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Conformational Differences between Various Myoglobin Ligated States As Monitored by ^{1}H NMR Spectroscopy[†]

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ABSTRACT: In paramagnetic metmyoglobin, cyanomyoglobin (CNMb), and deoxymyoglobin, His-36 has a high p*K* (~8), and the NMR titration behavior of the H-2 resonance is perturbed, due to the presence at low pH of a hydrogen bond with Glu-38, which is broken at high pH. The His-36 H-4 resonance shows no shift with p*K* ~ 8 because of two opposing chemical shift effects but monitors the titration of nearby Glu-36 (p*K* = 5.6). In diamagnetic derivatives [(carbon monoxy)myoglobin (COMb) and oxymyoglobin (oxyMb)], the titration behavior of His-36 H-2 and H-4 resonances is normalized (p*K* ~ 6.8). The very slight alkaline Bohr effect in sperm whale myoglobin (Mb) is interpreted in terms of the p*K* change of His-36 from deoxyMb to oxyMb and compensating p*K* changes in the opposite direction of other unspecified groups. In sperm whale COMb at 40 °C, the distal histidine (His-64) and His-97 have p*K* values of 5.0 and 5.9. The meso proton resonances remote from these groups do not show a titration shift, but the nearby γ-meso proton (p*K* = 5.3) responds to titration of both histidines, and the upfield Val-68 methyl at −2.3 ppm (p*K* = 4.7) witnesses the titration of nearby His-64. At 20 °C, the latter resonance is reduced in size, and a second resonance occurs at −2.8 ppm, which is insensitive to pH and, hence, more remote from His-64. Both resonances arise from two conformations of Val-68 in slow equilibrium. In oxyMb at 20 °C, only the latter resonance is observed, presumably because of the steric restrictions imposed by the hydrogen bond between ligand and His-64 in oxyMb, which is not present in COMb. In oxyMb the p*K* of His-97 (5.6) is similar to that of the meso proton resonances (5.5) and to the p*K* of other pH-dependent processes, including the very small acid Bohr effect. It is likely that these processes are controlled by the titration of His-97. The differences in the NMR titration behavior between the CO and oxygen complexes of Mb and leghemoglobin (Lb) are explained in terms of the larger and more flexible heme pocket in Lb and the absence in Lb of any titrating histidine other than the distal histidine.

In the preceding paper (Carver & Bradbury, 1984), we assigned many aromatic and hyperfine-shifted resonances in the ^{1}H NMR spectra of metMb,[1] hydroxyMb, CNMb, deoxyMb, COMb, and oxyMb. We have therefore identified many protons in various parts of the molecule including the meso heme protons in oxyMb and COMb (Bradbury et al., 1982)

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[1] Abbreviations: NMR, nuclear magnetic resonance; Mb, myoglobin; metMb, ferric aquomyoglobin; hydroxyMb, ferric hydroxymyoglobin; CNMb, ferric cyanomyoglobin; deoxyMb, ferrous deoxymyoglobin; COMb, ferrous (carbon monoxy)myoglobin; oxyMb, ferrous oxymyoglobin; N_3Mb, ferric azidomyoglobin; Hb, hemoglobin; deoxyHb, ferrous deoxyhemoglobin; oxyHb, ferrous oxyhemoglobin; COHb, ferrous (carbon monoxy)hemoglobin; Lb, leghemoglobin; deoxyLb, ferrous deoxyleghemoglobin; oxyLb, ferrous oxyleghemoglobin; COLb, ferrous (carbon monoxy)leghemoglobin.

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Table I: Chemical Shifts and p*K* Values at 20 °C of Histidine-36 in Various Ligated States of Sperm Whale Myoglobin[a]

	H-2 resonances				H-4 resonances		
derivative	δ_A	δ_B	Δ	p*K*	δ_A	δ_B	p*K*
metMB	8.40	7.18	1.22	8.03	6.94	6.66	5.67
CNMb	8.07	7.02	1.05	8.11	7.01	6.95	5.58
deoxyMb	8.24	7.48	0.76	7.94	6.78	6.74	
COMb	8.67	7.68	0.99	6.99	7.18	6.96	6.56
oxyMb	8.62	7.72	0.90	6.85	7.39	6.97	6.60

[a] δ_A and δ_B are the chemical shifts of the resonance at the acid and the alkaline limits, respectively, and Δ is the total chemical shift change on deprotonation.

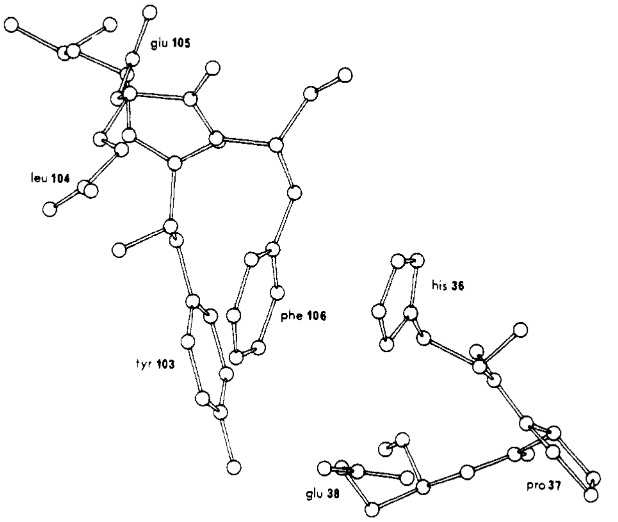

FIGURE 1: Environment of His-36 in metMb as determined from the X-ray crystal structure coordinates of Takano (1977a).

by means of their ^1H NMR resonances. In this paper we provide additional data on near-heme resonances and use the chemical shift and p*K* data for the histidine residues given in earlier papers to examine the nature of the changes undergone by Mb, particularly in the heme region, as a result of the binding of different ligands to the heme iron atom. The data presented are also used to compare the heme pockets Mb and Lb.

Results and Discussion

Behavior of His-36. In the paramagnetic derivatives metMb, CNMb, and deoxyMb (also N_3Mb; Hayes et al., 1975), δ_A and δ_B values for His-36 in Table I are, in general, smaller than the corresponding values for the diamagnetic derivatives (COMb and oxyMb). This is due to the closer proximity of Phe-106 in the paramagnetic derivatives (Figure 1) which causes an upfield ring current shift on His-36 H-2 and H-4 resonances. The smaller than usual shift on deprotonation of His-36 H-2 in deoxyMb (Table I) and the complete absence of a shift of His-36 H-4 [Table I and Carver & Bradbury (1984)] are probably due to the movement away on increasing pH of the ring of His-36 from the close environment of Phe-106, which causes a downfield shift of ~0.3 ppm, that compensates exactly for the upfield H-4 shift due to deprotonation. Similar type of behavior is also observed for CNMb and metMb, but in the latter case, the pictue is further complicated by the occurrence of the hemic acid dissociation (p*K* ~ 9), which causes a further upfield shift (~0.4 ppm) of His-36 H-2 and a slight downfield shift of His-36 H-4 [resonance 17; Figure 3a of Carver & Bradbury (1984)].

The balancing of two opposing chemical shift changes on the titration of His-36 H-4, together with a subsidiary shift at low pH, produces the unusual result that the p*K* obtained from the H-2 resonances (~8) is very different from that obtained from the H-4 resonances (~5.6). Ribbing & Rüterjans (1980a,b) have observed titration effects that are difficult to explain with the His-G2 H-2 resonance of monomeric *Chironomus* deoxyHb and COHb. In that case, Steigemann & Weber (1979) found that in the crystal there is a hydrogen bond between His-G2 and the C-terminal Met-H22 that raises the p*K* of His-G2 and a nearby phenylalanine ring (Phe-H19) causes an upfield shift of its H-2 resonance. In our system, the H-2 resonance monitors the titration of His-36, which is not observed by the H-4 resonance (because of compensation of two opposite effects), but the H-4 resonance is affected by the titration of nearby Glu-38. His-36 H-2 is apparently more remote from Glu-38 than the H-4 proton, because the H-2 does not undergo any shift at this pH. This result is consistent with the small shift (p*K* ~ 5) of the C-5 resonance of one of six histidine resonances in horse metMb (Wilbur & Allerhand, 1977), which agrees with the behavior of His-36 H-4, p*K* 4.8 [Table II of Carver & Bradbury (1984)]. The results of Wilbur & Allerhand (1977) for the p*K* values of the other five histidine residues fit those of Tables I and II of Carver & Bradbury (1984). The postulated high p*K* of 5.6 for Glu-38 in metMb and CNMb may be due to the nonpolar nature and consequent inaccessibility of the region to solvent as shown by the slow rate of deuteration at pH 7 of His-36 H-2 (Botelho & Gurd, 1978).

The high p*K* of His-36 observed in metMb, CNMb, and deoxy Mb (Table I) and also in N_3Mb (Hayes et al., 1975) is due to the presence of a hydrogen bond between His-36 and Glu-38, which also prevents the carboxymethylation of metMb at pH 6.8 in solution. However, carboxymethylation occurs in the crystal at pH 6.8 and also in solution at pH 9 (Hugli & Gurd, 1970a,b; Nigen & Gurd, 1973; Botelho & Gurd, 1978). In the crystal structure (Figure 1), His-36 and Glu-38 are separated by a hydrogen-bonded water molecule (Takano, 1977a,b). Thus, in paramagnetic derivatives there are two structures, the "low-pH" hydrogen-bonded structure and the "high-pH accessible" structure, which is also similar to that observed in the crystal at pH 6.8. The structure in the diamagnetic derivatives (COMb and oxyMb) is similar but not the same as the high-pH accessible structure, because of the fact that the chemical shift values in Table I would indicate that the ring of Phe–106 is closer and/or more closely parallel to the His-36 ring in the paramagnetic than in the diamagnetic derivatives. In the latter (COMb and oxyMb), there is normalization of chemical shifts and p*K* values for both H-2 and H-4 resonances.

The normalization of the titration characteristics of His-36 in oxyMb and COMb indicates that there must be a conformational difference in this region between the paramagnetic and diamagnetic species, since in the latter His-36 no longer interacts with Glu-38 and Phe-106. This change cannot be related to conformational changes associated with movement of the iron atom into the heme plane with conversion from high-spin deoxyMb to low-spin oxyMb and COMb, since no change in the pK of His-36 was observed for a smaller shift of the iron atom in going from high-spin metMb to low-spin CNMb and N_3Mb. More subtle effects than the shift of the iron atom are required to explain the observed difference in the behavior of His-36 between deoxyMb and oxyMb and COMb.

A similar difference has been observed in human deoxyHb in which His-146β (pK value 8.0; Kilmartin et al., 1973) forms a hydrogen bond to the carboxyl group of Asp-94β and lies in a pocket between the F and H helices (Perutz, 1970). In COHb, the hydrogen bond is broken, and the pK of His-146β is 7.1 (Kilmartin et al., 1973). Similarly, His-G2 in *Chironomus* deoxyHb has a pK of 7.9 along with an upfield shift for its H-2 resonance (Ribbing & Rüterjans, 1980a,b). In the oxy form, however, the pK of His-G2 is 7.0, and the chemical shift of its H-2 resonance is normal. However, no changes were observed in the environment of His-G2 in the crystal structures of *Chironomus* oxyHb as compared with deoxy Hb (Weber et al., 1978). Possible reasons for this are discussed by Ribbing & Rüterjans (1980b), and similar arguments may apply to Mb; i.e., the Mb molecule preferentially crystallizes in the "high-pH" conformation of His-36, and therefore, no differences are observed in this region between the crystal structure of deoxyMb and oxyMb.

Alkaline Bohr Effect. In the hemoglobins discussed above, the change in pK of the particular histidine between the deoxy and oxy derivatives is associated with the alkaline Bohr effect, viz., in increase in oxygen affinity and concomitant release of protons due to the lowering of pK's of various ionizable groups with increasing pH above 6. Thus, in human Hb the alkaline Bohr effect is considered to be due largely to the change in pK of His-146β (Kilmartin & Wooten, 1970). For human Mb, the oxygen affinity increases about 15% from pH 7 to 9 at 37 °C (Rossi Fanelli & Antonini, 1958). A similar change is observed in this pH range at 37 °C for horse Mb (La Mar et al., 1978; Antonini & Brunori, 1971) but not for sperm whale Mb (La Mar et al., 1978), although the conformational changes considered to be responsible for the observed alkaline Bohr effect in horse Mb are present in both species (La Mar et al., 1978). The large difference in pK of His-36 between the deoxy (pK = 7.9, Table I) and oxy (pK = 6.8) derivatives of sperm whale Mb is similar to the pK difference of His-146β between these two states in Hb. In Hb, however, there is a large alkaline Bohr effect, whereas it is virtually absent in Mb. The small alkaline Bohr effect in horse Mb can be explained by a change in pK of one ionizable residue by 0.05 unit upon oxygenation; hence, any slight alkaline Bohr effect in sperm whale Mb cannot be due to His-36 alone, and there must also be present one or more ionizable groups that raise their pK's upon oxygenation to compensate.

In tetrameric hemoglobins variation in pK's of histidine residues other than His-146β (Brown & Campbell, 1976; Russu et al., 1980, 1982) contribute to the alkaline Bohr effect as well as the lowering of the pK of Val-1α in going from the deoxy to ligated state of Hb (Garner et al., 1975; Matthew et al., 1977; Suzuki et al., 1974). In general, the pK's of the histidine residues that have normal titration characteristics are not appreciably different in deoxyMb and oxyMb (Carver & Bradbury, 1984). The N-terminal valine or glycine residues of various ferric myoglobins have been shown to have pK's in the range 7.2–8.0 (Garner et al., 1973; Garner & Gurd, 1975; Artyukh et al., 1977, 1979; Wilbur & Allerhand, 1977), but data are not available for the ferrous derivatives. If the pK value of the N-terminal residue in deoxyMb is lower than that in oxyMb or COMb by a significant amount, then this would offset the effect of the protons released by His-36 upon oxygenation. Dunker (1982) proposed that the proline NH group has a pK of 2–14 dependent on its distortion from planarity and that different conformations of proline in deoxyHb and oxyHb may contribute to the alkaline Bohr effect. Since in all myoglobins Pro-37 is conserved, it would witness the conformational changes of His-36 from deoxyMb to oxyMb and could undergo a change in planarity and hence pK to offset that of His-36. Thus, although there are two conformers of Mb present in the alkaline pH range that involve change in pK's of His-36 and possibly compensating changes in the N-terminal and other residues, the conformers have very similar oxygen affinities so that overall a very small alkaline Bohr effect is observed.

Variation with pH of Near-Heme and Meso-Heme Protons in COMb. In the ^{1}H NMR spectra of COMb and oxyMb, the resonances of meso-heme protons [α, β, γ, δ; Figure 12 of Carver & Bradbury (1984)] were assigned by selective deuteration (Bradbury et al., 1982). The pK value for the shift of the γ-meso proton resonance shown in Figure 2 is 5.3 at 20 °C and 5.1 at 40 °C. The α- and δ-meso proton resonances do not move with pH, but the β-meso proton resonance shifts slightly, particularly at 20 °C. The meso-proton resonances in horse and pig COMb are readily assigned as they are observed at similar chemical shift positions and exhibit similar movements with pH. The γ-meso proton in horse and pig COMb at 40 °C gave pK values of 5.5 and 5.4, respectively.

From the crystal coordinates of sperm whale COMb (Hanson & Schoenborn, 1981), the distances between the γ-, α-, β-, and δ-meso protons and the C-4 proton of His-64 are 6.6, 7.8, 7.6, and 7.7 Å, respectively. Furthermore, the distance of the γ-meso proton to His-97 H-4 is 5.1 Å compared with 8.4, 5.9, and 8.1 Å for the α-, β-, and δ-meso protons, respectively. Thus, the γ-meso proton is in close proximity to both His-64 and His-97, and the β-meso proton is reasonably near to His-97. In Figure 3, it is shown that the propionic acid at position 7 is folded back on the proximal side of the heme and hydrogen bonded to His-97. The protonation of His-97 with decreasing pH could alter this hydrogen-bonding interaction, and the subsequent structural rearrangements would be expected to be sensed by nearby groups, in particular the γ- and β-meso protons. Thus, the more remote α- and δ-meso protons do not show a titration shift (Figure 2), since shifts due to the titration of nearby groups fall off rapidly with distance in model systems (Bradbury & Fenn, 1969). Furthermore, the pK's exhibited by the titration of the γ-meso proton in sperm whale, horse, and pig COMb at 40 °C (5.1, 5.5, and 5.4, respectively) fall in general between the corresponding pK's of His-64 H-4 (5.0, 5.0, and 5.4, respectively) and the pK's of His-97 (5.9, 5.9, and 5.8, respectively). The small shift of the β-meso proton resonance (Figure 2) probably arises from the deprotonation of His-97 to which it is much closer than His-64 (see above).

A resonance observed at ~2.3 ppm has been assigned by Shulman et al. (1970) and Patel et al. (1970) to one of the methyl groups of Val-68(E11), and its ring current shift of >3 ppm is consistent with its proximity to the porphyrin plane (Figure 3). The resonance has an area equivalent to three protons and is absent in metMb, CNMb, and deoxyMb. Its titration shift with pH (Figure 4) gives a pK of 4.7, which is similar to that of His-64 (pK = 5.0) in sperm whale COMb at 40 °C and implies that Val-68, because of its proximity to His-64 [Table III in Carver & Bradbury (1984)], is witnessing changes associated with the titration of the distal histidine. In horse and pig COMb, only very small shifts at low pH are

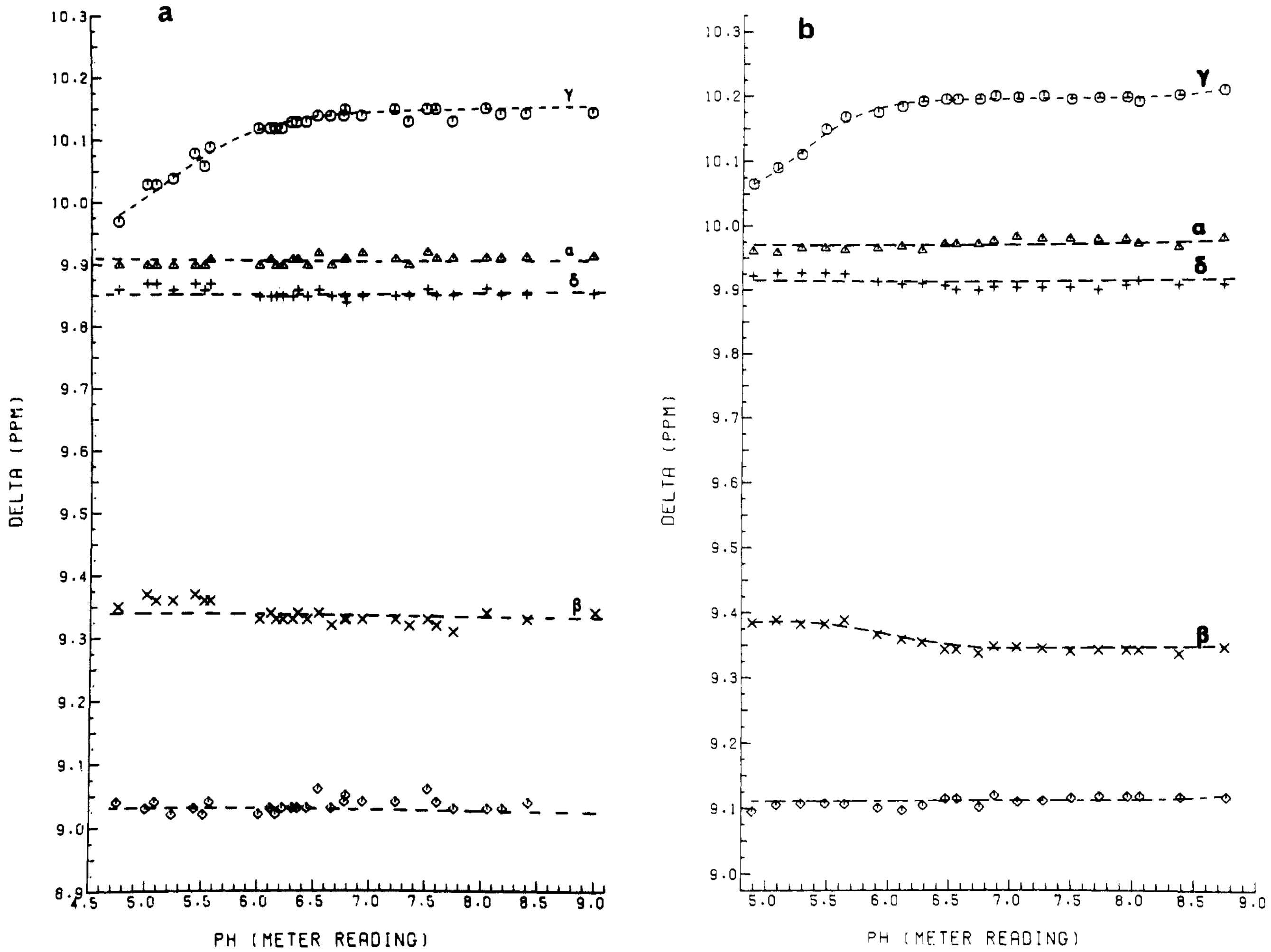

FIGURE 2: pH variation of the meso-heme proton resonances in the ^{1}H NMR spectrum of sperm whale COMb at (a) 40 and (b) 20 °C.

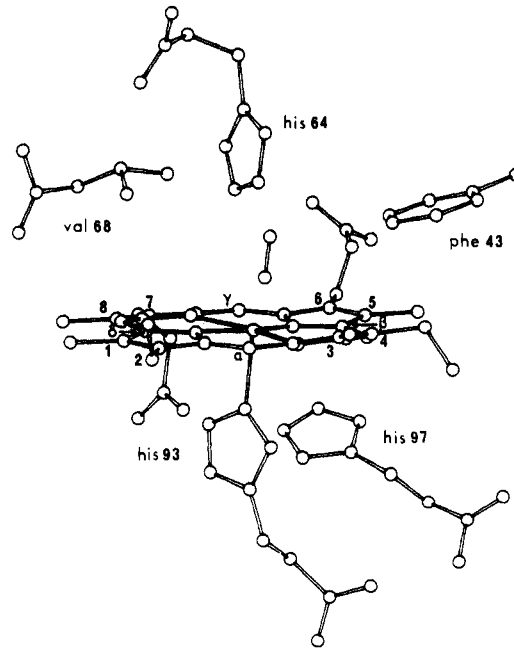

FIGURE 3: Heme environment in COMb determined from the crystal structure coordinates of Hanson & Schoenborn (1981).

observed for Val-68 upfield methyl resonance, which implies that any conformational changes associated with deprotonation of His-64 are not as large in horse and pig COMb or else that Val-68 is further away from His-64 in horse and pig COMb than in sperm whale COMb.

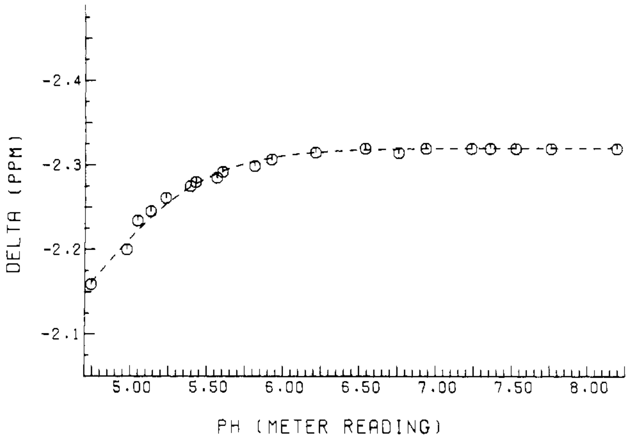

FIGURE 4: Variation with pH of the chemical shift of the upfield methyl resonance of Val-68 of sperm whale COMb at 40 °C.

In COMb at 20 °C, two resonances are observed at δ ~2.3 and ~2.8, each less than three protons in area along with a broad resonance at ~1.6 ppm (Figure 5). The variation in chemical shift position of these resonances with pH and temperature is given in Figures 6 and 7. The resonance at ~2.8 ppm is only observed below 25 °C (Figure 7) and at pH values above ~5.7 at 20 °C (Figure 6). This resonance increases in area with increasing pH and decreasing temperature compared with that of the resonance at ca. −2.3 ppm, which has been assigned to one of the methyl groups of Val-68. The resonance at ca. −2.8 ppm has the same chemical shift as the methyl resonance of Val-68 in sperm whale oxyMb (see be-

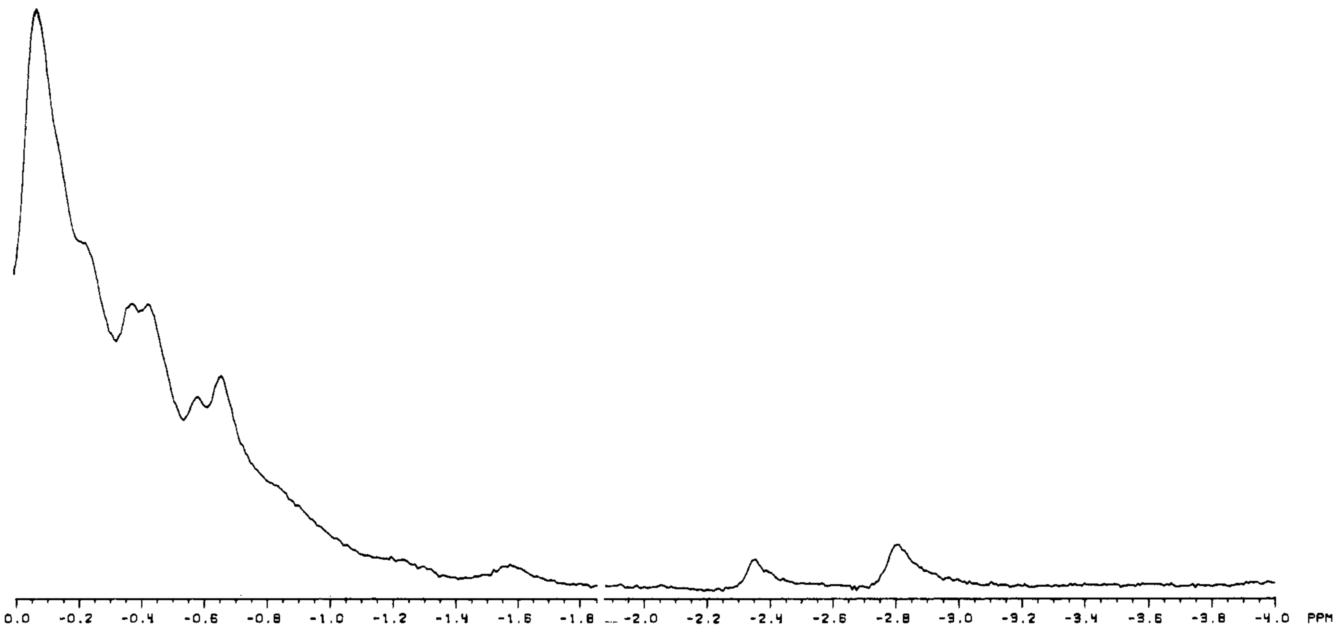

FIGURE 5: Upfield region of the normal ^{1}H NMR spectrum of sperm whale COMb at pH 6.76 and 20 °C.

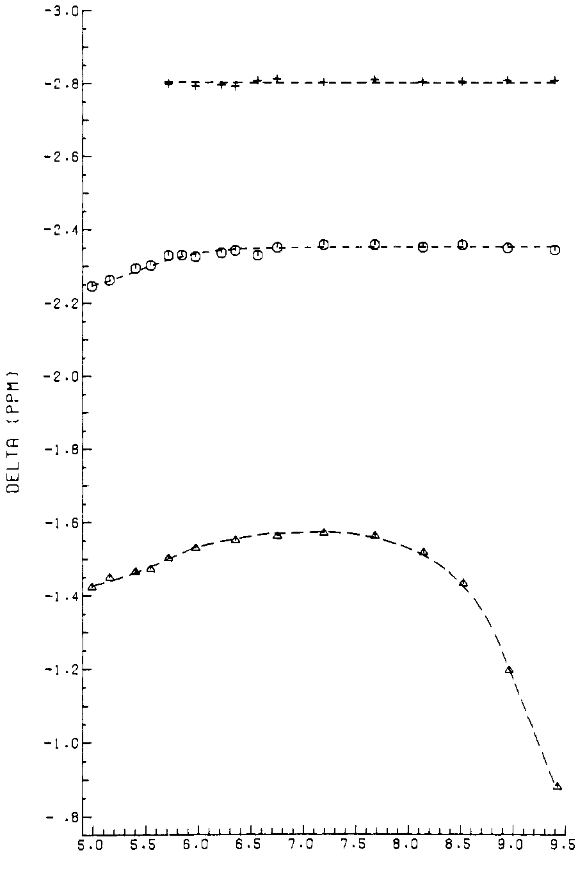

FIGURE 6: pH variation of the upfield resonances shown in Figure 5. The two upfield resonances are assigned to a methyl group of Val-68, and the downfield resonance is assigned to a near-heme proton.

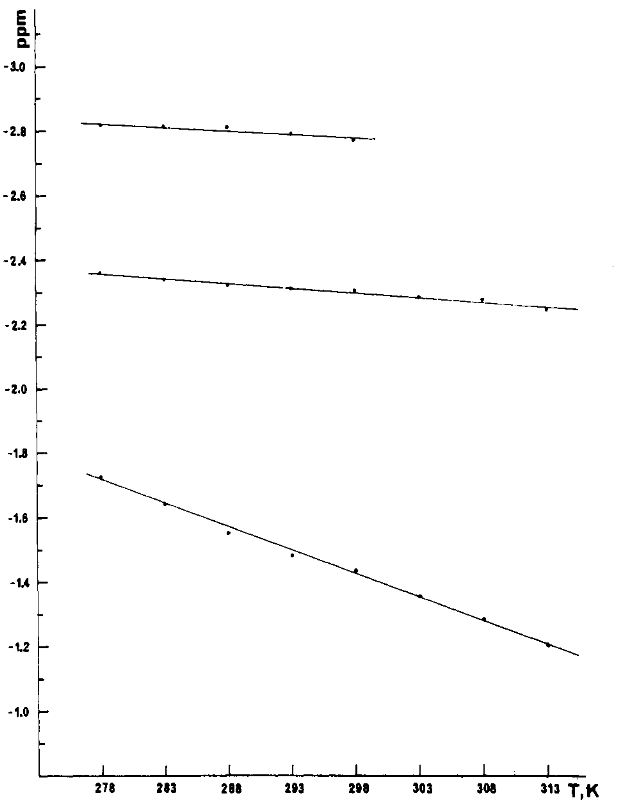

FIGURE 7: Plot of chemical shift vs. temperature (T) for the resonances shown in Figure 6 (pH 5.60, measured at 40 °C).

low). Under the conditions of low temperature and pH >5.6, Val-68 in COMb exists in a conformational equilibrium that is slow on the NMR time scale and gives rise to two separate resonances at δ −2.3 and −2.8.

At low pH, the resonance at δ ca. −2.3 shows the same upfield shift with increasing pH that was ascribed to the titration of the distal histidine as that observed at 40 °C (Figure 4). The extra resonance at ca. −2.8 ppm, however, is not observed until deprotonation of His-64 is virtually complete and, hence, does not show any variation in its chemical shift position with pH (Figure 6). Its disappearance at higher temperature (Figure 7) shows that there is a positive ΔH and, hence, also a positive ΔS for the formation of the −2.3 ppm conformer (since $\Delta G = \Delta H - T\Delta S$ must be negative for a spontaneous process). The slow equilibrium that produces the two resonances of Val-68 may be due to two conformations of Val-68, that at δ −2.8 being nearer to the normal from the center of the iron atom than that at δ −2.3. Another possibility would be two different conformations for the deprotonated form of His-64, observed by the stationary methyl group of Val-68, in which the chemical shift difference between −2.3 ppm and −2.8 ppm is due to the change in the ring current

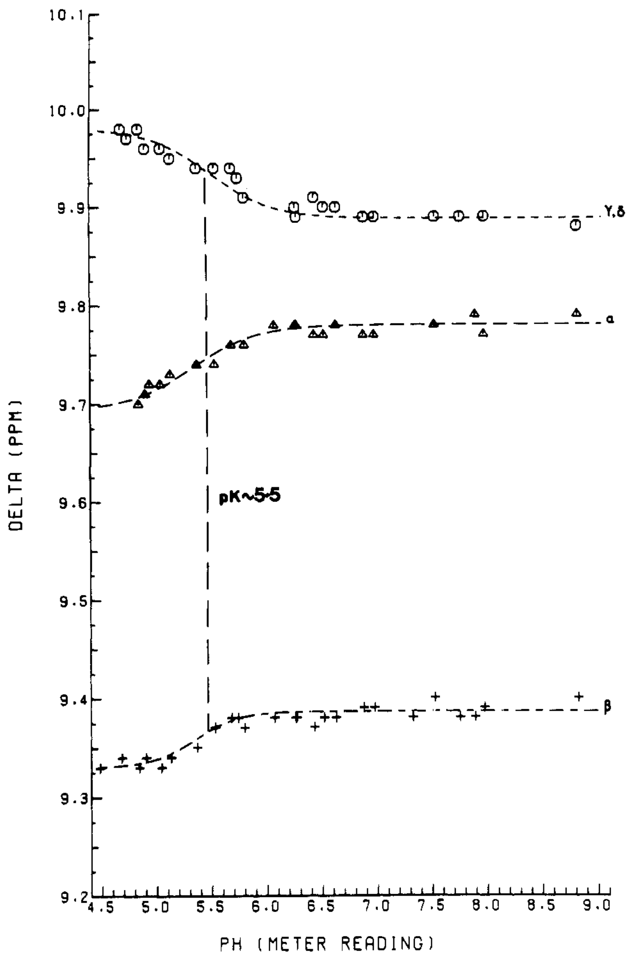

FIGURE 8: pH variation of the meso-heme resonances in the ^{1}H NMR spectrum of sperm whale oxyMb at 20 °C.

of His-64. This seems unlikely, however, because the γ-meso proton that also witnesses the titration of His-64 (see above) does not show any evidence of two states of His-64 at high pH and 20 °C (Figure 2b). The broad resonance of δ −1.6 in sperm whale COMb (and oxyMb, see below) at 20 °C shows a large downfield shift at high pH (Figure 6) and exhibits a large temperature change (Figure 7), implying that it belongs to a near-heme resonance.

Variation with pH of Near-Heme and Meso-Heme Protons in OxyMb. In sperm whale oxyMb, the meso-proton resonances have been assigned by selective deuteration (Bradbury et al., 1982), and their shift with pH (Figure 8) gives a p*K* of ~5.5 for all resonances. In the spectrum of oxyMb, a methyl resonance of Val-68 at δ −2.78 is observed, which is invariant with pH (Figure 9). The broad single-proton resonance at δ ca. −1.6 in COMb at 20 °C (Figure 5) due to a near-heme proton was also observed in oxyMb. This resonance gave a p*K* of 5.6, and at high pH it broadened out and disappeared (Figure 9). The differing pH responses of the meso-heme proton resonances in oxyMb and COMb may not be related to major structural change in the heme–globin contacts since the δ_B values of the δ-, α-, and β-meso proton resonances are very similar in both derivatives, although the γ-meso proton resonance is found in oxyMb ca. 0.25 ppm upfield of its position in COMb. Since for COMb the γ-meso proton is in close proximity to both His-64 and -97 (see above), any alteration in the position of one or both of these residues in oxyMb would affect the chemical shift position of the γ-meso proton.

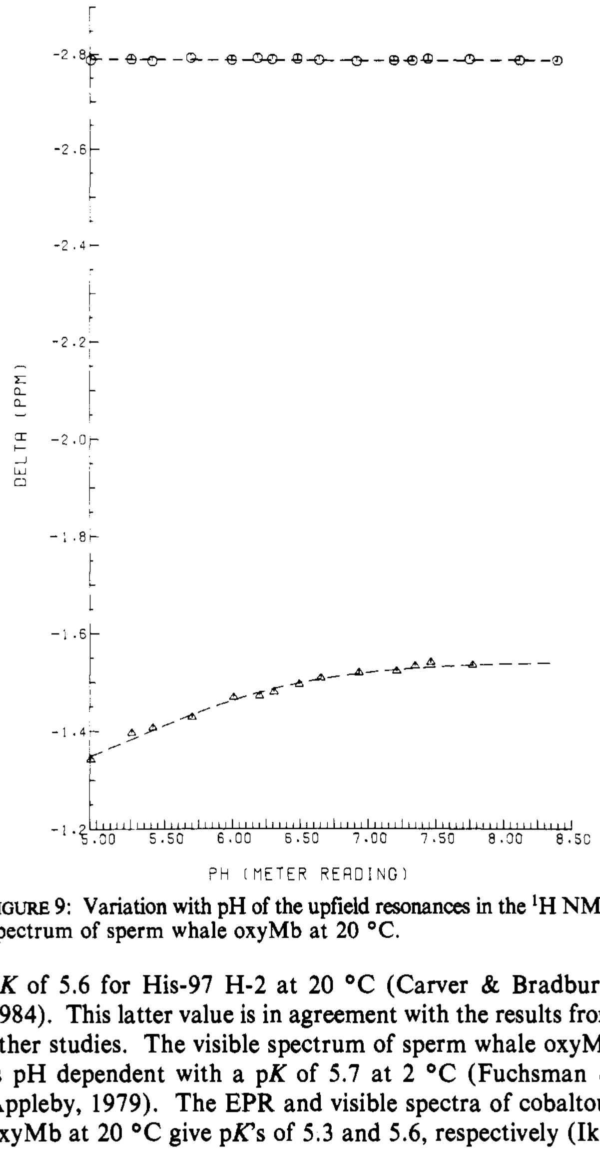

FIGURE 9: Variation with pH of the upfield resonances in the ^{1}H NMR spectrum of sperm whale oxyMb at 20 °C.

In oxyMb, the p*K*'s of 5.5 of the meso-heme proton resonances and the near-heme resonance are very similar to the p*K* of 5.6 for His-97 H-2 at 20 °C (Carver & Bradbury, 1984). This latter value is in agreement with the results from other studies. The visible spectrum of sperm whale oxyMb is pH dependent with a p*K* of 5.7 at 2 °C (Fuchsman & Appleby, 1979). The EPR and visible spectra of cobaltous oxyMb at 20 °C give p*K*'s of 5.3 and 5.6, respectively (Ikeda-Saito et al., 1977). The oxygen affinity of cobaltous Mb at 15 °C decreases with decreasing pH with a p*K* of 5.4. Ikeda-Saito et al. (1977) attributed this acid Bohr effect to the protonation of His-64 rather than to His-97, because of the absence of such behavior in *Glycera* Hb, in which His-64 is replaced by leucine. *Glycera* Hb, however, also does not have a histidine residue at the same near-heme position as His-97 in Mb (Padlan & Love, 1974), and therefore, their results are not inconsistent with the assignment of these effects to His-97 in Mb. An acid Bohr effect in Mb was observed by La Mar et al. (1978), and Ikeda-Saito et al. (1977) postulated that in Mb it involved an increase in the oxygen dissociation rate constant [confirmed by Doster et al. (1982), who found that the oxygen association rate constant is independent of pH over a wide temperature range) and was due to the distal histidine.

Since His-97 titrates with a p*K* value of 5.6 (Carver & Bradbury, 1984), it is probably responsible for the observed NMR changes in oxyMb. However, the lack of observation of titrating resonances for His-64 leaves open the possibility that it exhibits a p*K* similar to His-97, and hence, His-64 may contribute to the acid Bohr effect. Support for the role of His-97 comes from La Mar et al. (1978), who observed shifts with p*K*'s of 5.7 in the hyperfine-shifted resonances of sperm whale deoxyMb, which were attributed to titration of His-97 and not His-64, because of the absence of a "link" (hydrogen bond) between His-64 and the heme group in deoxyMb (Takano et al., 1977b). Such a link is present between His-97 and the propionic acid side chain at position 7 of the heme group (Figure 3). This is supported by the absence of a pH dependence of the hyperfine-shifted resonances of deoxyLb (Trewhella, 1980), in which there is a distal histidine but no histidine comparable to His-97 in Mb and, hence, no link through which an effect could be manifested.

The presence of titrating near-heme resonances with pK's of ~5.7 in deoxyMb (La Mar et al., 1978), CNMb, COMb, and oxyMb [this paper and Carver & Bradbury (1984)] suggests that the residue responsible for such phenomena is involved in similar hydrogen-bonding interactions in all these various derivatives. In crystals of metMb (Watson 1969; Takano, 1977a), deoxyMb (Takano, 1977b), COMb (Hanson & Schoenborn, 1981), and oxyMb (Phillips, 1980), His-97 is hydrogen bonded to the propionic acid side chain at position 7 in the heme ring. The distal histidine (His-64), however, has a variable environment in the above derivatives. In metMb (Watson, 1969; Takano, 1977a) and oxyMb (Phillips & Schoenborn, 1981), it is hydrogen bonded to the nearby water and oxygen ligands, respectively, whereas in deoxyMb (Takano, 1977b) there is no interaction with the heme group and in COMb (Hanson & Schoenborn, 1981) there is no hydrogen bond with the CO ligand. It would therefore be expected that His-97 would exhibit a similar p*K* in all derivatives whereas His-64, because of its different hydrogen-bonding interactions, would exhibit a variable p*K*. The constant p*K* of ~5.7 observed in all derivatives is therefore ascribed to His-97.

Comparison of the Heme Environments in COMb and OxyMb. The upfield Val-68 methyl resonances behave very differently in oxyMb and COMb. In oxyMb, a single resonance at δ −2.78 is observed (Figure 9), whereas in COMb at pH >5.6 and 20 °C there are two resonances at δ −2.8 and δ ca. −2.3 (Figure 6) and only one resonance (δ ca. −2.3) at 40 °C (Figure 4). The −2.3 ppm resonance from the Val-68 methyl in COMb is close enough to His-64 to shift with a p*K* of 4.7 as His-64 (p*K* = 5.0) titrates. It is also the preferred form at higher temperatures, where there is more motional freedom in the molecule. The resonance at δ −2.8 arises from a methyl group of Val-68 which does not respond to the titration of His-64; hence, this methyl group is further away from His-64 than is the "methyl group" that gives the δ −2.3 resonance. It appears that the steric restrictions imposed by the hydrogen bond between oxygen and His-64 in oxyMb have forced Val-68 to adopt a single conformation with a resonance at δ −2.78, whereas in COMb at 20 °C the lack of a hydrogen bond allows more motional freedom and, hence, the occurrence of two different conformations.

Large differences in the NMR behavior of oxyMb and COMb are also shown by the pH dependence of the meso-heme proton resonances of oxyMb (Figure 8) and COMb (Figure 2). The association rate constant for binding CO to Mb increases with decreasing pH with a p*K* of 5.7 (Doster et al., 1982), which implies that it is linked to the titration of His-97, whereas the association rate constant for oxygen binding is independent of pH. For COMb, the dissociation rate constant has not been measured, while the dissociation rate constant of oxyMb increases with decreasing pH at 37 °C (La Mar et al., 1978). Such differences in NMR behavior and ligand binding must be related to structural differences between the heme pockets of COMb and oxyMb, which may arise from several factors.

Changes in the orientation of His-97 relative to the heme group would affect differently the electronic distribution within the heme ring in COMb and oxyMb. The different chemical shifts of the γ-meso proton resonance in oxyMb and COMb are consistent with this. Any movement of His-97 would also affect the strength of the hydrogen bond between this residue and the propionic acid side chain. The distance from the His-97 N-3 to the carboxyl oxygen atoms of the propionic acid side chain is about 3.3 Å in oxyMb and 3.7 Å in COMb (Phillips, 1980; Hanson & Schoenborn, 1981); hence, the His-97 hydrogen bond is stronger in oxyMb than in COMb. In oxyMb compared with COMb, this would cause greater perturbations to the electronic structure of the heme ring upon protonation of His-97 at its N-1 position and result in the observed pH variations of the meso-heme proton resonances in oxyMb. The differing nature of the oxygen and carbon monoxide ligands and their interactions with the distal amino acids side chains are also relevant. The iron–oxygen bond favors bent end-on geometry in model compounds and in Mb (Phillips, 1978, 1980), whereas the iron–carbon monoxide bond favors straight end-on geometry (Peng & Ibers, 1976) but is forced to adopt bent end-on geometry in COMb because of steric constraints (Norvell et al., 1975; Hanson & Schoenborn, 1981). Furthermore, a hydrogen bond occurs between the distal histidine and oxygen ligand at all pH values (Yonetani et al., 1974; Ikeda-Saito et al., 1977; Phillips & Schoenborn, 1981) but is absent in COMb (Norvell et al., 1975). Also, the charge separation of CO when bound to the iron atom is not as great as that with oxygen (Chang & Traylor, 1975), and this difference is considered responsible for the observed differences between the pH dependences of the CO and oxygen association rate constants (Doster et al., 1982). Thus, the above factors may all contribute to and/or result from differences in the heme pocket, observed by NMR and other techniques, betwen COMb and oxyMb.

Comparison of Oxy and Carbon Monoxy Complexes of Mb and Lb. The meso proton resonances in COLb do not shift with pH (R. N. Johnson, J. H. Bradbury, and C. A. Appleby, unpublished results) whereas the comparable resonances in oxyLb shift about 0.5 ppm with pH with a p*K* of ~5.5 (Appleby et al., 1983). They have been assigned in oxyLb and COLb by selective deuteration (Bradbury et al., 1982) and by using nuclear Overhauser effects (Mabbutt & Wright, 1983).

The H-2 resonance of the distal histidine in COLb occurs at $\delta_B \sim 5.2$ and undergoes a titration shift of 2.6 ppm (p*K* ~ 4.1) to give a normal unperturbed acid-limiting chemical shift. The large shift was attributed by Johnson et al. (1978) to the imidazole ring moving away from the heme ring current upon protonation, yet this movement is not monitored by any of the meso-proton resonances. By contrast, in COMb there is a normal titration shift (Carver & Bradbury, 1984) of the perturbed H-4 distal histidine resonance, and the movement of the distal histidine on protonation is considered to be much smaller than that in COLb (Fuchsman & Appleby, 1979). The γ-meso heme resonance in COMb, however, does shift (Figure 2) in response to the titration of His-64. These differences in behavior may be caused by the distal histidine being closer to the heme in COMb than in COLb. Furthermore, the much smaller heme pocket in COMb compared with COLb would not allow as much movement in COMb of the imidazole ring on protonation as in COLb. Similarly, the large differences in chemical shifts of the meso-proton resonances between COLb and oxyLb and the absence of large differences between COMb and oxyMb (Bradbury et al., 1982) are further evidence for a much more flexible heme pocket in Lb compared with Mb.

In oxyLb the pK of ~5.5 observed for the meso-proton resonances is attributed to the titration of the distal histidine (there is no other titrating histidine in Lb). Protonation of the distal histidine causes a hydrogen bond with the oxygen ligand to form and the rate of dissociation of the ligand to be lowered (Ikeda-Saito et al., 1981; Appleby et al., 1983). Thus, in oxyLb there is a stronger interaction with the ligand at low pH, whereas in COLb at low pH the distal histidine moves away from the bound carbon monoxide.

In oxyLb, the titration shift of the meso-proton resonances is ~0.5 ppm (Appleby et al., 1983) whereas in oxyMb it is only ~0.1 ppm. In oxyLb the δ and β resonances move upfield and the γ and α resonances downfield, whereas in oxyMb the γ and δ resonances move upfield and the α and β resonances downfield with increasing pH. These differences can be related to the variation in oxygen affinity of Mb and Lb at low pH. The oxygen affinity of cobalt-substituted Mb at 15 °C decreases about 0.6 times on lowering the pH from 7.0 to 4.8 (Ikeda-Saito et al., 1977), whereas the oxygen affinity of cobalt-substituted Lb increases about 4-fold in a similar pH range (Ikeda-Saito et al., 1981), and a similar result is obtained for native ferrous Lb (Appleby et al., 1983). In oxyLb, the hydrogen-bonding interaction present at low pH would be transferred via the centrally placed iron-oxygen bond to the porphyrin plane and result in similar shifts with pH for the resonances of the diagonally opposed δ- and β-meso protons and similarly for the γ- and α-meso proton resonances. On the other hand, in oxyMb it is likely that protonation of His-97 is responsible for the observed change in oxygen affinity at low pH. His-97 is hydrogen bonded to the propionic acid group at position 7 on pyrrole ring 1, which is flanked by the methine bridges that contain the δ- and γ-meso protons (Figure 3). These protons therefore experience identical perturbations to their environment by protonation of His-97, transmitted through the hydrogen bond to pyrrole ring 1. The other two meso protons (α and β) experience an opposite effect, which balances that observed for the δ- and γ-meso proton resonances (Figure 8). The smaller chemical shift changes observed for the meso-proton resonances of oxyMb compared with oxyLb are consistent with the much less intimate association between the oxygen ligand and the acid Bohr donating group, i.e., His-97 in oxyMb as compared with the distal histidine in oxyLb.

Acknowledgments

We thank Dr. A. J. Jones and W. M. Begg of the National NMR Centre and Dr. G. A. Jenkins for obtaining the NMR spectra, M. W. Parker for the titration curves in Figure 8, Dr. Meta Sterns for help with the computer preparation of Figures 1 and 3, and Drs. S. E. V. Phillips, B. P. Schoenborn, and T. Takano for providing coordinates of oxyMb, COMb, deoxyMb, and metMb. We thank the Australian Research Grants Scheme for supporting this work.

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Photochemically Induced Nuclear Polarization Study of Exposed Tyrosines, Tryptophans, and Histidines in Postsynaptic Neurotoxins and in Membranotoxins of Elapid and Hydrophid Snake Venoms[†]

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ABSTRACT: The accessibility of surface tyrosines, histidines, and tryptophans in snake venom neurotoxins (short and long) and in membranotoxins to excited triplet 10-(carboxyethyl)-flavin was studied by photochemically induced dynamic nuclear polarization at 270 MHz. Trp-29 is accessible in the short neurotoxins—erabutoxins a, b, and c and cobrotoxin—and also in the long neurotoxins—α-cobratoxin and α-bungarotoxin. Tyr-25 is practically inaccessible in all neurotoxins. Tyr-39 in cobrotoxin and Tyr-55 in α-bungarotoxin are accessible. His-6 (revised sequence) is inaccessible in the erabutoxins while His-26 is only very weakly accessible. His-22 of α-cobratoxin is inaccessible as are His-4 and -68 in α-bungarotoxin and His-4 of cobrotoxin. His-33 of cobrotoxin is accessible. The rigidity order α-bungarotoxin $\geq$ α-cobratoxin $\geq$ erabutoxins, with respect to the unfolding effect of 7 M urea, was deduced in this study. In the membranotoxins studied (cardiotoxin and its analogues I, II, and IV as well as cytotoxin I and II), the two tyrosines Tyr-25 and Tyr-58 are only weakly accessible. Tyr-14 is completely accessible and so is in all probability Tyr-29. These studies allow deductions to be made about the accessibilities in analogous systems. Thus, the accessibility of His-33 and the inaccessibility of His-4 in cobrotoxin can be used to deduce the conformations of these residues in a large group of neurotoxins including the α-toxin of *Naja nigricollis*, neurotoxin II of *Naja naja oxiana*, and neurotoxins I and III of *Naja mossambica mossambica*. As a generalization of this study, we suggest that in snake venom toxins residues distant only one or two peptide units from a disulfide bond are inaccessible, while more distant residues have accessible side chains. Significant differences were observed between solution and crystal conformations in α-bungarotoxin and α-cobratoxin.

The solution conformations of snake venom toxins (Tu, 1977; Yang, 1978; Karlsson, 1979; Low, 1979; Strydom, 1979) are of obvious importance in determining their physiological effects, their binding at the target organs, and their inactivation by specific immunoglobulins (Boquet, 1979). Very recently, much detailed information on many aspects of solution conformations of the snake venom toxin proteins has been provided by high-resolution nuclear magnetic resonance studies [see, e.g., Arseniev et al. (1981), Endo et al. (1982), Miyazawa et al. (1983), Steinmetz et al. (1981), Fung et al. (1979), and Hider et al. (1982)]. These studies considered parameters such as pH, nuclear proximity, and inter peptide residue interactions, temperature, and group mobility, as well as effects of chemical modifications and their influence on chemical shifts, signal structure, and exchange rates.

In this paper we describe a different approach to the study of solution conformations of these molecules. This approach is based on the application of the triplet-dye-induced nuclear polarization method (protein photo-CIDNP)[1] [see, e.g., Muszkat et al. (1981, 1983) and Berliner & Kaptein (1981) and references cited therein] to the study of surface conformations and surface residue exposure of the snake venom toxins.

Briefly speaking, the protein photo-CIDNP method consists of a normal proton high-resolution NMR experiment modified by a simultaneous process of chemically reversible radical pair formation. The availability of suitable radical pair forming processes allows the application of this method to three peptide residues, tyrosine, histidine, and tryptophan. The radical pair formation process in question involves the reversible transfer of a hydrogen atom H from the aromatic hydroxyl of a tyrosine residue (AOH) or an imidazolyl NH of a histidine residue

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[1] Abbreviations: photo-CIDNP, photochemically induced dynamic nuclear polarization; SDS, sodium dodecyl sulfate.

0006-2960/84/0423-4913$01.50/0