Resonance Raman Studies of Acetylheme-Reconstituted Myoglobins. Characterization of 2- versus 4-Substituent/Protein Interactions†

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ABSTRACT: Resonance Raman (RR) spectra are reported for the deoxy, oxy, and metcyano species of sperm whale myoglobin (Mb) reconstituted with deuteroheme, 2-acetyldeuteroheme, 4-acetyldeuteroheme, and 2,4-diacetyldeuteroheme. The functional inequivalence of the 2- and 4-positions of the heme moiety is manifested in the vibrational frequencies of the carbonyl and certain heme skeletal modes. The RR data indicate that the protein influences the porphyrin π-system primarily through the 2-substituent. In the deoxyMbs, the 2-acetyl group is more conjugated into the π-macrocycle than the 4-acetyl group. In the ligated Mbs, the extent of conjugation of the 2-group is less than in the deoxy forms. This result indicates that ligand binding differentially alters the π-electronic structure for 2- vs 4-substituted systems. The proximal histidine–iron stretching vibration of all four deoxyMbs occurs at 222 ± 1 cm^{-1}, which indicates that the substituent-induced changes in the π-electronic structure do not result in large changes of the electron density in the axial ligand bonds. RR spectra recorded immediately after heme reconstitution indicate the presence of two inequivalent forms of the protein. ^1H NMR spectroscopy indicates that these two forms are the normal and reversed heme orientational isomers previously described by La Mar and co-workers [La Mar, G. N., Budd, D. L., Viscio, D. B., Smith, K. M., & Langry, K. C. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5755–5759]. The RR spectra demonstrate that in the deoxy species the 2-acetyl group of the normal form is conjugated into the macrocycle to approximately the same extent as the 4-acetyl group of the reversed form and vice versa. The iron–histidine stretching frequency of the reversed form of all four proteins is different than that of the normal form.

The oxygen-binding heme protein myoglobin (Mb)[1] has been extensively studied by a variety of biochemical, spectroscopic, and physical techniques (Antonini & Brunori, 1971; Chang, 1979). Despite these efforts, certain questions remain concerning the factors which control the O_2 binding affinity. It has been shown that Mbs reconstituted with hemes containing 2- and/or 4-substituents different from those of native protoheme exhibit altered O_2 affinities (Sono & Asakura, 1975; Kawabe et al., 1982; Chang et al., 1984). In addition, Mbs reconstituted with 2/4-structural isomers exhibit different affinities. The differences in affinity observed for these heme isomers cannot be attributed to the intrinsic structural inequivalence of the 2- and 4-positions of the porphyrin macrocycle because spectroscopic studies of metalloporphyrins in vitro have shown that the electronic structures of 2/4-isomers are essentially identical (Willems & Bocian, 1984, 1985). Thus, the altered affinities must be due to site-specific substituent/protein interactions. The protein could influence the affinity directly by mediating the electronic interaction between the 2- and/or 4-groups and the π-system of the macrocycle, thus altering the electron density on the iron ion. Alternatively, interactions between the protein and the 2- and/or 4-groups might indirectly influence the affinity by globally changing the heme environment (for example, by perturbing the proximal histidine–heme iron bond strength and/or the interaction between the distal histidine and O_2). The relative importance of direct vs indirect mediation of the O_2 affinity by 2- and/or 4-substituent/protein interactions has not been determined.

The characterization of the factors that influence the O_2 binding affinity of reconstituted Mbs is complicated by the apparent ability of the heme to insert into the apoprotein not only in the normal orientation but also in a form rotated 180° about the α,γ axis of the heme (heme orientational disorder) (La Mar et al., 1978, 1983, 1984; Miki et al., 1981; Docherty & Brown, 1982; Ahmad & Kincaid, 1983; Jue et al., 1983; Lecomte et al., 1985). These two orientational isomers are formed in approximately equal amounts upon reconstitution; with time, the system equilibrates and the more stable normal orientation predominates. The protein environment around the 2/4-positions of the heme is different in the two rotational isomers. This inequivalence may influence the relative O_2 binding affinities of the two forms (Livingston et al., 1984; Gersonde et al., 1986; Light et al., 1987).

Resonance Raman (RR) spectroscopy has proven to be an extremely useful probe of the heme environment in heme proteins (Adar & Erecinska, 1974; Adar, 1975; Spiro & Burke, 1976; Felton & Yu, 1978; Babcock & Salmeen, 1979; Kitagawa et al., 1979; Tsubaki et al., 1980; Choi et al., 1982b; Choi & Spiro, 1983; Spiro, 1983; Babcock, 1987). RR studies of modified heme-reconstituted Mbs have revealed various substituent group/protein interactions (Tsubaki et al., 1980; Choi et al., 1982a,b; Choi & Spiro, 1983). Surprisingly, only one RR study has appeared in which 2- vs 4-substituent/protein interactions were specifically probed (Tsubaki et al.,

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[1] Abbreviations: Mb, myoglobin; RR, resonance Raman; DH, deuteroheme; 2-AcDH, 2-acetyldeuteroheme; 4-AcDH, 4-acetyldeuteroheme; 2,4-AcDH, 2,4-diacetyldeuteroheme; DP, deuteroporphyrin IX diacid; 2-AcDP, 2-acetyldeuteroporphyrin IX diacid; 4-AcDP, 4-acetyldeuteroporphyrin IX diacid; 2,4-AcDP, 2,4-diacetyldeuteroporphyrin IX diacid; DMb, 2-AcMb, 4-AcMb, and 2,4-AcMb, myoglobin reconstituted with deuteroheme, 2-acetyldeuteroheme, 4-acetyldeuteroheme, and 2,4-diacetyldeuteroheme, respectively; NMR, nuclear magnetic resonance; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate.

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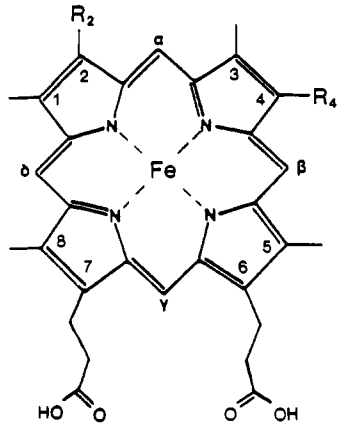

	R_2	R_4
deutero	H	H
2-acetyl	$CO-CH_3$	H
4-acetyl	H	$CO-CH_3$
2,4-diacetyl	$CO-CH_3$	$CO-CH_3$

FIGURE 1: Schematic representation and atomic labeling scheme of the hemes incorporated into sperm whale Mb.

1980). This study employed Mb reconstituted with spirographis (2-formyl-4-vinyldeuteroheme), isospirographis (2-vinyl-4-formyldeuteroheme), and 2,4-diformyldeuteroheme. Tsubaki et al. concluded that in the protein matrix the formyl group in the 2-position is more conjugated into the porphyrin π-system than is the group in the 4-position. This result, together with the observation that the O_2 affinity for the former isomer is lower than that for the latter (Asakura & Sono, 1974), suggests that electronic effects may play a role in mediating the affinity. However, no clear relationship was found between the affinity and the vibrational frequencies of either the proximal histidine–iron bond or the iron–O_2 bond. This result implies that O_2 binding is not controlled by electronic factors alone.

In this paper we report a RR study of deoxyMb, oxyMb, and metcyanoMb reconstituted with hemes selectively modified at the 2- and 4-positions of the macrocycle. These hemes are shown in Figure 1 and include deuteroheme (DH), 2-acetyldeuteroheme (2-AcDH), 4-acetyldeuteroheme (4-AcDH), and 2,4-diacetyldeuteroheme (2,4-AcDH). This series of molecules is particularly attractive for study because site-specific 2/4-substituent/protein interactions can be monitored without the complications introduced by a second bulky substituent group. The sensitivity of the acetyl carbonyl vibrational frequency to the heme environment also allows us to probe the effects of heme orientational disorder. Collectively, these studies provide a more comprehensive picture of the nature of 2- vs 4-substituent/protein interactions.

Materials and Methods

The free base dimethyl ester of deuteroporphyrin IX (DP) was purchased from Midcentury (Posen, IL) and used as received. 2-Acetyldeuteroporphyrin IX (2-AcDP), 4-AcDP, and 2,4-AcDP were prepared and separated according to published procedures (Brockmann et al., 1968; Miller & Rapaport, 1977). Deesterification and iron insertion were accomplished by standard methods (Dolphin et al., 1976; Furhop & Smith, 1978).

Sperm whale Mb was obtained from Sigma (St. Louis, MO) and used as received. The acid/2-butanone procedure (Teale, 1959) was used to remove the heme. Immediately after heme removal, the apoprotein solution was dialyzed vs dilute $NaHCO_3$ (40 mg/L) and then several times vs distilled water. Any denatured protein was removed by centrifugation. The solution was finally dialyzed vs 0.05 M KH_2PO_4/Na_2HPO_4 buffer, pH 6.9. ApoMb solutions were kept for a maximum of 4 days before being discarded. The concentration was determined by using the known $\epsilon_{280} = 15.9\ cm^{-1}\ M^{-1}$ (Harrison & Blout, 1965). Reconstitution was accomplished by dropwise addition of a stoichiometric amount of the desired heme (dissolved in minimal 0.1 M KOH and diluted with buffer) to the stirring apoMb solution. The reconstituted Mbs were then filtered and allowed to equilibrate in the metaquo form. The corresponding metcyano proteins were formed by addition of excess KCN to the metaquo species. The deoxyMb and oxyMb complexes were prepared by reducing the metaquo protein with sodium dithionite under an N_2 or O_2 atmosphere, respectively. All of the protein preparations were performed in a cold room maintained at 4 °C.

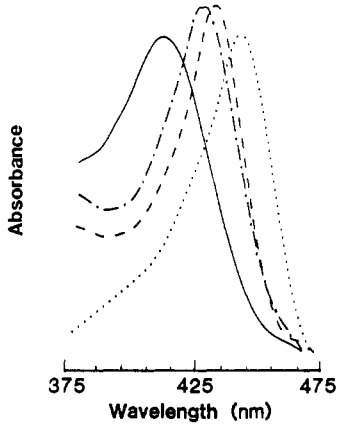

FIGURE 2: Soret-region absorption spectra of reconstituted deoxyMbs: (—) DMb, λ_{max} = 422 nm; (- - -) 2-AcMb, λ_{max} = 438 nm; (—·—) 4-AcMb, λ_{max} = 435 nm; (···) 2,4-AcMb, λ_{max} = 449 nm. Concentration of the proteins was ~0.5 mM in KH_2PO_4/Na_2HPO_4 buffer, pH 6.9.

Resonance Raman spectra of the various Mb species were recorded on a Spex 1403 double monochromator equipped with a photon-counting detection system and a thermoelectrically cooled Hamamatsu R928P photomultiplier tube. B-state excitation was provided by the discrete outputs of either a Coherent K-2000 K^+ or a Coherent INNOVA-15UV Ar^+ laser. The incident power at the sample was ~10–15 mW. Spectra were acquired at 1-cm^{-1} intervals at a spectral slit width of ~4 cm^{-1}. The protein solution was placed in a spinning cell in order to prevent photodegradation. Typical concentrations of the Mb samples were ~0.05–0.1 mM. Sodium sulfate (~0.5 M) was used as an internal standard.

^1H nuclear magnetic resonance (NMR) spectra of the metcyanoMb complexes were recorded at 38 °C on a General Electric GN300 narrow-bore spectrometer operating at 300 MHz (quadrature phase detection). The solvent signal was suppressed by using a binomial pulse sequence. A typical spectrum consisted of 4000 transients (collected in double-precision mode) over a 21-kHz bandwidth. The NMR spectra were acquired immediately after addition of KCN to aliquots of metaquo protein that had been allowed to equilibrate for times of 1, 4, 8, or 24 h after reconstitution. The samples were ~0.5–1.0 mM in 90% H_2O/10% D_2O (0.05 M PO_4^{3-} buffer, pH 6.9). 2,2-Dimethyl-2-silapentane-5-sulfonate (DSS) was used as an internal reference.

Results

Absorption Spectra. The Soret region absorption spectra of the deoxy species of Mb reconstituted with DH, 2-AcDH,

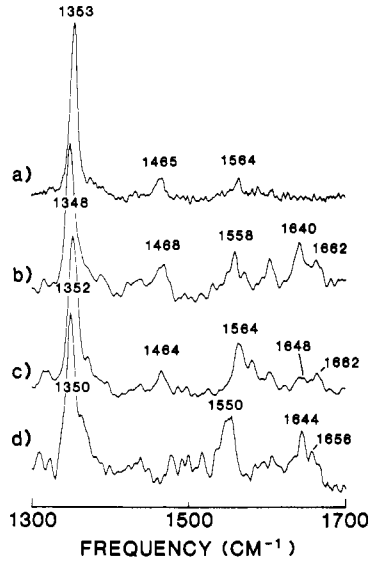

FIGURE 3: High-frequency regions of Soret-excitation RR spectra of (a) deoxyDMb (λ_{ex} = 413.1 nm, acquired 1 day after reconstitution), (b) deoxy-2-AcMb (λ_{ex} = 413.1 nm, acquired 2 days after reconstitution), (c) deoxy-4-AcMb (λ_{ex} = 413.1 nm, acquired 3 weeks after reconstitution), and (d) deoxy-2,4-AcMb (λ_{ex} = 457.9 nm, acquired 1 day after reconstitution).

4-AcDH, and 2,4-AcDH (DMb, 2-AcMb, 4-AcMb, and 2,4-AcMb, respectively) are shown in Figure 2 and are typical of those observed for high-spin ferrous heme proteins (Adar, 1978). The Soret maxima for the three acetylheme-reconstituted Mbs (2-AcMb, 438 nm; 4-AcMb, 435 nm; 2,4-AcMb, 449 nm) are to the red of the Soret maximum observed for DMb (422 nm), as is the case for the protein-free heme derivatives in solution. The Soret maxima for both the oxy and metcyano forms exhibit the same relative ordering but are blue shifted relative to the deoxy form. The difference in Soret maxima for the two different monoacetyl Mbs is noteworthy because the two heme complexes exhibit identical absorption spectra in vitro. Thus, the spectral differences observed for 2-AcMb vs 4-AcMb must be attributed to substituent/protein interactions.

RR Spectra. The high-frequency regions of the Soret-excitation RR spectra of the four reconstituted deoxyMbs are presented in Figure 3. The frequencies for several pertinent RR bands are summarized in Table I. The frequencies of the analogous modes of the oxy and metcyano forms of certain Mbs are also included in the table. The spectra that are shown in Figure 3 and the data that are presented in the first three columns of Table I were obtained from protein samples that had been allowed to equilibrate as the metaquo species for at least 1 day following reconstitution. In general, the RR spectra are similar to those observed for other heme proteins in the same oxidation and/or spin states (Choi et al., 1982b; Spiro, 1983). RR spectra were also acquired for the high- and low-spin, ferrous, and ferric hemes in solution (not shown). Particularly noteworthy is the observation that 2-AcDH and 4-AcDH exhibit identical RR spectra in solution for a given spin and/or oxidation state. These spectra are not, however, identical with those of DH or 2,4-AcDH. Similar observations have been reported for the Ni(II) complexes of these four DP derivatives (Willems & Bocian, 1984). Furthermore, comparison of the RR spectra of the nickel derivatives with those of the iron derivatives in any spin and/or oxidation state reveals that all trends which are observed in the frequencies of analogous modes of the different nickel derivatives are nearly identical with those that are observed in the frequencies of the same modes of the different iron derivatives. These trends

Table I: Comparison of RR Frequencies (cm^{-1}) of Ligated and Unligated Mbs

	equilibrated[a]			unequilibrated[b]	
	deoxy	oxy (Δ_{deoxy})[c]	metcyano (Δ_{deoxy})[c]	deoxy	metcyano (Δ_{deoxy})[c]
DMb					
ν_2	1564	1583 (+19)	1583 (+19)	1566	1583 (+17)
ν_4	1353	1376 (+23)	1374 (+21)	1353	1372 (+19)
ν(Fe–His)	223	*d*	*d*	217	*d*
ν(Fe–CN)			449		450
2-AcMb					
ν(C=O)	1640	*d*	*d*	1648	*d*
ν_2	1558	1579 (+21)	1578 (+20)	1560	1578 (+18)
ν_4	1348	1375 (+27)	1373 (+25)	1350	1372 (+22)
ν(Fe–His)	222	*d*	*d*	>222[e]	*d*
ν(Fe–CN)			451		451
4-AcMb		*f*			
ν(C=O)	1648		*d*	1644	*d*
ν_2	1564		1582 (+18)	1560	1583 (+23)
ν_4	1352		1374 (+22)	1352	1372 (+22)
ν(Fe–His)	220		*d*	>222[e]	*d*
ν(Fe–CN)			452		452
2,4-AcMb					*f*
ν(C=O)	1656	*d*	*d*	1654[g]	
	1644			1642	
ν_2	1550	1580 (+30)	1578 (+28)	1550	
ν_4	1350	1373 (+23)	1373 (+23)	1352	
ν(Fe–His)	222	*d*	*d*	>222[e]	
ν(Fe–CN)			452		

[a] RR spectra obtained at least 1 day after reconstitution. [b] RR spectra obtained less than 6 h after reconstitution. [c] $\Delta_{deoxy} = \nu_{ligated} - \nu_{deoxy}$. [d] Not observed. [e] Unequilibrated forms exhibit higher frequency than equilibrated forms (see text). [f] RR spectra not obtained for this complex. [g] Obtained via spectral deconvolution (see text).

serve as the reference point for trends that are observed in the RR spectra of the various reconstituted proteins. Pertinent features of the RR spectra of the various Mbs and the relationship of these spectra to those of the model compounds are summarized below.

(1) Deoxy Derivatives. The RR spectra of the deoxyMbs (Figure 3) exhibit several modes which can be used as indicators for protein-induced perturbations in the properties of the heme moieties. In particular, both the acetyl carbonyl and the iron histidine stretching modes are observed for the deoxy species (Table I). The observation of these RR bands along with those due to heme skeletal modes allows the assessment of the influence of the protein on different regions of the heme moiety.

(a) Carbonyl Stretching Modes. The most notable feature in the high-frequency region of the RR spectra is the acetyl carbonyl stretching mode, ν(C=O). This vibrational mode is observed as a relatively intense band at 1640 cm^{-1} for 2-AcMb and a much weaker, extremely broad band centered near 1648 cm^{-1} for 4-AcMb. [It should be noted that the band observed near 1660 cm^{-1} is not a carbonyl vibration but rather is a combination mode which gains intensity through interaction with the carbonyl stretch (Tsubaki et al., 1980).] RR studies of these 2/4-isomers in nonaqueous, non-hydrogen-bonding solvents have shown that the frequencies and intensities of ν(C=O) are identical for the two derivatives (Willems & Bocian, 1984). Thus, the difference in ν(C=O) for 2-AcMb vs 4-AcMb must be protein induced, as is the difference in the Soret absorption maximum of the two species.

For 2,4-AcMb, a prominent band is observed at 1644 cm^{-1} while a weaker shoulder appears at 1656 cm^{-1}. On the basis of their relative frequencies and intensities, these two bands are assigned to the in-phase and out-of-phase carbonyl stretches, respectively (Willems & Bocian, 1984). For metallo 2,4-AcDPs in solution, the in-phase and out-of-phase com-

ponents of ν(C=O) are separated by $\sim$7 cm^{-1} and the average frequency of the two components is upshifted ($\sim$3 cm^{-1}) relative to ν(C=O) of the monoacetyl complexes. The observation that the difference in frequency between the two carbonyl modes of 2,4-AcMb (12 cm^{-1}) is larger than that for the derivative in vitro (7 cm^{-1}) suggests that the protein either enhances the coupling between the two modes or diminishes the coupling and changes the intrinsic frequency of one group relative to the other. Enhanced coupling would be expected if the conjugation of one or both of the carbonyl groups is increased in the protein relative to that which occurs in solution, whereas diminished coupling could result if one of the two carbonyl groups were hydrogen bonded.

(*b*) *Heme Skeletal Modes.* Comparison of the RR spectra reveals that certain analogous high-frequency porphyrin skeletal modes of the various reconstituted Mbs occur at significantly different frequencies (see Figure 3). In particular, the ν_2 (C_bC_b) stretching vibrations appear at 1564 (DMb), 1558 (2-AcMb), 1564 (4-AcMb), and 1550 cm^{-1} (2,4-AcMb), while the ν_4 (C_aN) stretching modes are observed at 1353, 1348, 1352, and 1350 cm^{-1}, respectively. RR studies of metalloporphyrins in vitro have shown that ν_2 occurs at the same frequency for DP, 2-AcDP, and 4-AcDP and is 4–5 cm^{-1} lower for 2,4-AcDP, whereas the ν_4 modes of all four derivatives are observed at the same frequency (Willems & Bocian, 1984). The nearly identical frequencies observed for the skeletal modes of DMb and 4-AcMb indicate that the electronic structures of DH and 4-AcDH are nearly identical in the protein matrix, as is the case for these two hemes in solution. On the other hand, the substantially lower frequencies of the ν_2 and ν_4 modes of 2-AcMb relative to the analogous vibrations of both DMb and 4-AcMb indicate that the protein perturbs the π-electronic structure of 2-AcDH relative to that of DH and 4-AcDH. These observations imply that the heme moiety interacts with the protein through the 2-substituent but not the 4-substituent.

The skeletal-mode frequencies that are observed for 2,4-AcMb are also consistent with the assessment that the protein interacts with only one of the two acetyl groups. Both ν_2 and ν_4 for this species are lower than would be expected if the π-electronic structure of the heme is unperturbed (relative to DMb) by intercalation into the protein. However, the downshifts for the skeletal modes of 2,4-AcMb are less than might be expected if both acetyl groups were influenced by the protein to the extent that the 2-group of 2-AcMb is influenced. In particular, the frequency of ν_4 for 2,4-AcMb (1350 cm^{-1}) approaches that of ν_4 for 2-AcMb (1348 cm^{-1}) but is not lower. On the basis of the results obtained for 2-AcMb and 4-AcMb, it seems reasonable to assume that the perturbed acetyl group of 2,4-AcMb is at the 2-position.

(*c*) *Other Vibrational Modes.* The low-frequency RR spectra of the various reconstituted Mbs (not shown) reveal that the other porphyrin skeletal modes of these systems are not significantly different in frequency. In addition, the proximal histidine–iron stretching frequencies, ν(Fe–His), of all four species are observed at 222 $\pm$ 1 cm^{-1}, as is the case for native, spirographis, isospirographis, and 2,4-diformyl Mbs (Tsubaki et al., 1980). These results indicate that protein-induced changes in the π-electronic structure of the heme are not directly manifested in the Fe–His bond strength.

(*2*) *Oxy and Metcyano Derivatives.* The vibrational frequencies of the oxy complexes of DMb, 2-AcMb, and 2,4-AcMb are in general quite similar to those of the corresponding metcyano complexes (Table I). (RR spectra could not be obtained for oxy-4-AcMb due to severe photoinstability of the protein. Given the similarity of the vibrational frequencies of the oxy and metcyano forms of DMb, 2-AcMb, and 2,4-AcMb, the frequencies of oxy-4-AcMb are also probably similar to those of metcyano-4-AcMb.) The RR spectra of the ligated forms of the reconstituted Mbs are less informative than those of the deoxy species. For the ligated forms, neither the ν(C=O) nor the ν(Fe–His) modes are observed. The former mode is apparently weak and/or obscured by the strong porphyrin skeletal mode ν_{10}. The latter mode is expected to be near 270 and 315 cm^{-1} for the oxy (Walters & Spiro, 1982) and metcyano (Kerr et al., 1985) forms, respectively. However, ν(Fe–His) is weak for ligated forms and falls in a spectral region that is heavily congested. In this context, the ν(C=O) and ν(Fe–His) modes are also unobserved in the RR spectra of the oxy forms of spirographis, isospirographis, and 2,4-diformyl Mbs (Tsubaki et al., 1980).

(*a*) *Heme Skeletal Modes.* The frequencies of ν_2 and ν_4 of metcyano-4-AcMb are essentially identical with those of the corresponding modes of the ligated DMbs. For the ligated 2-AcMbs, the frequency of ν_4 is also similar to that of the ligated DMbs and metcyano-4-AcMb while the frequency of ν_2 of the former species is lower than that of the latter two. The trends in the frequencies of the skeletal modes of the ligated forms of DMb, 2-AcMb, and 4-AcMb generally parallel those for the deoxy species with the exception that the frequency of ν_4 for the ligated 2-AcMbs is the same as that of ν_4 for the metcyano-4-AcMb and DMbs rather than lower. These observations suggest that, in the ligated state, the heme moiety interacts with the protein through the 2-substituent as is the case for the deoxyMbs. However, the extent of the protein-induced perturbation appears to be somewhat different for the six- vs five-coordinate heme.

The skeletal-mode frequencies that are observed for the ligated 2,4-AcMbs are in general similar to those that are observed for ligated 2-AcMbs. In particular, the ν_2 modes of the mono- and diacetyl complexes occur at essentially identical frequencies. This result can be contrasted with that which is observed for either the deoxy protein species or model hemes. The frequency of ν_2 for deoxy 2,4-AcMb is substantially lower than that of deoxy-2-AcMb (8 cm^{-1}) while for model complexes it is somewhat lower (5 cm^{-1}). Collectively, these observations suggest that the protein perturbs the π-electronic structure of 2,4-AcDH in ligated protein complexes in a substantially different fashion than in the deoxy complex.

(*b*) *Other Vibrational Modes.* Although the ν(Fe–His) modes are not observed for any of the ligated species, the iron–cyanide stretching mode, ν(Fe–CN) is observed at 451 $\pm$ 1 cm^{-1} for all four reconstituted proteins (Table I). The frequency that is observed for ν(Fe–CN) of the metcyano Mbs is similar to that which has previously been reported for monomeric insect hemoglobins (Gersonde et al., 1987). The similarity in ν(Fe–CN) for the various protein species indicates that this axial vibration is not sensitive to protein-induced changes in the π-electronic structure of the heme.

(*3*) *Time Dependence of the RR Spectra.* The high-frequency regions of the Soret-excitation RR spectra of deoxy-2-AcMb and deoxy-4-AcMb which were recorded at various times after reconstitution are shown in Figures 4 and 5, respectively. As can be seen, these RR spectra change with time. The changes are most pronounced in the region of the carbonyl modes. The RR spectrum of deoxy-2-AcMb initially exhibits a strong band at 1640 cm^{-1} and a shoulder at 1648 cm^{-1} (Figure 4a). Six hours after reconstitution, the higher frequency shoulder is decreased in intensity relative to the lower frequency band (Figure 4b). After 3 weeks, only a single sharp

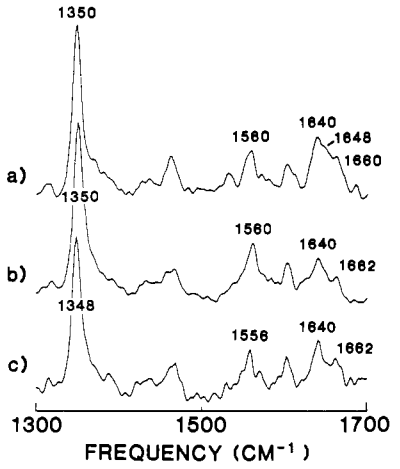

FIGURE 4: High-frequency regions of Soret-excitation (λ_{ex} = 413.1 nm) RR spectra of 2-AcMb obtained as a function of time after reconstitution. Spectra were acquired on the deoxy species which had equilibrated as the metaquo protein for (a) 3 h, (b) 6 h, and (c) 3 weeks after reconstitution.

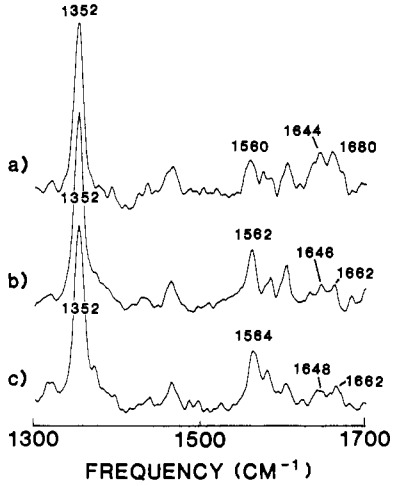

FIGURE 5: High-frequency regions of Soret-excitation (λ_{ex} = 413.1 nm) RR spectra of 4-AcMb obtained as a function of time after reconstitution. Spectra were acquired on the deoxy species which had equilibrated as the metaquo protein for (a) 2 h, (b) 6 h, and (c) 2 days after reconstitution.

peak is observed at 1640 cm^{-1} (Figure 4c). A similar but opposite trend is observed in the carbonyl region of the RR spectrum of deoxy-4-AcMb (Figure 5). For this species, a relatively sharp band is observed at 1644 cm^{-1} 2 h after reconstitution (Figure 5a). With time, this band decreases in intensity, and eventually only a broad maximum at 1648 cm^{-1} remains (Figure 5b,c). The frequencies of the ν_2 bands of deoxy-2-AcMb and deoxy-4-AcMb also exhibit a time dependence. This mode of the former complex appears to downshift slightly (1560 to 1558 cm^{-1}) as the system equilibrates while that of the latter upshifts (1560 to 1564 cm^{-1}). In contrast, the frequency of the ν_2 mode of deoxy-DMb does not change with time.

The time dependence of the high-frequency region of the Soret-excitation RR spectrum of deoxy-2,4-AcMb is shown in Figure 6. After 6 h of equilibration, the carbonyl region of the RR spectrum appears as a broad, poorly resolved envelope centered at 1640 cm^{-1} (Figure 6a). Spectral simulations indicate that this envelope is due to two sets of two bands. One pair of bands occurs at 1656 and 1644 cm^{-1} (the frequencies observed at equilibrium). The other two bands are also separated by approximately 12 cm^{-1} but are slighly downshifted (2–3 cm^{-1}) relative to the first pair. With time, the broad envelope evolves into the two bands that are observed at

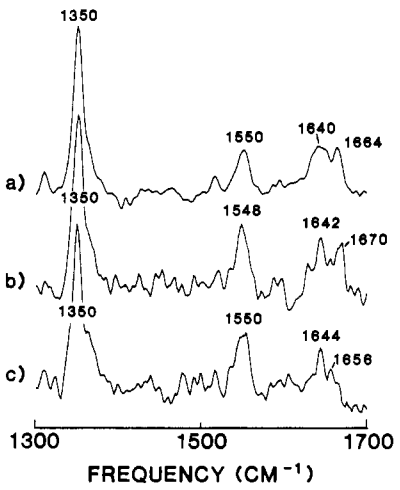

FIGURE 6: High-frequency regions of Soret-excitation (λ_{ex} = 457.9 nm) RR spectra of 2,4-AcMb obtained as a function of time after reconstitution. Spectra were acquired on the deoxy species which had equilibrated as the metaquo protein for (a) 6, (b) 8, and (c) 30 h after reconstitution.

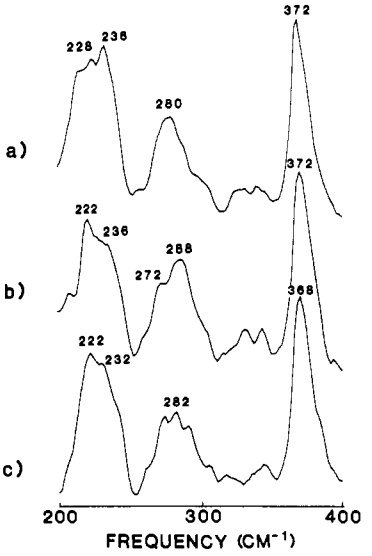

FIGURE 7: Low-frequency regions of Soret-excitation (λ_{ex} = 457.9 nm) RR spectra of 2,4-AcMb obtained as a function of time after reconstitution. The experimental conditions are the same as those given in Figure 6.

equilibrium. As is the case for deoxy-DMb, by ν_2 mode of deoxy-2,4-AcMb does not exhibit any particular time dependence. This behavior can be contrasted with that observed for the ν_2 modes of deoxy-2-AcMb and deoxy-4-AcMb.

The behavior of ν(Fe–His) for deoxy-2,4-AcMb as a function of time after reconstitution is shown in Figure 7. This mode initially appears as a broad envelope with a maximum at 236 cm^{-1} (Figure 7a). With time, the higher frequency side of the envelope decreases in intensity (Figure 7b). At equilibrium, a band at 222 cm^{-1} is predominant (Figure 7c). The ν(Fe–His) modes of deoxy-2-AcMb, deoxy-4-AcMb, and deoxy-DMb also exhibits a time dependence (not shown). For the two monoacetyl systems, ν(Fe–His) for the unequilibrated form occurs at a higher frequency than for the equilibrated form. In contrast, ν(Fe–His) for the unequilibrated form of DMb is observed at 217 cm^{-1}, which is lower than the frequency observed for the equilibrated form (223 cm^{-1}).

The time dependence of the RR spectra was also investigated for the ligated derivatives of DMb, 2-AcMb, and 4-AcMb. RR spectra of the oxy form of freshly reconstituted proteins could not be obtained due to severe photoinstability;

therefore, RR spectra were obtained only for the metcyano derivatives of these species. The pertinent vibrational data are compared with that observed for the unequilibrated deoxy species in the last two columns of Table I. The ν(C=O) and ν(Fe–His) modes, which are the most sensitive to the equilibration phenomenon, are not observed for the unequilibrated forms of the metcyanoMbs. The frequencies for the skeletal modes of the unequilibrated forms appear to be similar to those of the equilibrated forms. However, on the basis of studies of the deoxy species, these modes are not expected to be particularly sensitive to the equilibration phenomenon. Similarly, ν(Fe–CN) does not exhibit any pronounced time dependence.

Time Dependence of the NMR Spectra. In order to investigate the equilibration phenomenon further, ^{1}H NMR spectra were obtained for the various Mbs as a function of time after reconstitution (not shown). The NMR spectra of the metcyano derivatives were recorded, owing to the superior spectral quality relative to that of the deoxy species (La Mar, 1979). In addition, the complexation of cyanide has been reported to "freeze" the equilibration process (La Mar et al., 1978; Ahmad & Kincaid, 1983). For all four reconstituted Mbs examined, NMR spectra recorded immediately after heme reconstitution exhibited two sets of heme methyl resonances. The paramagnetic shifts of the resolvable heme methyl resonances of metcyanoDMb and metcyano-2,4-AcMb were found to be identical with those reported by La Mar and co-workers, who have previously investigated the equilibrium phenomenon in these species (La Mar & Krishnamoorthi, 1983). NMR spectra recorded at later times showed that one set of methyl resonances was substantively diminished in intensity relative to the other. The time evolution of the intensities of the NMR signals appeared to parallel the time evolution of the intensities of the RR bands. These observations indicate that the time dependence of the RR spectra reflects the equilibration process which has been described by La Mar and co-workers (La Mar et al., 1978, 1983, 1984; Jue et al., 1983; Lecomte et al., 1985).

DISCUSSION

Specific 2- vs 4-Substituent/Protein Interactions. The RR studies reported here clearly demonstrate the protein-induced inequivalence of the 2- and 4-positions of the heme moiety in the reconstituted Mbs. The RR data further indicate that the protein perturbs the heme moiety through interactions with the substituent at the 2-position. The frequencies of the carbonyl and skeletal modes of deoxy-2-AcMb are systematically lower than those of deoxy-4-AcMb. Similarly, the frequencies of the skeletal modes of the ligand forms of the former derivative are lower than those of the latter. These downshifts are consistent with a larger degree of porphyrin/carbonyl conjugation for the acetyl group in the 2-position vs that in the 4-position. A lowering of ν(C=O) for 2-AcMb via hydrogen bonding can be ruled out because this type of interaction is not consistent with the downshifts observed in the skeletal modes of the macrocycle. Indeed, the X-ray crystal structures of native deoxy- and oxyMb show that no residues capable of hydrogen bonding are in the vicinity of the 2-position of the heme (Takano, 1977; Phillips, 1980). The observation that the skeletal-mode frequencies of deoxy- (ligated) 2-AcMb are lower than those of deoxy- (ligated) DMb further indicates that the protein host increases the extent of conjugation of the acetyl group relative to that which occurs in nonaqueous, non-hydrogen-bonding solvents.

The determination of the extent of conjugation of the 2-acetyl group of 2,4-AcMb in the deoxy relative to the ligated forms appears to be more complicated than that for the other reconstituted proteins. For the deoxy complex, the extent of conjugation of the 2-group is larger than that of the 4-group and also larger than that which occurs in solution. This increased conjugation of the 2-group is manifested as a larger splitting of the coupled carbonyl modes (12 cm^{-1}) than that which occurs in solution (7 cm^{-1}) (Willems & Bocian, 1984). On the other hand, the frequencies of the skeletal modes of ligated 2,4-AcMb are not sufficiently lower than those of ligated DMb to indicate that the extent of conjugation of the 2-group of the ligated protein is lower than that of the 4-group. This result is interesting in view of the consistent behavior of the skeletal frequencies for both the deoxy and ligated forms of 2-AcMb and 4-AcMb and suggests that the disubstituted heme may reside in a slightly different position in the protein pocket than either of the monosubstituted hemes.

The observation that the protein environment alters the extent of conjugation of the 2-group is interesting because X-ray crystallographic studies of native deoxy- and oxyMb show that relatively few protein residues are in the vicinity of the 2-position, whereas a hydrophobic cluster consisting of leucine-32, phenylalanine-43, and leucine-104 surrounds the 4-position (Takano, 1977; Phillips, 1980). In view of the protein environment around the two different sites, it might have been expected that the influence of the protein on the 2/4-groups would be opposite to that which is observed. In particular, the presence of protein contacts around the 4-group might have been expected to increase the extent of conjugation of the acetyl moiety relative to that which occurs in solution. The fact that the opposite behavior is observed suggests that long-range electrostatic and/or dispersive interactions between the 2-acetyl group and the protein may be more important than steric interactions for determining the orientation of this substituent. The spectral data indicate that these interactions may either increase or decrease the extent of conjugation of the group.

The general trends observed in the RR spectra of the reconstituted Mbs indicate that the largest protein-induced perturbation occurs in the electronic structure of the carbonyl group itself. The electronic structure of the π-system of the macrocycle is also perturbed, although to a much lesser extent than that of the carbonyl group. Presumably, the changes that occur in the π-structure of the ring occur indirectly as a result of the change in the extent of conjugation of the 2-substituent. This conclusion is substantiated by the observation that vibrational frequencies for 2-AcMb (relative to 4-AcMb) are successively less perturbed as the distance from the conjugating substituent increases: $\Delta\nu(C{=}O) > \Delta\nu(C_bC_b) > \Delta\nu(C_aN)$. The observation that ν(Fe–His) is the same for all reconstituted deoxyMbs (as well as for native, spirographis, isospirographis, and 2,4-diformyl Mbs) further suggests that the electron density at the metal center experiences little, if any, perturbation as a result of changes in the porphyrin π-structure that originate at the periphery of the ring.

Heme Orientational Disorder. X-ray crystallographic studies of native sperm whale Mb have shown that the heme moiety assumes a unique orientation in the protein pocket (Takano, 1977; Phillips, 1980). Nevertheless, La Mar and co-workers have shown that the initial product after reconstitution consists of a 1:1 mixture of two different heme orientational isomers (La Mar et al., 1978, 1983, 1984; Jue et al., 1983; Lecomte et al., 1985). The RR spectra reported here for the various reconstituted deoxyMbs provide additional insight into the nature of the 2/4-substituent/protein interactions that occur in the reversed orientation of the heme. In

particular, ν(C=O) for the unequilibrated form of deoxy-2-AcMb occurs at ~1648 cm^{-1}, a frequency identical with that observed for the equilibrated form of deoxy-4-AcMb. Conversely, ν(C=O) for the unequilibrated form of deoxy-4-AcMb is observed at 1644 cm^{-1}, a frequency which is lower than that of the equilibrated form and which approaches that of the equilibrated form of deoxy-2-AcMb (1640 cm^{-1}). These observations suggest that the protein environment around the acetyl group in the unequilibrated form of the 2-isomer is similar to (although not identical with) that of the equilibrated form of the 4-isomer and vice versa. The observation that the carbonyl and skeletal frequencies of the unequilibrated form of deoxy-2,4-AcMb are similar to those of the equilibrated form lends further support to this conclusion. This behavior of the vibrational modes is as expected if equilibration exchanges the positions of the two carbonyl groups in the protein matrix. In the unequilibrated form, the 4-group is more conjugated than the 2-group and approximately as conjugated as the 2-group of the equilibrated form. Collectively, these data lend strong support to the proposal that the unequilibrated form of the protein contains the heme moiety in an orientation rotated 180° about the α,γ axis of the heme. Indeed, it is difficult to imagine how unequilibrated forms which contain the heme in arbitrary locations in the protein pocket could give rise to the complementary behavior observed for the vibrational modes of the two monoacetylheme-reconstituted Mbs.

The most substantial change in the electronic structure of the heme moiety that occurs when the prosthetic group is in the reversed orientation appears to be in the nature of the proximal histidine–iron bond. For the unequilibrated forms of deoxy-2-, deoxy-4-, and deoxy-2,4-AcMb, ν(Fe–His) is higher than that of the respective equilibrated species. This trend can be contrasted with the behavior that is exhibited by ν(Fe–His) for deoxyDMb. For this derivative, ν(Fe–His) of the unequilibrated form is observed at a lower frequency than for the equilibrated form. The shift to higher frequency of ν(Fe–His) for the reversed orientation for all of the acetylheme-reconstituted deoxyMbs implies that the bonding between the metal ion and the proximal histidine is increased relative to that which occurs in the normal orientation. On the other hand, the bonding is apparently decreased for the reversed orientation of deoxyDMb. The fact that the extent of conjugation of the acetyl group of the unequilibrated 2-isomer is similar to that of the equilibrated 4-isomer and vice versa suggests that electronic effects are not responsible for the different ν(Fe–His) frequencies of the normal and reversed forms. The higher frequencies observed for the reversed forms of deoxy-2-, deoxy-4-, and deoxy-2,4-AcMb probably result from heme/protein interactions that either place the iron ion and histidine residue in closer proximity or alter the tilt angle between this residue and the heme plane. These interactions may occur between the protein and any of the substituents on the heme. The lower frequency observed for the reversed form of deoxyDMb could occur if the prosthetic group slips further into the hydrophobic pocket. This change in heme location could lengthen the Fe–His bond and/or alter the His/heme tilt angle.

Implications for O_2 Binding Affinities. The RR studies reported here demonstrate that protein-induced changes in the extent of electronic interaction between the 2-conjugating group and the porphyrin π-system do not result in a large change in the electron density at the metal ion. The fact that ν(Fe–His) is the same for all the reconstituted deoxy species indicates that the density in the Fe–His σ bond is essentially unaltered by substituent changes at the periphery. The lack of influence of the substituent on the charge density in the axial bonds is also reflected in the similarity of ν(Fe–CN) for the various reconstituted Mbs. In this regard, previous RR studies have shown that the iron–oxygen stretching frequency, ν(Fe–O_2), is insensitive to the substitution at the 2/4-positions of the heme (Tsubaki et al., 1980). Nevertheless, the O_2 binding affinity has been shown to vary with such substitutions (Sono & Asakura, 1974; Kawabe et al., 1982; Chang et al., 1984).

It is possible that the different O_2 binding affinities that are exhibited by 2/4-substituted isomers are a result of substituent-induced changes of the charge density in the metal π orbitals. Indeed, examination of Table I shows that, upon ligand binding, the skeletal modes of 2-AcMb and 2,4-AcMb undergo somewhat larger shifts than do those of 4-AcMb. The relative differences in spectral shifts, Δ_{deoxy}, that are exhibited by the 2/4-substituted Mbs are indicative of relative differences in the heme π-electronic system of the ligated vs deoxy proteins. However, there is no pattern in the values of Δ_{deoxy} for the vibrational modes of the different 2/4-substituted Mbs. In particular, $\Delta_{deoxy}(\nu_4)$ is larger than $\Delta_{deoxy}(\nu_2)$ for 2-AcMb and 4-AcMb whereas the reverse is true for 2,4-AcMb. Thus, it appears that no simple correlation can be made between the spectral shifts and any differences that might exist between the O_2 binding affinities of the 2/4-substituted systems.

Although differential changes that may influence the O_2 affinity are induced in the π-electronic systems of 2- vs 4-substituted Mbs upon ligand binding, other factors may also be important. In particular, steric and/or other types of interactions between the 2/4-groups and the protein could induce differential alterations in the environment of the binding site for the ligated vs deoxy forms. The observation that ν(Fe–His), ν(Fe–CN), and ν(Fe–O_2) are insensitive to 2/4-substituent modifications strongly suggests that the environment around histidine-93 (proximal site) is unperturbed by such modifications. Thus, the changes in the nature of the binding site would have to occur in the vicinity of histidine-64 (distal site). Examination of the X-ray crystallographic data for native deoxy- and oxyMb (Takano, 1977; Phillips, 1980) reveals that the 2-position substituent is in the vicinity of valine-68 and leucine-72. These amino acids are only four and eight residues removed from the distal histidine, respectively. In contrast, the amino acids that surround the 4-substituent are 20–30 residues removed from this histidine. These structural features suggest that any perturbations at the distal site would have to originate from substituent/protein interactions at the 2-position. This conclusion is appealing because our RR data indicate that the protein interacts with the 2-substituent. The fact that the 2-substituent is relatively unencumbered by protein residues suggests that simple steric interactions are not the origin of the changes in the distal site. Indeed, the observation that the O_2 affinity is not correlated with the size of the 2-substituent lends further support to this argument (Sono & Asakura, 1974; Kawabe et al., 1982). Possibly, the long-range electrostatic and/or dispersive interactions which influence the conformation of the 2-substituent also alter the conformation of the protein matrix in the vicinity of this group. These perturbations may be felt at the distal site because the residues that surround the 2-substituent are not far removed from the distal histidine. Additional studies are clearly needed to explore this issue.

It is interesting to speculate whether the factors that influence the O_2 binding affinity in reconstituted Mbs containing the heme in the normal orientation also mediate the affinity of the disordered form. It has been reported that sperm whale Mb which contains protoheme in the reversed orientation

exhibits a higher O_2 affinity than native Mb (Livingston et al., 1984). While the validity of these measurements on Mb has been questioned (Gersonde et al., 1986; Light et al., 1987), different affinities have also been reported for the two orientational isomers of monomeric insect hemoglobins (Kerr et al., 1985; Gersonde et al., 1986). The observation that the Fe–His bond strength is substantively altered upon heme reversal suggests that the protein environment around the proximal site is different from that in the normal form. Such structural changes may completely dominate any subtle perturbations at the distal site that result from 2-substituent/protein interactions. These issues cannot be resolved without additional binding studies of Mb reconstituted with specific 2/4-mono- and disubstituted hemes.

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