Spin State and Axial Ligand Bonding in the Hydroxide Complexes of Metmyoglobin, Methemoglobin, and Horseradish Peroxidase at Room and Low Temperatures†

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ABSTRACT: Absorption and resonance Raman spectra using Soret excitation of alkaline metmyoglobin (metMb), methemoglobin (metHb), and horseradish peroxidase (HRP) were obtained at room and low temperature. At 298 K both metMb and metHb exhibit two isotope-sensitive bands assigned to high- and low-spin ν(Fe–OH) stretching modes, respectively, which are correlated with the spin-state population. The low-spin stretch occurs 60 cm^{-1} to higher energy than the corresponding high-spin vibration. When the temperature is lowered, only the low-spin species is observed. HRP exhibits at both 298 and 20 K only the low-spin ν(Fe–OH) stretching mode, which occurs 50 cm^{-1} to lower energy than the corresponding modes observed in the globins. This is explained in the context of a strong hydrogen bond between the hydroxyl ligand and the distal His42 and/or Arg38. Lowering temperature causes in all of the examined proteins a strengthening of the Fe–OH bond and a contraction of the core of about 0.01 Å, as determined by the upshifting of the low-spin ν(Fe–OH) stretching mode and the core size marker bands. Both effects are ascribed to an increase of the packing forces.

Heme proteins such as globins, cytochromes, catalases, and peroxidases can bind a wide range of small molecules to their Fe atom. Ligand binding induces changes in the Fe spin state, in the porphyrin structure, and in the protein conformation, which are relevant for the study of the biological function of these heme proteins. Metmyoglobin (metMb)[1] (Asher & Schuster, 1979; Desbois et al., 1979), methemoglobin (metHb) (Asher et al., 1977), and horseradish peroxidase (HRP) (Foote et al., 1987; Sitter et al., 1988) bind a hydroxyl group at alkaline pH. The alkaline forms of metMb and metHb have magnetic susceptibility (Beetlestone & George, 1964) and resonance Raman (RR) spectra (Asher & Schuster, 1979; Desbois et al., 1979; Asher et al., 1977) intermediate between those of high- and low-spin heme, these being the two species in a thermal spin-state equilibrium (Beetlestone & George, 1964; George et al., 1964). In contrast, HRP is characterized by an almost complete low-spin heme (Sitter et al., 1988).

The RR high-spin iron–hydroxide stretching modes at room temperature in alkaline metMb and metHb have been identified (Asher & Schuster, 1979; Desbois et al., 1979; Asher et al., 1977), as has the low-spin iron–hydroxide mode in alkaline HRP (Sitter et al., 1988). On the contrary, no iron–axial ligand modes have been observed for the low-spin species of the alkaline forms of the globins. Data reported by Sitter et al. (1988) showed that the ν(Fe–OH) stretching mode of the low-spin HRP occurs at nearly the same frequency as for the high-spin ν(Fe–OH) of metMb and metHb, raising some doubts (Rodgers et al., 1992) about its origin, which could be from the 7% high-spin species present in solution (George et al., 1964).

In this paper we report the identification of the high-spin and low-spin Fe–OH stretching modes in metMb and metHb and the low-spin Fe–OH stretching mode in HRP by isotopic substitution with ^{18}O and ^{2}H, obtained at both room and low temperature (20 K). The low-spin ν(Fe–OH) stretching mode in the globins is about 65 cm^{-1} higher than the corresponding mode found for the high-spin form, whereas the low-spin ν(Fe–OH) stretching mode in HRP has a frequency which is about 15 cm^{-1} higher than the corresponding high-spin mode of the globins, but about 50 cm^{-1} downshifted with respect to their low-spin forms.

MATERIALS AND METHODS

D_2O (99.9% D), DCl (99.5% D), and NaOD (99% D) were purchased from Aldrich; $H_2{}^{18}O$ (95% ^{18}O) was purchased from Iso-Yeda Co. Ltd.

Horse heart myoglobin (metMb) and horseradish peroxidase (HRP-C, type VI-A) were purchased from Sigma and used without further purification. Human oxyhemoglobin (Hb-O_2) was a generous gift from Professor M. Coletta (Department of Molecular, Cellular, and Animal Biology, Camerino University). Methemoglobin (metHb) at alkaline pH was prepared by oxidation of Hb-O_2 with excess potassium ferricyanide and purified on a CM-50-Sephadex column previously equilibrated and eluted with borate buffer at pH 10.4.

The OD^- derivative of metHb was prepared by freeze-drying the buffered alkaline solution and redissolving the freeze-dried material in D_2O. The hydroxide complex of metMb was prepared by dissolving the proteins in 0.025 M borate buffer solution near the desired pH value and then adjusting the pH to the final value by slowly titrating with NaOH or NaOD and using rapid stirring. The hydroxide complex of HRP was obtained by dissolving the protein in diluted NaOH or NaOD (pH 12; pD 12.5) solution. The $H_2{}^{18}O$ samples of metMb and HRP were prepared in the same way as the sample in D_2O, redissolving the proteins in $H_2{}^{18}O$ borate buffer.

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[1] Abbreviations: metMb, metmyoglobin; metHb, methemoglobin; oxyHb, oxyhemoglobin; HRP, horseradish peroxidase isoenzyme C; CCP, cytochrome *c* peroxidase; RR, resonance Raman; 6-c, 6-coordinate heme; PP, protoporphyrin IX; HS and LS, high and low spin, respectively.

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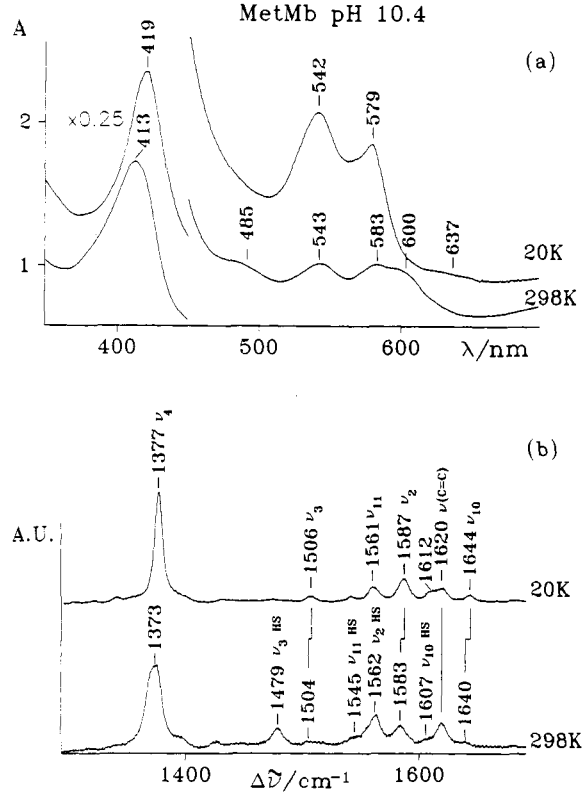

FIGURE 1: (a) Absorption spectra of alkaline metMb in 0.025 M borate buffer, pH 10.4, taken at 298 and 20 K. (b) High-frequency-region resonance Raman spectra of alkaline metMb in 0.025 M borate buffer, pH 10.4, at 298 and 20 K. Experimental conditions: λ_{exc}, 413.1 nm; laser power, 15 mW at the sample; resolution, 5 cm^{-1}; collection interval, 3 s/0.5 cm^{-1}. HS indicates the bands that are due to the high-spin heme; all the others are due to the low-spin species.

Table 1: Resonance Raman Frequencies (cm^{-1}) of the Core Size Marker Bands for the Low-Spin Hemes Observed at Room and Low Temperature for Alkaline MetMb, MetHb, and HRP Compared with Those Obtained for the Low-Spin Model Compound $(ImH)_2PPFe^{III}$ [a]

	MetMb		MetHb		HRP		$(ImH)_2PPFe^{III}$,
	298 K	20 K	298 K	20 K	298 K	20 K	298 K
ν_3	1504	1506	1503	1509	1506	1511	1502
ν_{11}	*b*	1561	1564	1568	1565	1568	1562
ν_2	1583[c]	1587	1584	1588	1584	1590	1579
ν_{10}	1640	1644	1636	1644	1640	1645	1640

[a] Choi et al. (1982). [b] Overlapped with the bands due to the high-spin species. [c] Overlapped with ν_{38} of the high-spin species.

Heme concentrations for the resonance Raman (RR) experiments were typically 10^{-4} M, whereas for the absorption spectra the samples were diluted about 10-fold.

The RR spectra were obtained with excitation from the 413.1-nm line of a Kr ion laser (Coherent). The back-scattered light from a slowly rotating NMR tube was collected and focused into a computer-controlled double monochromator (Jobin-Yvon HG2S) equipped with a cooled photomultiplier (RCA C31034 A) and photon-counting electronics. The RR spectra were calibrated with indene as a standard for the high-frequency region and with indene and CCl_4 for the low-frequency region. The frequencies were accurate to ± 1 cm^{-1} for the intense isolated bands.

The absorption spectra were recorded with a Cary 5 spectrophotometer.

Low temperature was obtained using a closed-cycle He cryotip with an automatic temperature control. The RR

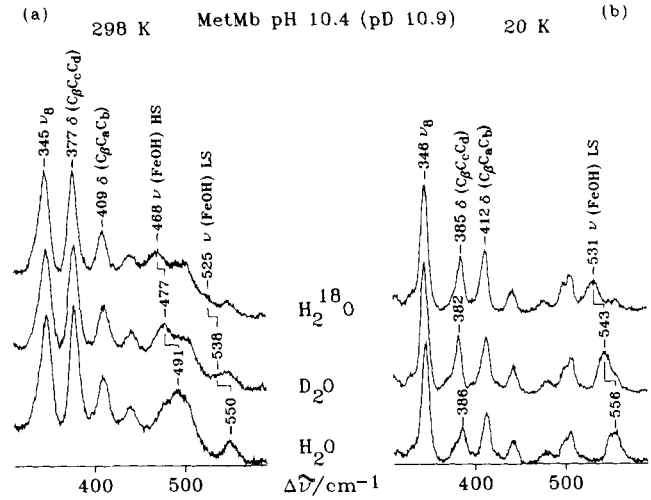

FIGURE 2: Low-frequency-region resonance Raman spectra of alkaline metMb in 0.025 M borate buffer, pH 10.4 or pD 10.9, at 298 K (a) and 20 K (b), obtained in H_2O, D_2O, and $H_2{}^{18}O$. Experimental conditions are as given in the caption to Figure 1, except a 6 s/0.5 cm^{-1} collection interval was used.

spectra were measured after transferring the protein solutions with a syringe to a small groove in the copper cold finger of the cryostat at 180 K under a nitrogen flow. The temperature was then slowly decreased to 20 K under vacuum, and RR spectra were obtained at this temperature.

The absorption spectra at low temperature were obtained using a sealed quartz microcuvette (20 μL, 0.25 cm). The samples were transferred to the cuvette at room temperature, and the sealed cuvette was mounted on the cold finger of the cryotip. The temperature was then slowly decreased under vacuum. The same apparatus was also used to obtain the RR spectra. Identical results were obtained in the high-frequency region using the two methods. The low-frequency region showed a background due to the thick quartz windows.

RESULTS

Metmyoglobin. Figure 1a shows the absorption spectra of metMb at alkaline pH obtained at 298 and 20 K. With decreasing temperature the Soret band shifted from 413 to 419 nm, and the bands at 485 and 600 nm, which have been assigned to charge-transfer bands of the high-spin complex (Makinen & Churg, 1983; Asher & Schuster, 1979), disappeared completely. A new weak band was detected at 637 nm, and small blue shifts were observed for the α and β bands. These results indicate that the hydroxide derivative of metMb, which exists in a thermal spin-state equilibrium at room temperature with a 30:70 mixture of low- and high-spin species (Beetlestone & George, 1964; Asher & Schuster, 1979), has been converted to a completely low-spin heme at 20 K. In agreement with this finding, the RR spectrum at 20 K (Figure 1b) showed the characteristic porphyrin vibrational frequencies (Table 1) for a low-spin six-coordinate heme (Choi et al., 1982). The transition from the high- to the low-spin heme is gradual and reversible as detected by the absorption spectra at intermediate temperatures. In particular the intensity of the 600-nm band gradually decreased as the temperature was lowered (data not shown).

Figure 2a shows the RR spectra excited at 413.1 nm in the 350–650-cm^{-1} region of metMb at alkaline pH in H_2O, D_2O, and $H_2{}^{18}O$ buffered solutions at 298 K. The isotopic substitution affected the frequencies of the two bands at 491 and 550 cm^{-1}, which were downshifted by 14 and 12 cm^{-1} in

Table 2: Resonance Raman Frequencies (cm^{-1}) of the ν(Fe–OH) Stretching Modes of Alkaline MetMb, MetHb, and HRP Obtained in H_2O, D_2O, and $H_2{}^{18}O$ at 298 and 20 K[a]

	MetMb			MetHb			HRP	
	298 K		20 K,	298 K		20 K,	298 K,[b]	20 K,
	HS	LS	LS	HS	LS	LS	LS	LS
H_2O	491	550	556	492	553	558	503	507
D_2O	477	538	543	479	544	547	509	511
$H_2{}^{18}O$	468	525	531				484	492

[a] HS, high spin; LS, low spin. [b] Sitter et al. (1988).

D_2O, and by 23 and 25 cm^{-1} in $H_2{}^{18}O$, respectively. The frequencies of the overlapped porphyrin bands at about 500 and 550 cm^{-1}, did not change. The corresponding spectra at 20 K are shown in Figure 2b. Most bands were upshifted by 2–9 cm^{-1} and changed in intensity at low temperature (namely, the bands at 377, 409, 439, and 501 cm^{-1} at room temperature). The most striking feature, however, is the disappearance of the isotope-sensitive band in the 490-cm^{-1} region and the concomitant increase of the band at 556 cm^{-1} in H_2O, which was downshifted by 13 and 25 cm^{-1} in D_2O and $H_2{}^{18}O$, respectively (Table 2). In addition the band at 386 cm^{-1} shifted to 382 cm^{-1} in D_2O. The band at 490 cm^{-1} was previously assigned to the high-spin Fe–OH stretching mode (Desbois et al., 1979; Asher et al., 1979). The strong isotope-sensitive band at 550 cm^{-1}, which shifted to 556 cm^{-1} at low temperature, is therefore assigned to the corresponding low-spin Fe–OH stretch. It is interesting to note that both bands appeared in the spectrum at room temperature at 491 and 550 cm^{-1} in agreement with the existence of the thermal spin-state equilibrium between the high- and low-spin species.

Methemoglobin. The hydroxide complex of metHb also exists in a spin-equilibrium at room temperature, but the low-spin species is favored (55:45 mixture of low- and high-spin species) (George et al., 1964) as compared to metMb. Lowering the temperature shifted the equilibrium completely to the low-spin species as shown from the absorption spectra of alkaline metHb at 298 and 20 K (Figure 3a) . The band pattern at room temperature is very similar to that of metMb, except for the longest wavelength band at about 600 nm, which is decreased in intensity. At 20 K both of the shoulders at 482 and 600 nm which are due to charge- transfer bands of the high-spin complex disappeared, and a new weak band appeared at 635 nm. The RR spectrum in the high-frequency region of hydroxy-metHb at room temperature (Figure 3b) displayed a complex series of overlapping bands assigned to both high- and low-spin hemes (Choi et al., 1982). In agreement with the absorption spectrum at low temperature, the corresponding RR spectrum showed the presence of almost complete low-spin heme with the porphyrin marker bands at higher frequency than the corresponding bands observed at room temperature (Table 1).

The low-frequency-region RR spectra of metHb at room and low temperature in H_2O and D_2O are shown in Figure 4. At room temperature two isotope-sensitive bands were detected at 492 and 553 cm^{-1}. They downshifted to 479 and 544 cm^{-1} in D_2O, respectively, and were therefore assigned to the high-spin (Asher et al., 1977) and low-spin Fe–OH stretching modes, respectively. Asher and Schuster (1979) localized the high-spin Fe–OH stretch at 495 cm^{-1} by using a 600-nm exciting line. When the temperature was lowered only one isotope-sensitive band was detected at 558 cm^{-1}, which downshifted by 11 cm^{-1} in D_2O and is therefore assigned to the low-spin ν(Fe–OH) stretching mode (Table 2). Additional effects have been observed at both room and low temperature

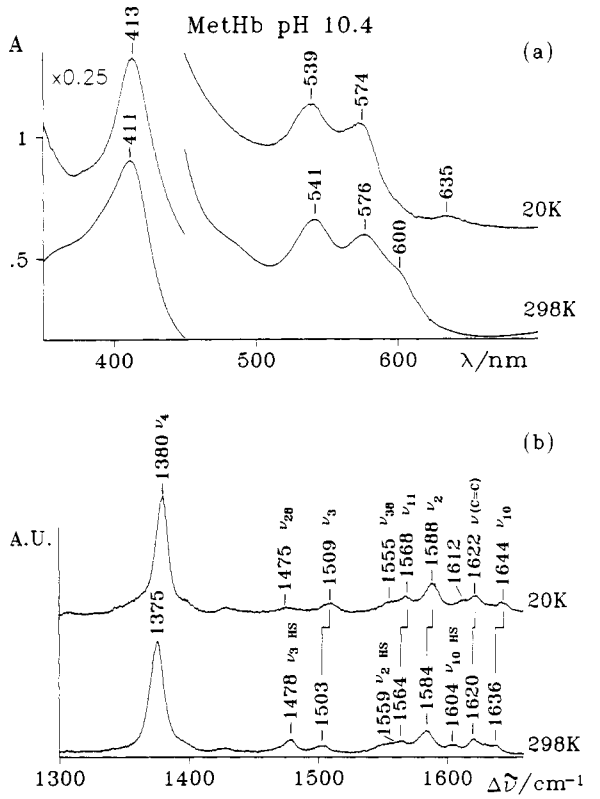

FIGURE 3: (a) Absorption spectra of alkaline metHb in 0.025 M borate buffer, pH 10.4, at 298 and 20 K. (b) High-frequency-region resonance Raman spectra of alkaline metHb in 0.025 M borate buffer, pH 10.4, at 298 and 20 K. Experimental conditions are as given in the caption to Figure 1. HS indicates the bands that are due to the high-spin heme; all the others are due to the low-spin species.

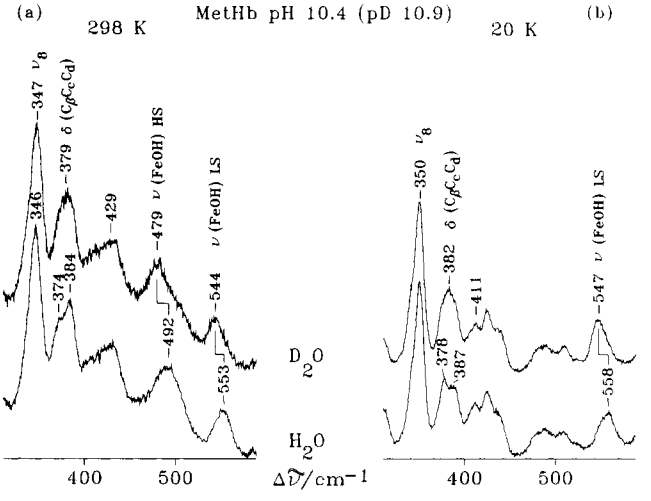

FIGURE 4: Low-frequency-region resonance Raman spectra of alkaline metHb at 298 K (a) and 20 K (b), obtained in H_2O and in D_2O in 0.025 M borate buffer, pH 10.4 or pD 10.9. Experimental conditions are as given in the caption to Figure 1, except a 6 s/0.5 cm^{-1} collection interval was used.

when the samples are dissolved in D_2O. The band at 384 cm^{-1} was downshifted to 379 cm^{-1} at room temperature, whereas at 20 K the band shifted from 387 to 382 cm^{-1}.

Horseradish Peroxidase. The absorption spectra of HRP at alkaline pH were recorded at 298 and 20 K (Figure 5a). With lowering temperature the shoulder at about 605 nm disappeared and the α and β bands shifted down by 2 nm. A weak band at 638 nm was present at both temperatures. No changes were observed in the coordination and spin states at room and low temperature. The corresponding RR spectra

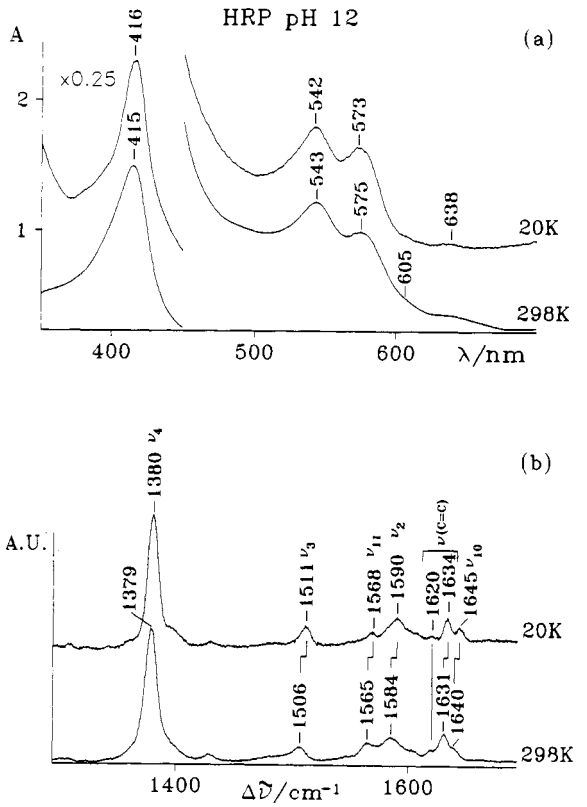

FIGURE 5: (a) Absorption spectra of alkaline HRP in NaOH, pH 12, at 298 and 20 K. (b) High-frequency-region resonance Raman spectra of alkaline HRP inNaOH, pH 12, at 298 and 20 K. Experimental conditions are as given in the caption to Figure 1.

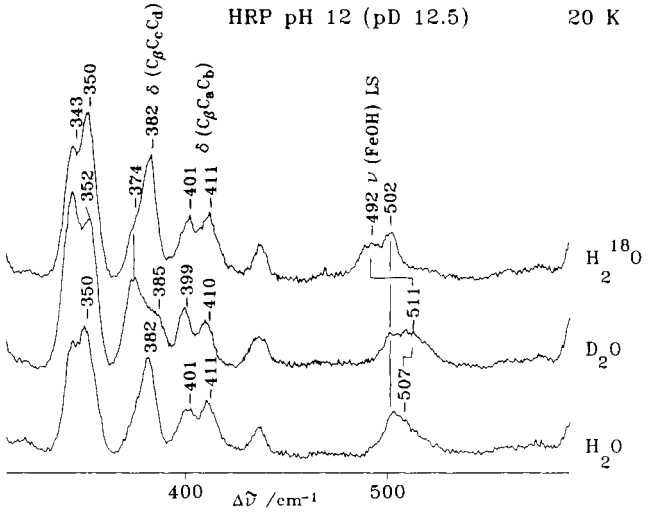

FIGURE 6: Low-frequency-region resonance Raman spectra of alkaline HRP at 20 K obtained in H_2O, D_2O and $H_2{}^{18}O$, with NaOH, pH 12, or NaOD, pD 12.5. Experimental conditions are as given in the caption to Figure 1, except a 6 s/0.5 cm^{-1} collection interval was used.

in the high-frequency region are shown in Figure 5b. Both the RR and absorption spectra are characteristic of mainly low-spin heme. The lower temperature caused an overall upshift by 3–6 cm^{-1} of the RR core size marker bands (Table 1), and of the ν(C=C) band at 1631 cm^{-1} by 3 cm^{-1}.

Figure 6 shows the low-frequency-region RR spectra of HRP at pH 12 in H_2O, D_2O, and $H_2{}^{18}O$ buffered solutions at 20 K. The shoulder observed at 507 cm^{-1} shifted to lower wavenumbers by 15 cm^{-1} in $H_2{}^{18}O$, but upshifted by 4 cm^{-1} in D_2O (Table 2). Furthermore, in the deuterated sample the band at 382 cm^{-1} was split into two bands at 385 and 374 cm^{-1}, and the bands at 350 and 411 cm^{-1} decreased in intensity. Similar results have been obtained by Sitter et al. (1988) on alkaline HRP at room temperature. These authors assigned the isotope-sensitive band at 503 cm^{-1} to a low-spin Fe–OH stretch which therefore corresponds to the shoulder observed at 507 cm^{-1} in the low-temperature spectrum.

DISCUSSION

ν(Fe–OH) Stretching Modes. The hydroxide complexes of metMb and metHb exist at room temperature in thermal spin-state equilibrium between high- and low-spin species. At room temperature the high spin is favored, albeit with a different ratio for the two proteins. The present experiments showed that at low temperature the proteins are converted to exclusively low-spin heme. At room temperature two ν(Fe–OH) stretching modes were observed, at about 490 and 550 cm^{-1} for the high- and low-spin species, respectively. At low temperature only the low-spin Fe–OH exists, and its frequency is upshifted by about 5 cm^{-1} with respect to the corresponding band observed at room temperature (see below). The frequency is lower for the high-spin than for the low-spin complex due to partial occupation and partial antibonding character of the $d_z{}^2$ orbital in the high-spin case.

At room temperature alkaline HRP is essentially low-spin, the high-spin species accounting for about 7% (George et al., 1964). Sitter et al. (1988) have identified the low-spin ν(Fe–OH) at 503 cm^{-1}. Accordingly, with decreasing temperature no relevant changes were observed in either the absorption or the RR spectra which could be ascribed to a spin-state change. The ν(Fe–OH) is assigned to the shoulder at about 507 cm^{-1} on the basis of the isotopic shifts. Hence HRP shows a low-spin ν(Fe–OH) stretching mode at much lower frequency and weaker intensity than the corresponding bands observed for metMb and metHb.

Recently Rodgers et al. (1992) identified two Fe–OH stretching modes in the Raman spectra of a model heme complex. The two bands were assigned to a low- and a high-spin species in thermal equilibrium. The low-spin species was the dominant species at 10 ° C; the high-spin population increased as the temperature increased. On the basis of the low-spin force constants for their model complex, Rodgers et al. have predicted the appearance of a low-spin ν(Fe–OH) stretching mode of a protein–hydroxide ion adduct near 550 cm^{-1}. This is in perfect agreement with the frequency of the bands we found for the low-spin complexes of metMb and metHb. Furthermore, the observed downshifts by 11–13 cm^{-1} (for metMb and metHb) and 25 cm^{-1} (for metMb) upon ^2H and ^{18}O isotopic substitution, respectively, are in perfect agreement with the biatomic oscillator Fe–OH model. The 50 cm^{-1} downshift in frequency, the strong intensity decrease, the 15 cm^{-1} downshift upon ^{18}O isotopic substitution, and the 4 cm^{-1} upshift upon ^2H isotopic substitution of the low-spin ν(Fe–OH) stretching mode in HRP are most likely due to a strong H-bond between the OH anion ligand and a distal residue. In fact, with an increase of the H-bond strength, a decrease of the force constant of the Fe-O bond together with a loss of its biatomic oscillator character (via vibrational coupling with the Fe–OH bending and torsion and the O···HX stretch modes) is expected. In addition the strength of the H-bond is predicted to weaken upon deuterium substitution (Saitoh et al., 1982). As a consequence, the Fe–OD stretch can experience a lesser downshift or even an upshift compared with the corresponding Fe–OH stretch, despite the increasing mass.

A downshift of the Fe–F stretching mode has been observed for the fluoride complexes as a result of the H-bonding interaction. The Fe–F stretching vibration in both metMb-F and metHb-F downshifted by 60 cm^{-1} at low pH compared to those at neutral pH, as a result of protonation of the distal histidine and an accompanying formation of a hydrogen bond to the F^- anion, thereby decreasing the Fe–F force constant (Asher et al., 1981). In the HRP-F complex at neutral pH the ν(Fe–F) stretching frequency has been detected at 385 cm^{-1} (Yu, 1986), 86 cm^{-1} lower than the one observed for metMb-F. In this case a strong hydrogen bond between the distal histidine and F^- has been postulated (Schonbaum et al., 1979).

Sitter et al. (1988) suggested the presence of an iron hydroxyl group that donates a hydrogen bond to the distal histidine in HRP. In fact this interaction would give the hydroxyl ligand partial oxo character resulting in the higher Fe–OH frequency than in high-spin alkaline metMb and metHb. On the other hand the presence of a low-spin Fe–OH around 550 cm^{-1} for these latter proteins clearly indicates the occurrence of a downshift of the Fe–OH stretching mode in HRP and not an upshift. Such a downshift could be induced by a strong H-bond between the oxygen atom of the hydroxyl group and a protonated residue. Therefore, although a hydrogen bond between HRP distal His42 and the bound hydroxyl ligand cannot be ruled out, the presence of a distal Arg (Arg38) (Welinder, 1985) in HRP means that the possibility of a strong H-bond between the partially negatively charged oxygen of the bonded hydroxyl with the positively charged guanidinium group of Arg38, i.e., that the hydroxyl group acts as a H-bond acceptor, also must be considered.

Plant peroxidases and cytochrome *c* peroxidase (CCP) are homologous, and it has been shown that in CCP the distal Arg is highly movable and can optimize its interaction with ligands. In particular, in the CCP-F complex the Arg48 guanidinium group moves about 2.5 Å toward the ligand to form a hydrogen bond or an ion pair with fluoride (Edwards & Poulos, 1990). No RR data are available for the CCP-F complex, but the fact that the distal catalytic His52 and Arg48 (His42 and Arg38 in HRP) are invariant and characteristic for plant peroxidases (Welinder, 1992) makes it likely that a similar mechanism is in operation in the two peroxidases upon complex formation with the F^- and OH^- ligands. The ligand stabilization by Arg48 plays an important role in the catalytic mechanism. It has been proposed that in the first step of the reaction cycle the bound ROO^- donates its proton to the unprotonated His52 and is stabilized by the positive charge of the guanidinium group of Arg48 and the formation of a H-bond to the ferryl oxygen atom (Edwards et al., 1987; Fulop et al., 1993). Therefore, ligand stabilization by Arg48 is coupled to His52 protonation. A second role of Arg, besides facilitating the heterolysis of the O–O bond, might be to maintain the His52 unprotonated in the protein as a result of the positive charge of the guanidinium group. The distal His operates as an acid–base catalyst by directing the transfer of the peroxide proton.

Isotopic Effects in the Low-Frequency Heme Modes. In alkaline metMb at room temperature no band shows isotopic sensitivity besides the ν(Fe–OH) stretching modes. On the contrary, the spectra of metMb at low temperature in D_2O clearly showed a downshift by 4 cm^{-1} of the band observed at 382 cm^{-1} in H_2O. This band corresponds to the band at 377 cm^{-1} observed at room temperature and assigned to the $\delta(C_\beta C_c C_d)$ propionate bending modes via propionate deuteration (S. Hu and T. G. Spiro, private communication). A similar effect was observed in metHb in D_2O at both room temperature, where the band at 384 cm^{-1} shifted to 379 cm^{-1}, and low temperature, where the band at 387 cm^{-1} shifted to 382 cm^{-1}. These bands could be assigned to the $\delta(C_\beta C_c C_d)$ propionate bending mode.

The propionate groups are hydrogen-bonded to adjacent side chains in both metMb (Evans & Brayer, 1990) and metHb (Ladner et al., 1977). Hence, an explanation for the frequency shift upon H/D exchange could involve an alteration in the structure of the hydrogen-bonded propionate groups induced by the different strengths of hydrogen and deuterium bonds. Anyway, it must be considered that the downshift upon deuteration was not observed in metMb at room temperature, where Fe–OH is essentially high-spin, and seems to be correlated with the occurrence of low-spin Fe–OH. Therefore, the $\delta(C_\beta C_c C_d)$ propionate bending modes might be coupled with a mode of the low-spin Fe–OH group.

Major spectral changes occur in HRP at alkaline pH upon deuterium substitution at both room (Sitter et al., 1988) and low temperature. The band at 382 cm^{-1} (381 cm^{-1} at room temperature) splits into two bands at 374 and 385 cm^{-1} (379 and 385 cm^{-1} at room temperature). A D_2O experiment with HRP isoenzyme A1 at room temperature gave similar results (Sitter et al., 1988). Moreover, in HRP at neutral pH, where the OH group is not present, the 381 cm^{-1} band loses its deuteration sensitivity (Sitter et al., 1988).

In HRP, H/D exchange also causes a small frequency downshift and a change in the relative intensity of the two bands at 401 and 411 cm^{-1} (20 K) (399 and 412 cm^{-1}, respectively, at room temperature). The assignment of the bands in this region is not yet established for HRP. For metMb the vibrational bending modes associated with the 2- and 4-vinyl groups give rise to bands near 410 cm^{-1} (Uchida et al., 1988). In CCP at pH 6 the strong band at 406 cm^{-1} was recently assigned to the vinyl bending $\delta(C_\beta C_a C_b)$ mode from its large 2,4-di(β-d_2) shift (G. Smulevich, S. Hu, K. R. Rodgers, D. B. Goodin, K. M. Smith, and T. G. Spiro, unpublished results). Although two bands are expected for the vinyl bending, only one band was discernible, probably owing to accidental frequency degeneracy. Concomitantly, in the high-frequency region two ν(C=C) stretching modes giving rise to a band at 1620 cm^{-1} were observed. At alkaline pH CCP undergoes a conformational change (Smulevich et al., 1991b) inducing an upshift to 410 cm^{-1} of the $\delta(C_\beta C_a C_b)$ vinyl bending modes and to 1628 cm^{-1} of the ν(C=C) stretching mode of the vinyl in position 2. For HRP at alkaline pH two ν(C=C) stretching modes have been identified on the basis of polarization measurements at 1620 and 1630 cm^{-1} (Terner & Reed, 1984). Therefore, it is very likely that at least one of the two bands observed at 401 and at 411 cm^{-1} is due to the $\delta(C_\beta C_a C_b)$ vinyl bending modes. As for the $\delta(C_\beta C_c C_d)$ propionate bending modes, tentatively assigned to the band at 382 cm^{-1} at low temperature (379 cm^{-1} at room temperature), the isotopic effect in D_2O can be ascribed to a coupling with a mode of the low-spin FeOH group, which in this case is involved in hydrogen bonding.

Effect of Temperature Lowering. From a comparison of the RR spectra in the high-frequency region at room and low temperature of the three proteins under investigation, it appears that the core size marker bands are upshifted at 20 K (Table 1), indicating that core contraction occurs. It is well established for a variety of heme systems that the frequencies of the RR core size marker bands are correlated with the porphyrin center to pyrrole nitrogen distance, Ct–N (Spiro & Li, 1988). The skeletal mode frequency versus the

porphyrin core size is expressed by the linear relationship $\nu_i = K_i(A_i - d)$, where ν_i represents the skeletal mode frequency (cm^{-1}), d is the heme core size (Ct–N, Å), and K_i and A_i are mode- dependent constants (cm^{-1}/Å) (Parthasarathi et al., 1987). We calculated for metHb the decrement of the d distance with temperature, Δd = 0.015, 0.011, 0.012, and 0.014, by taking the corresponding frequencies of ν_3, ν_{11}, ν_2, and ν_{10}, respectively. For HRP Δd = 0.012, 0.009, 0.018, and 0.009, respectively. Therefore the core size of the low-spin species of these proteins contracts by about 0.01 Å at low temperature. The effect is ascribed to the increase of the protein packing forces with decreasing temperature. According to the effect on the core size marker bands, with decreasing temperature an upshift of about 5 cm^{-1} was observed for the ν(Fe–OH) stretching mode, indicating a strengthening of the Fe–ligand bond. It is interesting to note that in HRP the strengthening of the Fe–OH bond and the contraction of the core at low temperature induce a different strength of the H-bond involving the hydroxyl group with respect to that at room temperature, as evidenced by a lesser upshift of the ν(Fe–OH) stretching mode upon deuterium substitution.

The ν(C=C) stretching modes appear also to be affected by lowering the temperature. In metHb both of the ν(C=C) stretching modes occur at 1620 cm^{-1} at room temperature (Rousseau et al., 1983) and are upshifted by 2 cm^{-1} at 20 K. In HRP at low temperature the separation between the vinyl peaks increases by 3 cm^{-1}, since the high-frequency mode shifts to 1634 cm^{-1}, indicating that the conformation of only one vinyl group changes. The same effect has been observed previously for HRP at neutral pH by lowering the temperature (Smulevich et al., 1991a).

The absorption spectra at room temperature of alkaline metMb, metHb, and HRP are characterized by a Soret band around 413 nm and α and β bands around 578 and 540 nm, respectively. In addition the globins also showed the presence of two extra bands around 485 and 600 nm. Both bands are due to z-polarized charge-transfer transition of the high-spin form (Makinen & Churg, 1983; Rodgers et al., 1992; Asher et al., 1977). Therefore it is not surprising that they disappear at low temperature when the thermal spin-state equilibrium is shifted toward a complete Fe–OH low-spin heme and that they are almost undetectable in alkaline HRP at room temperature. On the other hand, the low-spin hydroxyl proteins are characterized by a weak band around 635 nm, in the same range where the z-polarized charge- transfer band of the metHb-N_3^- complex has been observed (Smith & Williams, 1970; Eaton & Hochstrasser, 1968). Therefore it is very likely that the band at 635 nm of the low-spin OH complex has the same origin as the band assigned for the metHb-N_3^- complex. Further experiments are necessary to demonstrate that the Fe–axial ligand modes are enhanced upon excitation in this region, as is the case for the azide complex (Asher et al., 1977).

CONCLUSIONS

The resonance Raman and absorption data indicate that two Fe–OH forms exist at room temperature in metMb and metHb corresponding to high- and low-spin species in thermal spin-state equilibrium. Low-spin Fe–OH is stabilized at 20 K, giving rise to a strong Fe–OH stretching mode. In contrast, in HRP the low-spin is favored at both room and low temperature. The 50-cm^{-1} downshift in frequency of the ν(Fe–OH) stretching mode and the weakening of its intensity with respect to those observed for metMb and metHb, together with its anomalous upshift upon deuteration, clearly indicate that the OH ligand is strongly H-bonded with a distal side chain. We propose that the positively charged guanidinium of Arg38 is involved in the strong H-bond interaction with the oxygen atom of the hydroxyl group, which therefore acts as a proton acceptor. The low-spin species are characterized by a new absorption band at around 635 nm, which is assigned to a charge-transfer transition.

At low temperature the Raman data indicate a strengthening of the Fe–OH bonds and a contraction of the heme core size by about 0.01 Å. Both effects are ascribed to an increase of the packing forces at low temperature.

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