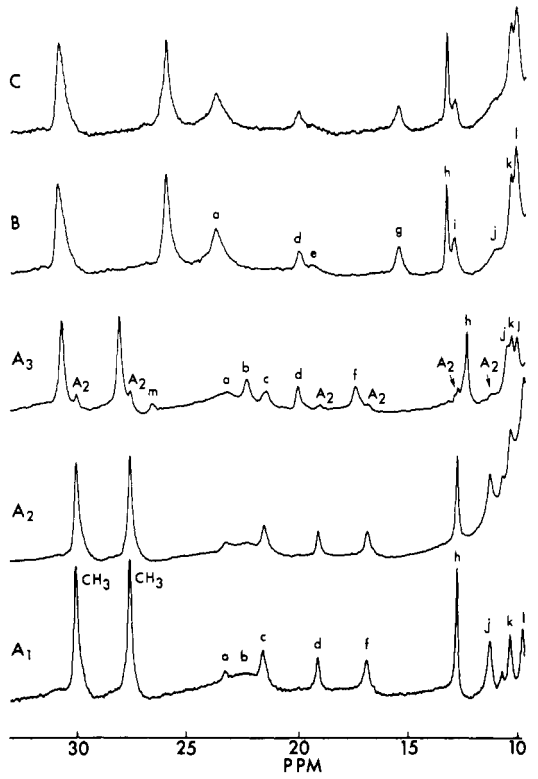

FIGURE 4: Downfield proton NMR spectra of ferric cyano-HRP isoenzymes. Conditions: 25 °C, D_2O solution, 0.10 M phosphate buffer, pD 6.6, KCN concentration typically 10 mM, enzyme concentrations 0.5–2.0 mM, DSS reference.

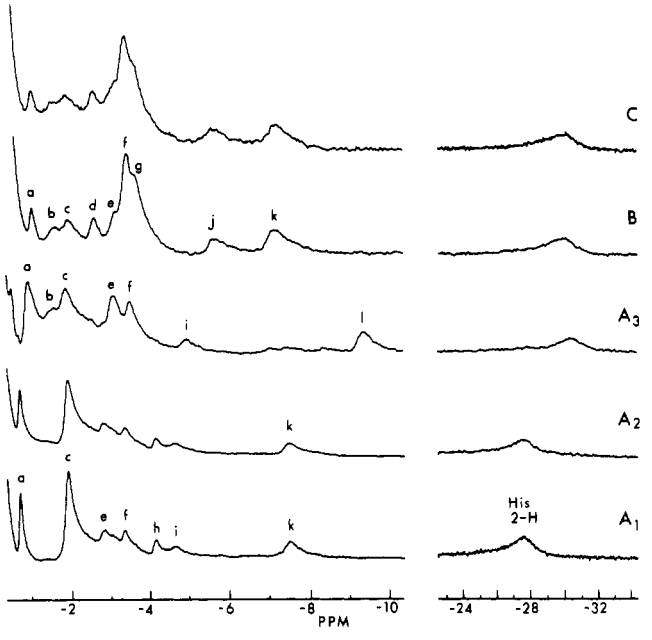

FIGURE 5: Upfield proton NMR spectra of ferric cyano-HRP isoenzymes. Conditions: 25 °C, D_2O solution, 0.10 M phosphate buffer, pD 6.6, KCN concentration typically 10 mM, enzyme concentrations 0.5–2.0 mM, DSS reference.

spectra. However, significant spectral differences are apparent for CN-HRP B/C as compared with the A isoenzymes. A survey of the downfield region in Figure 4 reveals a greater spread of heme signals for the B/C isoenzymes. This is most apparent for the 8,3-position ring methyl signals located at 30.9 and 26.0 ppm in CN-HRP B/C. This chemical shift spread of 4.9 ppm is to be compared with values of 2.5 and 2.7 ppm for respective downfield ring methyl signals of CN-HRP A_1/A_2 and A_3. Other downfield signals in the 25–15 ppm region are also spread over a considerably larger chemical shift range for CN-HRP B/C. A similar pattern of heme methyl chemical shift values is also apparent for the acidic P_1, P_2, and P_3 turnip peroxidase isoenzymes as compared with the CN-HRP B/C complex (Williams et al., 1975). Conservation of the heme region amino acid sequences of acidic root peroxidases is thus suggested.

Upfield proton NMR traces for CN-HRP complexes are included in Figure 5. Assignment of the proximal histidyl 2*H*-imidazole signal at −30 ppm has previously been offered for CN-HRP B/C (La Mar et al., 1982a). It is reasonably assumed that the broad signal in the −27 to −30 ppm region is from the 2*H*-imidazole proton in all cyanoisoenzymes. On the basis of model compound studies, the magnitude of the upfield shift for the proximal 2*H*-imidazole signal is qualitatively correlated with the degree of proximal imidazole hydrogen bonding. In this regard, the slight downfield shift of CN-HRP A_1/A_2 2*H*-imidazole signals to −27.7 ppm could be taken to imply a small but measurably diminished role for proximal ligand hydrogen bonding.

Porphyrin $-CH_2$ vinyl and $-CH_2$ propionate assignments in the −1.5 to −3.1 ppm spectral region of CN-HRP B/C have been made unambiguously by selective deuterium labeling of reconstituted iron(III) protoporphyrin (de Ropp et al., 1984). A corresponding set of upfield resonances in the −1.5 to −3.1 ppm region is not evident for CN-HRP A_1/A_2, but multiple signals are seen in the −2.8 to −4.2 ppm region. In this regard, it should be noted that a 4-vinyl CH_2 resonance is seen at −4.0 ppm in the ferric cyanocytochrome *c* peroxidase spectrum (Satterlee et al., 1983b). It is not clear as to whether vinyl signals are shifted further upfield or downfield for CN-HRP A_1/A_2, but significant perturbation is clearly evident among the isoenzymes. The differences could reasonably reflect varying degrees of vinyl group coplanarity with the porphyrin ring, as evidence has been presented favoring preferential orientation of porphyrin 2,4-vinyl substituents through the steric clamping mechanism (La Mar et al., 1983b).

Additional upfield signals observed in the proton NMR spectra of CN-HRP B/C do not represent those of methine or $-CH_2$ propionate residues (de Ropp et al., 1984) and thus must be due to amino acid substituents. Significant differences in the −0.5 to −10 ppm region for the native and cyano-complexed B/C, A_3, and A_1/A_2 isoenzymes infer substantial variation in protein conformation and/or amino acid composition near the heme center. Previous sequence studies conducted for HRP C and for the assumed proximal and distal histidine peptide regions of turnip peroxidase P_1, P_2, P_3, and P_7 isoenzymes show a remarkable degree of homology among the root peroxidases (Welinder & Mazza, 1977). Seven invariant amino acids are found in the proximal histidine region, and 11 invariant residues occur in the putative distal region of acidic turnip peroxidases and HRP C. However, the near-upfield proton NMR spectra for the cyano complexes are quite diverse (Williams et al., 1975). Important structural and functional roles for amino acids well removed from the proximal ligand and distal residues are suggested.

Acid–Base Dependence. Previously reported proton NMR spectra of native HRP B/C exhibit only subtle changes over the range from pD 5.5 to 10.5. signals broaden in acidic solution, and heme methyl chemical shifts change by as much as 1.0 ppm with an inflection centered at pD 5.9 (Morishima et al., 1977). A dramatic transition centered at pD 10.9 also occurs in which the proton NMR spectrum is converted from that of a high-spin to that of a low-spin iron(III) hemoprotein (Morishima et al., 1977; de Ropp et al., 1984). An analogous

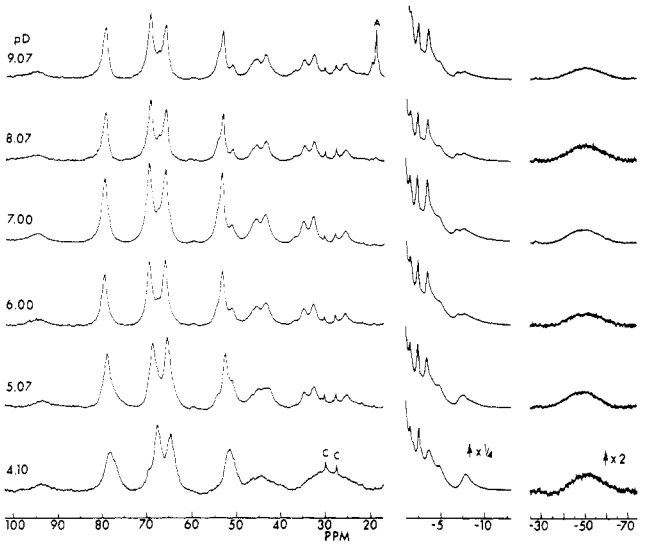

FIGURE 6: Proton NMR spectra of ferric native HRP A isoenzyme mixture (predominantly HRP A_1) at various pD values. Conditions: 25 °C, D_2O solution, 0.10 M phosphate buffer, 1 mM enzyme, signals referenced to DSS. Signals labeled C are from ring methyl resonances of cyano-HRP as a consequence of trace cyanide contamination.

Table V: Comparative Properties of Native HRP Isoenzymes

isoenzyme	peroxidase sp act.[a]	K_M[b] (mM)	pD of alkaline transition	proton NMR spectra
A_1	2.2	16.0	9.3 ± 0.1	identical
A_2	2.3	18.8	9.4 ± 0.1	
A_3	0.9	3.8	9.8 ± 0.1	
B	0.1	1.7		identical
C	0.1	1.6	10.9[c]	

[a] Moles of H_2O_2 utilized per minute per micromole of enzyme for *o*-dianisidine oxidation (Kay et al., 1967). [b] For H_2O_2 with *o*-dianisidine oxidation (Kay et al., 1967). [c] de Ropp et al. (1984).

acid–base dependence study is summarized in Figure 6 for a mixture of native HRP A isoenzymes (predominantly HRP A_1). Broadening of resonances is evident for the most acidic solution examined. Small chemical shift changes are also apparent in the low-pD region, but fit of the available data to a single-proton (deuteron) transition is not possible. Significantly, the far-downfield proximal histidyl imidazole N–H resonances are unchanged over the displayed pD range, thus indicating that the native histidyl–iron(III) adduct remains as the major species.

At more alkaline pD values, striking spectral changes occur. For the pD 9.07 spectrum in Figure 6, an intense new peak (labeled A) is noted at 18.8 ppm. The appearance of this signal and others at 19.6, 18.3, 14.9, 14.5, 11.72 (heme methyl?), and −3.5 (heme methyl?) ppm is reminiscent of the conversion to the low-spin "alkaline" HRP B/C spectrum. Assuming that the 18.8 ppm signal represents a single heme methyl resonance, examination of a separate HRP A_1 sample over the narrow range from pD 8.75 to 10.20 (not shown in Figure 6) allowed calculation of a pD 9.3 ± 0.1 alkaline transition. The process was shown to be reversible and to involve a single proton. Relative intensity ratios for pure HRP A_2 and A_3 in this pD region revealed alkaline transitions centered respectively at pD 9.4 and 9.8. The value for HRP A_2 is equivalent to the pH 9.3 transition measured for HRP A_2 redox potential changes (Hayashi & Yamazaki, 1978, 1979) and is to be contrasted with the pH 10.9 transition measured by NMR and potentiometric methods for HRP C. Table V provides a comparative summary of HRP isoenzyme catalytic and acid–base properties. A correlation clearly exists

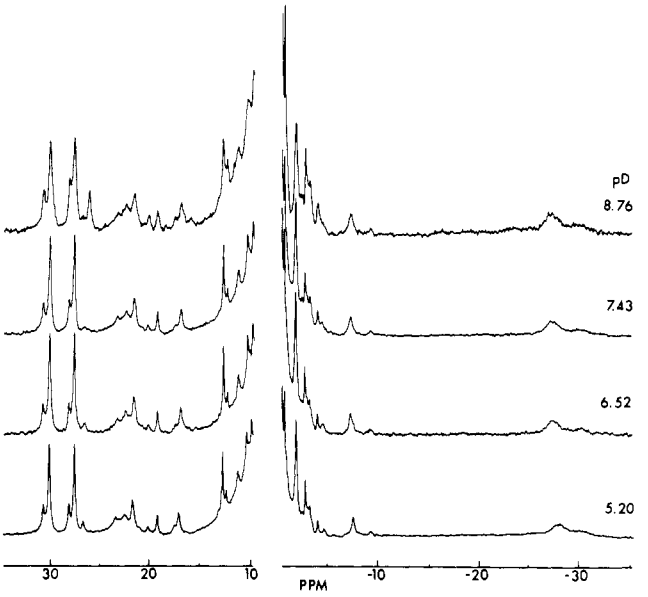

FIGURE 7: Proton NMR spectra of ferric cyano-HRP A isoenzyme mixture (predominantly HRP A_1) at various pD values. Conditions: 25 °C, D_2O solution, 0.10 M phosphate buffer, 1 mM enzyme, 10 mM KCN, signals referenced to DSS.

between specific activities, H_2O_2 K_M values, and the alkaline transition pD values. Conversion to the low-spin state in basic solution presumably is associated with deprotonation and coordination of a distal nitrogenous amino acid ligand (de Ropp et al., 1984). This residue must be intimately associated with peroxide activation. The conformational differences and/or amino acid substitutions responsible for distinctive proton NMR spectra among HRP isoenzymes must also dictate the appreciably lower pH alkaline transition in HRP A isoenzymes.

Figure 7 demonstrates that proton NMR spectra of (predominantly) CN-HRP A_1 are quite independent of pH (pD) in the physiological range. Broadening is apparent at elevated pH. At the highest pH shown, a new signal is clearly noted at 25.8 ppm. On the basis of earlier NMR examination of CN-HRP B/C (Morishima et al., 1977; de Ropp et al., 1984), it is reasonable that this resonance represents a heme ring methyl signal in the alkaline form of CN-HRP A_1. The HRP A_1 isoenzyme thus appears to behave in a manner analogous to that of the HRP B/C isoenzymes in that an alkaline transition occurs at approximately the same pH values for ferricyano and native forms (of a given isoenzyme). Less basic conditions are required to produce alkaline CN-HRP A_1 (midpoint ca. pD 9.5) as compared with alkaline CN-HRP B/C (pD 10.6). It should be noted that the high-pH form of native HRP A_1 with a distinctive heme methyl signal at 18.8 ppm must have a different coordination environment than that of alkaline CN-HRP A_1 which shows a signal at 25.8 ppm. Retention of a cyano ligand at elevated pH is thus suggested.

Conclusions

Given the sensitivity of iron porphyrin hyperfine NMR shifts to subtle changes in ligation and environment, it is not surprising that spectral differences occur for HRP isoenzymes. Chemical shift perturbation for heme protons is likely dictated in part by protein interaction with the porphyrin 2-vinyl side chain. Upfield region signal patterns are highly distinctive and must also reflect substitution of amino acids in the heme pocket of isoenzymes, or conformational changes near the heme induced by substitutions elsewhere in the polypeptides. Proton NMR spectra are identical for the A_1/A_2 and B/C sets

of isoenzymes despite measurable differences in amino acid and sugar composition within a given set (Shannon et al., 1966; Shih et al., 1971). Of particular significance is the fact that specific activities and alkaline pH transitions within the A_1/A_2 and B/C sets are also equivalent (Kay et al., 1967). Thus, changes in the hyperfine NMR spectra correlate with changes in the catalytic activity of the various isoenzymes. In other words, the hyperfine NMR spectrum is sensitive to those modifications of hemoprotein structure that modulate peroxidase activity. This important empirical realization provides the basis for understanding variations in hemoprotein biocatalytic properties at the atomic level once complete NMR assignments (particularly of upfield protein signals) are available.

Registry No. Peroxidase, 9003-99-0.

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