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Proton Nuclear Magnetic Resonance Studies of Myoglobin in H_2O

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Abstract. Exchangeable hydrogens in proteins can be identified by comparison of nuclear magnetic resonance spectra obtained in H_2O and in D_2O. In oxymyoglobin and myoglobin we have been able to observe resonances of the NH protons of the two tryptophans, as well as one resonance from arginine and one from histidine in the range -10 to -15 ppm downfield from 3-(trimethylsilyl)propanesulfonic acid (sodium salt). These resonances have been identified by chemical modifications coupled with considerations of crystallographic structure and the dependence of the resonances on the species (sperm whale, porpoise, horse) from which the myoglobin was obtained and on spin, pH, and temperature.

High-resolution proton nmr measurements are usually made in D_2O solution to simplify the spectra. Nmr investigations in H_2O permit one to observe resonances of exchangeable protons. Phillips and co-workers[1] have shown that for lysozyme in H_2O the NH resonance of the tryptophan residues can be observed between -10 and -11 ppm, indicating slow exchange with the solvent. It is well known that hydrogen bonds can shift proton nmr peaks several ppm to lower fields and also can reduce their rate of exchange with solvent.[2–4] Therefore, peaks from hydrogen-bonded protons of polar amino acids in H_2O might be shifted below -10 ppm and be narrow enough to be observed.

Previous proton nmr measurements of heme proteins have investigated the temperature-independent ring current effects of the porphyrin prosthetic group and the temperature-dependent hyperfine interactions of the unpaired iron spins on the protein resonances in D_2O.[5,6] The present nmr investigation of myoglobin derivatives in H_2O studies the effects of ligand, spin, pH, temperature, and variation due to the species from which the myoglobin is obtained upon the exchangeable proton resonances.

Experimental methods. Oxymyoglobin was prepared by a combination of the methods of Hugli and Gurd[7] and Hapner *et al.*[8] This involves homogenization in 70% saturated ammonium sulfate solution and subsequent chromatography on DEAE-Sephadex A-50 in 0.03 M 2-amino-2-methyl-1,3-propanediol buffer, pH 8.7. Globin was prepared by an adaptation[9] of Teale's[10] method, in which the heme is extracted from an acid solution of carbon monoxymyoglobin with methylethylketone. Myoglobin with one modified tryptophan residue was obtained by treatment with 2-hydroxy-5-nitrobenzyl bromide.[11]

For the nmr experiments approximately 0.001–0.01 M solutions of the myoglobin

derivatives in 0.1 M deuterated or protonated phosphate buffer, at the required pH, were prepared. High-resolution proton nmr spectra were recorded on a Varian HR-220 spectrometer equipped with a standard Varian variable temperature control unit. The temperature in the sample zone was determined from the chemical shifts of the resonances of ethylene glycol. Chemical shifts are expressed in ppm from the internal standard [3-(trimethylsilyl)propanesulfonic acid sodium salt], where shifts to low field are assigned negative values. A Fabritek 1062 computer of average transients was employed to improve the signal/noise ratio. The spectra represent time averaging of 1–4 hr.

Results and Discussion. The nmr spectra of diamagnetic proteins in D_2O give resonances between 0 and −9 ppm. Myoglobin (Mb) has 950 protons and the spectral envelope in this region prohibits a detailed analysis. This research concentrates on resonances which are shifted upfield and downfield from the 0 to −9 ppm region. The nmr spectra of diamagnetic oxymyoglobin, MbO_2 (sperm whale) in H_2O, at pH 7 and pH 9, in the regions −9 to −15 ppm and +2 to +4 ppm, are presented in Fig. 1. Comparison with spectra run in D_2O indicate that

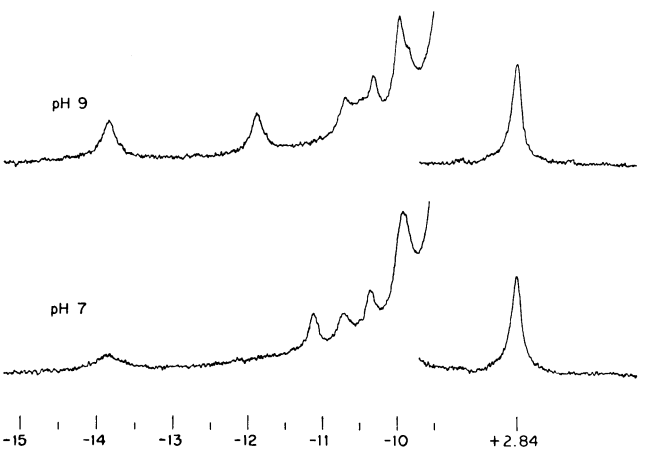

FIG. 1. 220 MHz proton nmr spectra of MbO_2 (sperm whale) in 0.1 M phosphate buffer in H_2O, 23°C at pH 7 and pH 9.

the resonances shown between −10 and −15 ppm arise from exchangeable protons, while nonexchangeable resonances account for the peaks at −9.7 ppm and +2.84 ppm.

Nonexchangeable hydrogens. It was necessary to measure the number of protons in a resolved nonexchangeable resonance in the spectrum of MbO_2 and to use it to calibrate the areas of the downfield exchangeable resonances. MbO_2 was converted to metmyoglobin cyanide (MetMbCN) and the +2.84 ppm resonance in MbO_2 determined to have an area of 2.7–2.8 protons when compared with the −26 ppm resonance in MetMbCN with an area known to correspond to three protons. (This calibration was suggested by Dr. B. Sheard.) Ring current shielding by the porphyrin ring accounts for the >3 ppm upfield shift of the +2.84 ppm MbO_2 resonance relative to the aliphatic region. This peak is absent in the paramagnetic Mb and MetMb, presumably because it is

shifted by interactions with the unpaired iron spins. This resonance is species independent. The resonance shifts from +2.86 ppm (MbO_2) to +2.41 ppm (MbCO) and +2.23 ppm (MbisoCN) on replacing the sixth ligand[6] suggesting its origin in a CH_3 group on the distal side. The W. E. Blumberg computer simulation (personal communication) of the MbO_2 spectrum indicates that the two CH_3 resonances of valine in fragment E11 (Val E11) appear at +0.28 ppm and +1.77 ppm, the latter being the highest field resonance in the simulated spectrum. This computer program uses the coordinates of the carbon atoms to calculate the proton spectra. The calculated Val E11 position would be at still higher fields if the proton coordinates were used. On the basis of its position and intensity the +2.86 ppm resonance in MbO_2 is assigned to one of the methyls of Val E11.

Exchangeable hydrogens: These resonances, observed in H_2O but not in D_2O, will be discussed in two groups. First those in the −10.5 to −11.5 ppm tryptophan region and second those between −11.5 and −14 ppm.

There are two tryptophans in myoglobin, at positions[11] A5 and A12. At pH 7 the spectrum of diamagnetic MbO_2 in the tryptophan region has three exchangeable resonances at −10.3, −10.7, and −11.15 ppm, with areas of 0.7 ± 0.1 of a proton (Fig. 1). The −10.3 and −11.15 ppm, MbO_2 resonances are also present in diamagnetic MbisoCN while the broader −10.7 ppm MbO_2 resonance, whose intensity varies with samples, is absent in MbisoCN. The exchangeable MbO_2 resonances between −10.0 and −11.5 ppm at pH 7 and pH 9 in H_2O were unchanged in sperm whale, porpoise, and horse; this is consistent with the invariancy of tryptophan at positions A5 and A12 with species.[12,13] As the pH is raised from 7 to 9 the line width and position of the −10.3 and −10.7 ppm exchangeable resonances in MbO_2 remains essentially unchanged while the −11.15 ppm exchangeable resonance shifts upfield by 0.7 ppm and broadens (Fig. 1). Variation of the temperature between +15 and +25°C has no effect on the line widths of these exchangeable MbO_2 resonances. The comparison of the nmr spectra in H_2O between −10.0 and −11.5 ppm of diamagnetic MbO_2 with paramagnetic met Mb and Mb is shown in Fig. 2 and the positions of the exchangeable resonances is given in Table 1. The three-dimensional models of myoglobin based upon the x-ray coordinates of Kendrew and Watson (private communication) locate the two tryptophans facing inwards at the sur-

TABLE 1. *Resonance positions (in ppm downfield from 3-(trimethylsilyl)propanesulfonic acid sodium salt) for exchangeable protons of myoglobin (sperm whale) derivatives in H_2O.*

	MbO_2* (S = 0)	MetMb* (S = 5/2)	Mb† (S = 2)
Peaks at pH 7.4	−13.85	−13.82	∼−13.8‡
	−11.15	−11.21	−11.35
	−10.69	−10.45	
	−10.31	− 9.95	−10.05
Additional peak at pH 9	−11.88	−11.80	∼−11.8‡

* 23°C.
† 25°C.
‡ These resonances were separated from porphyrin resonances by their temperature independence.

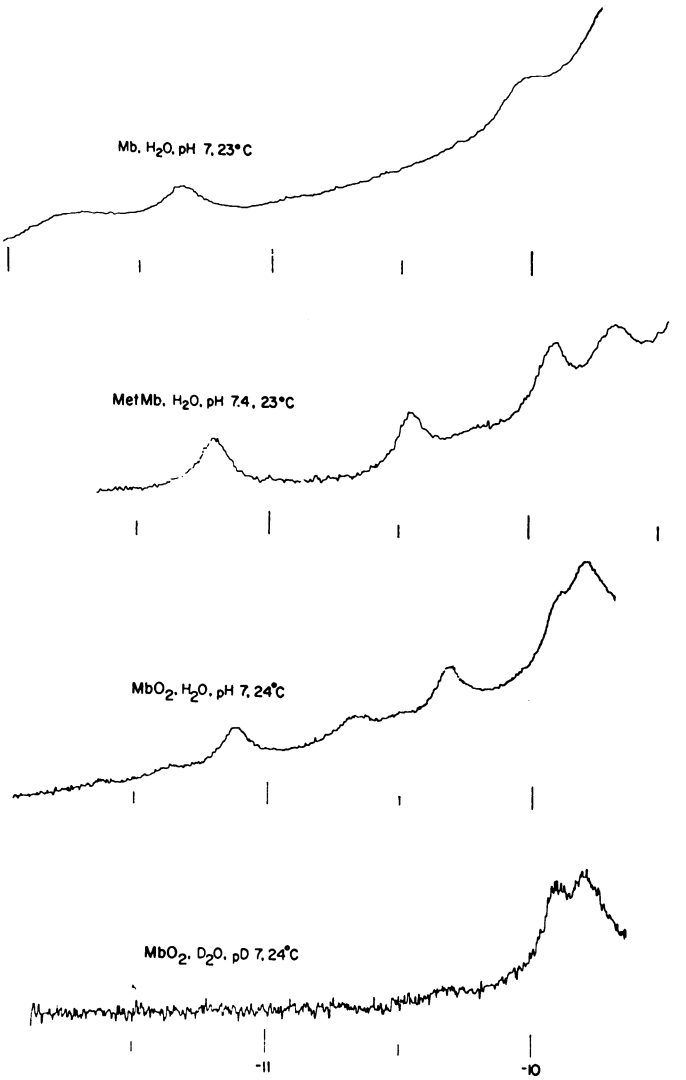

FIG. 2. 220 MHz proton nmr spectra of Mb (sperm whale) derivatives in 0.1 M phosphate buffer solutions. Porphyrin resonances appear at −9.8, −9.9 ppm in MbO_2 (bottom two spectra), −9.75 ppm in MetMb, and −11.75 ppm in Mb (*top*).

face and far from the heme. Thus, pseudocontact shifts and dipolar line broadening, which are distance-dependent, should be negligible in the paramagnetic forms. The largest differences between the spectrum of MbO_2 and those of MbisoCN and MetMb occur in the −10.7 ppm peak, which disappears in the last two compounds. The most likely explanation is that the −10.7 ppm peak arises from a proton nearer the iron than the two tryptophans, which are responsible for the peaks at −10.3 and −11.15 ppm. The small changes in the

positions of these peaks between MbO_2 and MetMb and the larger changes in Mb are probably caused by ligand-induced conformational changes as monitored at the A helix.

The MbO_2 spectrum at pH 9 in H_2O at 23°C indicates a 1.2 proton exchangeable resonance at −11.9 ppm (Fig. 1). Lowering the pH gradually to 7 results in line broadening and disappearance of this resonance at 23°C. A lifetime broadening mechanism is suggested from its reappearance in the pH 7 sample at 15°C. The −11.9 ppm MbO_2 peak is observable in paramagnetic MetMb and Mb with similar chemical shifts (Table 1) and exchange dependence on pH and temperature. The absence of shifts and/or line broadening suggests that the iron is >10 Å distant from this exchangeable proton because it can be calculated from the known electron spin relaxation times in MetMb that appreciable line broadening would occur at shorter distances from the iron.[13] From the three-dimensional myoglobin model, built in accordance with Kendrew and Watson's coordinates, we have listed in Table 2 the exchangeable side-chain hydrogens of Tyr, Arg, His, Lys, and Gln that are involved in hydrogen bonds more than 10 Å from the heme. Table 3 indicates the amino acids in myoglobins from sperm

TABLE 2. *Hydrogen-bonded exchangeable protons in liganded myoglobin from x-ray model for residues >10 Å from heme.*

Exchangeable hydrogens	
Tyr H22 (1)	Ilu FG5 CO
Arg CD3 (3)	Asp E3 CO_2^-
	Porphyrin CO_2^-
Arg G19 (2)	Glu A16 CO_2^-
	Asp B8 CO_2^-
His CD6 (1)	Arg CD3 CO
His EF5 (1)	Asp H17 CO_2H
Gln B7 (1)	Lys D6 CO
Lys E5 (1)	Glu E3 CO_2^-
Lys E20 (1)	Glu A16 CO_2^-
Lys EF2 (1)	Glu A2 CO_2^-

TABLE 3. *Myoglobin species sequence for hydrogen-bonded exchangeable hydrogens.*

	B7	CD3	CD6	E5	E20	EF2	EF5	G19	H22
Sperm whale	Gln	Arg	His	Lys	Lys	Lys	His	Arg	Tyr
Porpoise	Gln	Lys	His	Lys	Lys	Lys	His	Arg	Tyr
Horse	Glx	Lys	His	Lys	Lys	Lys	His	Lys	Tyr

whale, porpoise, and horse that satisfy these conditions. The species dependence of the −11.9 ppm resonance was studied, with the results shown in Fig. 3. This exchangeable resonance was present in the oxy derivative of sperm whale and porpoise myoglobin but was absent in that of horse in H_2O at pH 9. The −11.9 ppm resonance can be accounted for by a guanidinium NH resonance of Arg G19 (Table 2) involved in a hydrogen bond with either the CO_2^- group of Asp B8 or Glu A16 (Table 2). Since the arginine side-chain resonances of the protonated amino acid in aqueous solution appear at −6.7 ppm (primary) and −7.2 ppm (secondary),[14,15] the large downfield shift to −11.9 ppm of the Arg G19 resonance observed in MbO_2 is attributed to hydrogen bonding. Note that this resonance is present in globin (Fig. 3), indicating that the hydrogen bonding

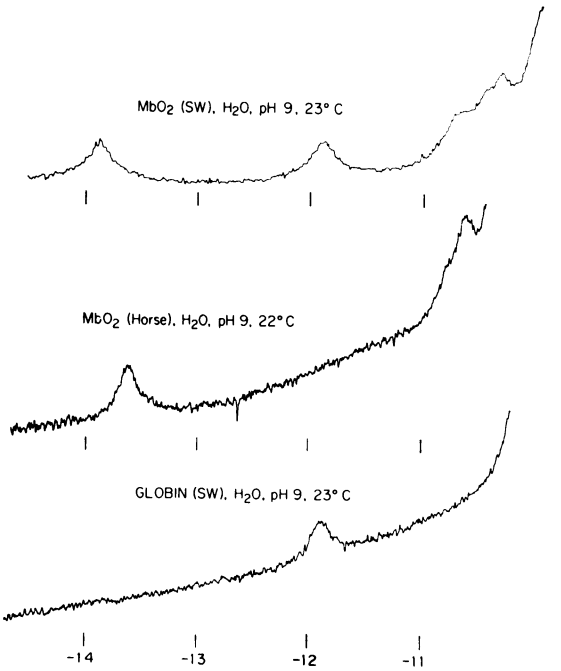

FIG. 3. 220 MHz proton nmr spectra (23°C) of MbO_2 from two species and of globin in 0.1 M phosphate/H_2O at pH 9. SW, sperm whale.

from the G helix residue to either the A or B helix is intact in globin. Since the −11.9 ppm exchangeable resonance broadens around pH 8, its lifetime in the hydrogen bond can be calculated. At pH 7.4, the full width determined at half height is 110 Hz, giving a lifetime of $\tau = 1/\pi\Delta\nu = 3$ msec in the hydrogen bond. This lifetime becomes shorter as the pH decreases. The arginine side chain exchangeable protons in the free amino acid undergo lifetime broadening with water *above* pH 7. On the other hand, the hydrogen bonded exchangeable side chain Arg G19 proton in myoglobin derivatives undergoes lifetime broadening *below* pH 8. A possible explanation of the broadening as the pH is lowered is that its lifetime is interrupted by protonation of the carboxylate. In this case we calculate that the specific rate constant for the protonation is $\sim 10^{10}$ sec^{-1}, which agrees very well with the values observed in model systems.[16]

The MbO_2 exchangeable resonance at −13.85 ppm in H_2O has an area of 1.4 protons at pH 9 and 0.8_5 proton at pH 7 (Fig. 1). (A broad shoulder at −14 ppm has been observed in a few myoglobin samples at pH 9.) This resonance broadens beyond detectability at pH 5. The resonance is present in sperm whale, porpoise, and horse myoglobin (Fig. 3). By contrast, it is absent in globin in H_2O at pH 7 and pH 9 (Fig. 3). The −13.85 ppm exchangeable resonance has similar chemical shift, pH dependence, and temperature dependence in the paramagnetic deoxy and metmyoglobins (Fig. 4 and Table 1), indicating, as mentioned above, that it is more than 10 Å distance from the iron. This resonance has also been observed in MbCN.[17] Certain residues can be eliminated as possible origins of the −13.85 ppm resonance by consideration of these experimental results. Arg CD3 is eliminated by the comparison among the

different myoglobin species and Tyr H22 is eliminated because the resonance is not observed in the oxy or deoxy forms of the α and β chains of hemoglobin, which contain this tyrosine. We cannot definitely eliminate Arg, Tyr, Lys, or Gln as assignments for this resonance. However, because their exchangeable resonances fall near -7 ppm it would require a very large shift by the hydrogen bond formation to move them down to -13.85 ppm. The imidazole NH protons in $CDCl_3$ have been observed[18] at -13.4 ppm so that His must be considered a likely origin of these low field resonances. In the protonated form, in trifluoroacetic acid,[14] the imidazole NH protons have been observed at -12.3 ppm. The imidazole NH protons of His CD6 and His EF5 (Table 2) form hydrogen bonds with carbonyl groups and are more than 10 Å from the heme. These two possibilities might be distinguished by their different susceptibilities to alkylation.[19] An assignment of the -13.85 ppm resonance must await further results.

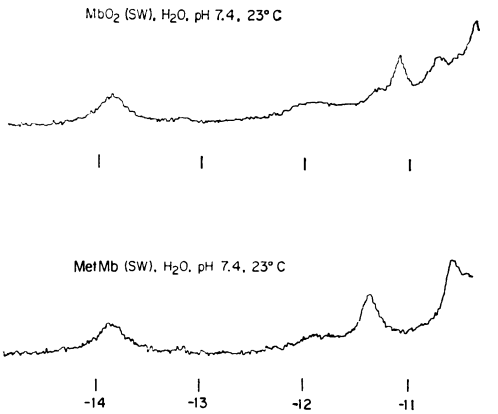

FIG. 4. 220 MHz proton nmr spectra of diamagnetic MbO_2 with paramagnetic MetMb at pH 7.4, 23°C in 0.1 M phosphate/H_2O.

Abbreviations: nmr, nuclear magnetic resonance; Mb, myoglobin; MbO_2, oxymyoglobin; MbCO, carbon monoxide myoglobin; MbCN, myoglobin cyanide; MbisoCN, myoglobin isocyanide; MetMb, metmyoglobin.

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