Resonance Raman Studies of the Heme Active Site of the Homodimeric Myoglobin from *Nassa mutabilis*: A Peculiar Case†

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ABSTRACT: A spectroscopic investigation by resonance Raman has been carried out at pH 7.0 in 0.1 M phosphate buffer on the cooperative homodimeric myoglobin from *Nassa mutabilis*. The study has been performed on the unligated ferrous form, as well as on the ligated species MbO_2 and MbCO, and on the ferric form met-Mb. Two ν(C=C) vinyl stretching modes have been observed in all the investigated forms, reflecting different degrees of vinyl conjugation with the porphyrin ring, as a consequence of a strongly asymmetric environment for the two side groups of the heme. Furthermore, the ferric form displays a hexacoordinate low-spin heme, which suggests the presence of an endogenous ligand bound to the Fe atom. The frequency of the ν(Fe−Im) stretching mode of Mb from *Nassa mutabils* shifts down by 4 cm^{-1} as compared with that of horse heart myoglobin, reflecting a protein-induced proximal strain as a result of heme−heme interaction due to the close proximity of the two hemes in the dimer. The lower frequency of the ν(Fe−Im) stretching mode agrees well with the lower affinity for oxygen binding found for *Nassa mutabilis* Mb and with the slight heme core expansion with respect to horse heart Mb, suggesting a critical role for the Fe−His bond on the heme's function and structure.

Myoglobin (Mb)[1] is a hemoprotein, which reversibly binds O_2, and it is present in the muscle cells of most eukaryotic organisms. It is likely involved in the regulation of the O_2 flux in many tissues (Wittenberg, 1966; Wyman, 1966), working as an oxygen reservoir because of its high ligand affinity (Antonini & Brunori, 1971). Unlike hemoglobin (Hb), Mb is usually monomeric, and its functional properties are not significantly affected by environmental conditions. However, homodimeric myoglobins have been found in the radular muscles of three gastropod molluscs of the prosobranchia subclass, namely, *Busycon c.* (Bonner & Larsen, 1977), *Cherithidea r.* (Takagi et al., 1983), and *Nassa mutabilis* (NM) (Geraci et al., 1977), the last only being characterized by cooperative O_2 binding. This is the first dimeric myoglobin endowed of such a functional property, similar to that observed in cooperative homodimeric Hbs reported for Arcid species, such as *Scapharca inaequivalvis* (Chiancone et al., 1981).

The similar functional behavior of oxygen equilibrium between *Nassa mutabilis* (NM) Mb and *Scapharca inaequivalvis* (SI) Hb, mentioned above, finds an additional common aspect in the observation of a concerted protonation for the N_ϵ of the imidazole of the proximal histidine of both proteins (Coletta et al., 1990, 1992). Such a feature, never observed for other multimeric vertebrate hemoproteins (Coletta et al., 1988), has been correlated in the case of *Scapharca inaequivalvis* to a different mode of heme−heme interaction, wherefore ligand binding cooperativity originates (Coletta et al., 1990). This hypothesis has been confirmed by the X-ray structure (Royer et al., 1989, 1990) of the CO-bound form of the homodimeric Hb from *Scapharca inaequivalvis* that shows an unusual assembly of subunits with the monomer−monomer interface formed by the heme-carrying E and F helices, and the two heme groups much closer (18.4 Å) (Royer et al., 1989) than in the $\alpha_1-\beta_2$ pair of horse Hb (25 Å) (Heidner et al., 1976). In addition, high-resolution data show that the propionate group of one heme interacts with two side chains from the F helix of the other subunit (Royer et al., 1989, 1990), a contact which has been suggested to represent the structural basis for the pH-dependent cooperativity (Coletta et al., 1990). The closely similar dynamic properties observed for Mb from NM and for Hb from SI raise the question whether the cooperativity originates in these hemoproteins from the same molecular mechanism, thus eliciting our interest in carrying out a detailed resonance Raman study with different excitation wavelengths and in polarized light of the deoxy-, O_2-bound and CO-bound derivatives, as well as of the met form of the Mb from *Nassa mutabilis*.

Resonance Raman (RR) spectroscopy has been extensively applied to heme-proteins (Spiro, 1985), since it provides information not only on the coordination and spin state of

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[1] Abbreviations: CTT hemoglobin, monomeric hemoglobin from insect larvae of *Chironomus thummi thummi*; Mb, myoglobin; Hb, hemoglobin; HbA, human hemoglobin; SI Hb, homodimeric hemoglobin from *Scapharca inaequivalvis*; NM, *Nassa mutabilis*; HH, horse heart; SW, sperm whale; HRP, horseradish peroxidase; NMR, nuclear magnetic resonance; RR, resonance Raman; 5-c and 6-c, 5-coordinate and 6-coordinate hemes; hs and ls, high and low spin; p, polarized band; dp, depolarized band; ap, anomalously polarized; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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the iron atom but also on the status of the bond between the Fe atom and the proximal ligand. In NM Mb, the heme Fe is bound to the proximal histidine residue, and in a previous paper (Coletta et al., 1992), it has been shown that the ν(Fe−Im) stretching mode of the deoxy NM Mb shifts down by 4 cm^{-1} with respect to the corresponding band observed for horse heart (HH) Mb (Kitagawa et al., 1979; Argade et al., 1984). This downshift, attributed to a weaker bond strength between the Fe atom and the proximal imidazole, is in agreement with a lower affinity for the oxygen binding.

In the present work, the spectra of NM Mb, compared with those obtained for monomeric Mbs, document other differences attributed to the heme−heme interactions. Furthermore, in view of the recent resonance Raman investigation of the homodimeric hemoglobin from *Scapharca inaequivalvis* (Song et al., 1993, Boffi et al., 1994; Spagnuolo et al., 1994), some comparison is carried out between the two cooperative homodimers.

MATERIALS AND METHODS

The homodimeric NM oxy-Mb was prepared as previously reported (Geraci et al., 1977). The met form was prepared by oxidation of the oxy form with an approximately 3-fold molar excess of potassium ferricyanide followed by gel filtration on a Biogel P6DG (BioRad) column equilibrated either with 0.1 M NaCl, 0.1 M phosphate, pH 7.0 (buffer P), or with 0.1 M NaCl, 0.1 M HEPES (buffer H), as required by the experiment. HH met-Mb was purchased from Sigma and used without further purification; the oxy derivative of HH Mb was prepared by reducing the met form with 10 μL of (10 mg/mL) freshly prepared solution of sodium dithionite (Merck) per 100 μL of Mb solution and then filtering through a Biogel P6DG column equilibrated with buffer P or H, as indicated. The deoxy derivatives of NM and HH Mbs were prepared by adding 10 μL of (10 mg/mL) a freshly prepared solution of sodium dithionite per 100 μL of deoxygenated buffered solution of oxy- or met-Mb. The CO derivative was obtained by gently flowing ^{12}CO (1 atm) (Rivoira) for 10 min over the surface of the reduced protein solution. The ^{13}CO (99%) (Matheson) derivative was obtained by introducing the gas over the Mb solution in an evacuated NMR tube.

Samples for RR, IR, and electronic absorption spectra were prepared in buffer P or buffer H to give 0.3−0.5 mM Mb concentration for RR and UV−visible absorption spectra and about 1 mM for IR spectra.

Absorption spectra were recorded with a Cary 5 spectrophotometer. Infrared spectra were recorded with a Bruker IF120HF FTIR spectrophotometer. The Mb-CO samples were transferred by a gas-tight syringe flushed with CO into a CaF_2 IR cell (0.1 mm path length) which had been previously flushed with CO. The corresponding buffer solution was used as reference.

The RR spectra were obtained at room temperature with excitation from the 406.7, 413.1, and 530.9 nm lines of a Kr^+ laser (Coherent, Innova 90/K) and the 457.9 nm line of an Ar^+ laser (Coherent, Innova 90/5). The back-scattered light from a slowly rotating NMR tube was collected and focused into a computer-controlled double monochromator (Jobin-Yvon HG 2S) equipped with a cooled photomultiplier (RCA C31034 A) and photon counting electronics. The RR spectra were calibrated with indene and CCl_4 as standards, and the frequencies are accurate to ± 1 cm^{-1} for the intense isolated bands.

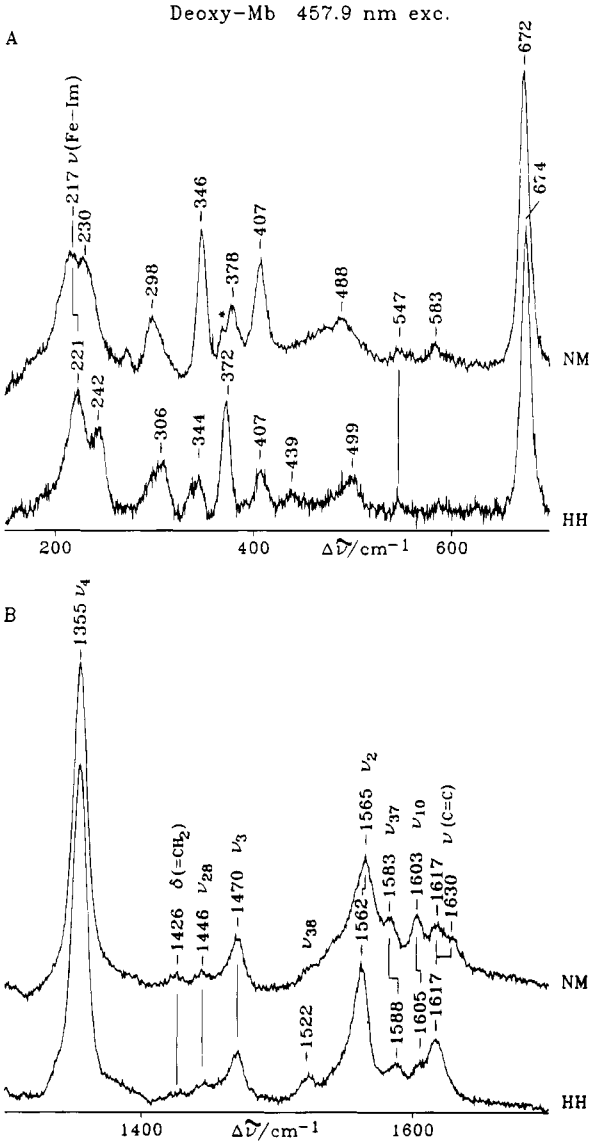

FIGURE 1: Resonance Raman spectra of deoxy forms of *Nassa mutabilis* (NM) and horse heart (HH) myoglobins, obtained with 457.9 nm excitation wavelength. Panel A shows the low-frequency region, and panel B shows the high-frequency region. Experimental conditions: 40 mW laser power at the sample; 5 cm^{-1} resolution; and 5 s/cm^{-1} collection interval. The asterisk indicates a plasma line.

Polarized spectra were obtained by inserting a polaroid analyzer between the sample and the entrance slit of the spectrometer. The depolarization ratios, $\varrho = I_\perp/I_\parallel$, of the bands at 318 and 459 cm^{-1} of CCl_4 were measured to check the reliability of the polarization measurements using a rotating NMR tube with 180° back-scattered geometry. The values obtained, $\varrho = 0.73$ and $\varrho = 0$, for the bands at 318 and 459 cm^{-1} compare favorably with the expected theoretical values $\varrho = 0.75$ and $\varrho = 0$, respectively.

RESULTS

Deoxy-Mb. Figure 1 compares the RR spectra excited at 457.9 nm in the low-(A) and high-frequency (B) regions of deoxy-Mbs from *Nassa mutabilis* (top) and horse heart (bottom). The spectrum in the low-frequency region of HH Mb is characterized by a strong band at 221 cm^{-1} assigned to the ν(Fe−Im) stretching mode (Kitagawa et al., 1979; Argade et al., 1984; Bangcharoenpaurpong et al., 1984). As

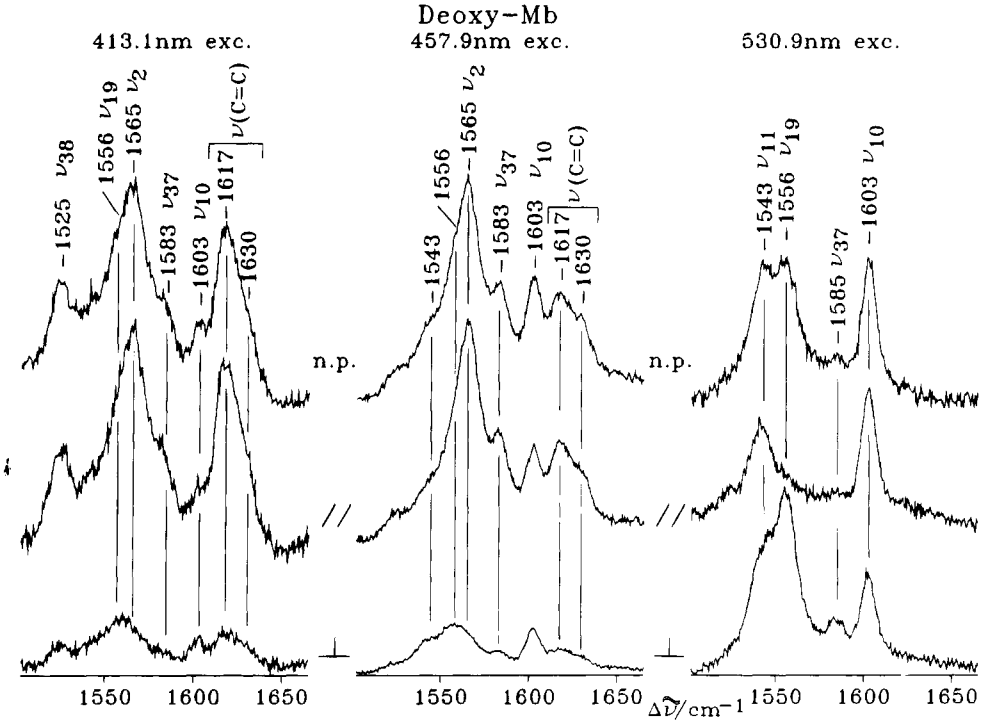

FIGURE 2: Resonance Raman spectra in polarized light of deoxymyoglobin from *Nassa mutabilis* (NM), obtained with different excitation wavelengths. Experimental conditions: 5 cm^{-1} resolution; 413.1 nm excitation, 30 mW laser power at the sample, 5 s/cm^{-1} collection interval for the nonpolarized spectrum (n.p.) and 10 s/cm^{-1} collection interval for the spectra in polarized light; 457.9 nm excitation, 50 mW laser power at the sample, 10 s/cm^{-1} collection interval for the n. p. spectrum and 6 and 12 s/cm^{-1} collection intervals for the spectra taken in parallel (∥) and perpendicular (⊥) polarized light, respectively; 530.9 nm excitation, 80 mW laser power at the sample, 5 s/cm^{-1} collection interval for n.p. spectrum and 8 s/cm^{-1} collection interval for the spectra taken in polarized light.

previously reported, this band is shifted down by 4 cm^{-1} in NM Mb (Coletta et al., 1992). In addition, other changes were observed in the spectrum of NM Mb, as compared to that of HH Mb. This latter showed a band at 306 cm^{-1} with a shoulder around 298 cm^{-1}. These two bands were assigned to a mode involving the out-of -plane motion of the methine carbon atoms, and to a bending mode of the vinyl substituents, respectively (Choi & Spiro, 1983). The two bands were still observed at about the same frequencies in NM Mb, but showed a reversed relative intensity with respect to HH Mb: the strongest band was observed at 298 cm^{-1} with a very weak shoulder at 306 cm^{-1}. In the region between 340 and 420 cm^{-1}, the bands at 346, 378, and 407 cm^{-1} in NM Mb correspond in frequency to those observed at 344, 372, and 407 cm^{-1} in HH Mb, but differ in their relative intensities. In the latter molecule, these bands were assigned as ν_8, a mode involving in-plane substituent group bending motion (Abe et al., 1978,; Choi & Spiro, 1983), as an out-of-plane bending mode of the propionate groups $\delta(C_\beta C_c C_d)$ (S. C. Hu, and T. G. Spiro, unpublished results), and as an in-plane vinyl bending mode $\delta(C_\beta C_a C_b)$, respectively (Choi & Spiro, 1983).

In the high-frequency region, the RR spectrum of NM Mb behaves similarly to that of HH Mb. Both proteins show core size marker bands characteristic of a 5-coordinate high-spin (5-c hs) heme with ν_3 at 1470, ν_2 at 1562 (HH) and 1565 (NM), and ν_{10} at 1605 (HH) and 1603 (NM) cm^{-1}, whereas a marked difference is observed in the vinyl region. Thus, HH Mb shows only one strong ν(C=C) stretching mode at 1617 cm^{-1}, while NM Mb shows two bands at 1617 and 1630 cm^{-1}.

In order to assign all the observed bands, we undertook a study with different excitation wavelengths and in polarized light. With the Soret excitation, the heme totally symmetric (A_{1g}) and the vinyl modes (which give rise to polarized bands, p) are enhanced via the *A*-term (Franck–Condon) mechanism, and the heme nontotally symmetric modes (B_{1g}) (dp) are enhanced via the Jahn–Teller mechanism. These latter modes are also enhanced together with the anomalously polarized modes (ap) (A_{2g}) via the *B*-term (vibronic mixing) mechanism with Q-excitation (Choi et al., 1982a; Spiro & Li, 1988). In addition, with excitation in the blue with respect to the Soret absorption maximum, the E_u, A_{2g}, and the vinyl stretching modes are enhanced, as previously observed by Palanappian and Terner (1989) for horseradish peroxidase (HRP).

Table 1: Resonance Raman Frequencies (cm^{-1}) of Deoxy-, Oxy-, and Met-Mb from *Nassa mutabilis*

mode	deoxy 5-c hs[a]	oxy 6-c ls[a]	met 6-c ls[a]
$\nu_{(C=C)}$	1630	1630	1630
$\nu_{(C=C)}$	1617	1620	1621
ν_{10}	1603	1638	1639
ν_{37}	1583	1604	1601
ν_2	1565	1581	1578
ν_{19}	1556	1594	1586
ν_{11}	1543	1563	1561
ν_{38}	1525	1547	1550
ν_3	1470	1503	1505
ν_{28}	1446		
$\delta_{(=CH_2)}$	1426	1429	1431
ν_4	1355	1375	1373
$\nu_{(Fe-Im)}$	217		
$\nu_{(Fe-O_2)}$		571	
$\nu_{(CO)}$	1947		
$\nu_{(Fe-CO)}$	517		
$\delta_{(Fe-CO)}$	580		

[a] 5-c hs, 6-c hs, and 6-c ls, 5-coordinate high-spin, 6-coordinate high-spin, and 6-coordinate low-spin heme, respectively.

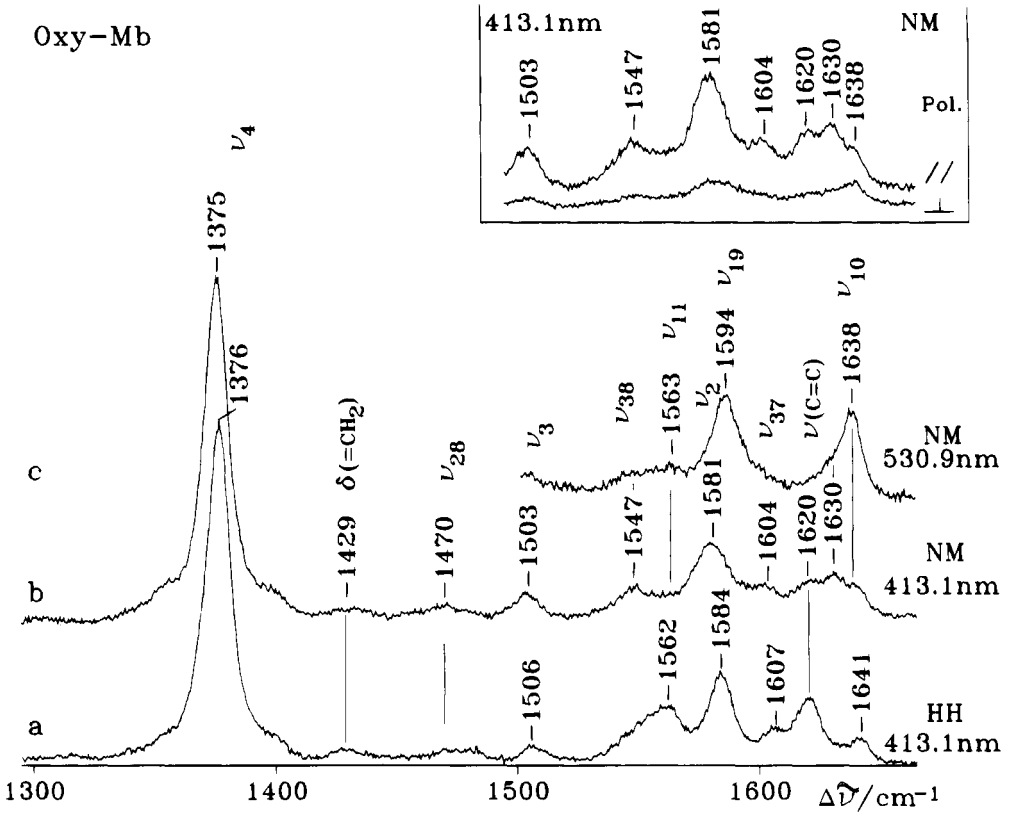

FIGURE 3: Resonance Raman spectra of the oxy forms of *Nassa mutabilis* (NM) and horse heart (HH) myoglobins in the high-frequency region. (a, b) 413.1 nm excitation, 8 mW laser power at the sample, 5 cm^{-1} resolution, 8 s/cm^{-1} collection interval (a), and 4 s/cm^{-1} collection interval (b); (c) 530.1 nm excitation, 40 mW laser power at the sample, 5 cm^{-1} resolution, and 3 s/cm^{-1} collection interval; insert: spectra in polarized light taken with 413.1 nm excitation; conditions are as given in the legend (a), 8 and 12 s/cm^{-1} collection intervals for the spectra taken in parallel (||) and perpendicular (⊥) polarized light. All the spectra are obtained using a cylindrical lens to minimize photolysis.

Figure 2 shows the resonance Raman spectra of deoxy-Mb from *Nassa mutabilis* taken with different excitation wavelengths (413.1, 457.9, and 530.9 nm) and in polarized light. On the basis of their different intensity enhancement with the excitation wavelength, and on their depolarization ratio (ϱ), the bands are assigned as follows: 1525 and 1583 cm^{-1} to ν_{38} and ν_{37} (E_u), respectively, 1543 and 1603 cm^{-1} to ν_{11} and ν_{10} (B_{1g}), respectively, 1556 cm^{-1} to ν_{19} (A_{2g}), and the two bands at 1617 and 1630 cm^{-1}, which are polarized and enhanced only with Soret excitation, to the two ν(C=C) stretching modes. It is interesting to note that these two bands appear to have different excitation profiles, the band at 1617 cm^{-1} being much more enhanced in the spectrum obtained with 413.1 nm excitation (Figure 2) than in the others. Complete assignments are reported in Table 1.

Oxy-Mb. Figure 3 compares the high-frequency region spectra of oxy-Mbs from NM (b, c) and HH (a) excited at 413.1 (a, b) and 530.9 (c) nm. The spectra of both compounds are very similar and characteristic of a 6-coordinate low-spin (6-c ls) heme. All the core size marker bands of NM are about 3 cm^{-1} lower in frequency with respect to the corresponding bands of HH. In addition, the spectrum of NM Mb is characterized by the appearance of a new band at 1630 cm^{-1}, assigned to a second vinyl stretching mode based on the spectra in polarized light obtained with 413.1 nm excitation (Figure 3, insert).

Figure 4 compares the low-frequency region spectra of oxy-Mbs from HH (A) and NM (B) of the unphotolyzed (bottom) and photolyzed (top) compounds. The band at 571 cm^{-1} observed in HH Mb has been assigned to the $\nu(Fe^{II}-O_2)$ stretching mode (Van Wart & Zimmer, 1985). NM Mb showed a band at the same frequency which disappeared upon increasing the laser power (top spectra); therefore, it has been assigned to $\nu(Fe^{II}-O_2)$. Substantial differences between the two spectra were observed. In particular, the band at 377 cm^{-1}, due to the propionyl bending mode, was much weaker in NM Mb than in HH Mb. The narrow band at 410 cm^{-1} of HH Mb, due to the accidentally degenerate vinyl bending modes, upshifted to 416 cm^{-1} and broadened in NM Mb.

Met-Mb. Figure 5 shows the Soret RR spectra in the low- (A) and high-frequency (B) regions of the freshly prepared oxidized form of met-Mbs from HH (top) and NM (bottom). The high-frequency region spectra are quite different; NM Mb is characteristic of a 6-c ls heme, whereas the HH Mb is 6-c hs heme. The assignment of the bands has been performed on the basis of the RR spectra taken with different excitation wavelengths and in polarized light, as shown in Figure 6. The complete assignment is reported in Table 1.

The high-frequency region RR spectrum of NM met-Mb (Figure 5B) resembles that of NM oxy-Mb (Figure 3, spectrum b), and the only difference is represented by the relative intensity of the doublet due to the vinyl stretching modes, the 1621 cm^{-1} component being stronger in met-Mb than in the oxy form. The low-frequency regions of the two compounds are almost identical (Figures 5A and 4B), and they differed only by the absence of the band due to the $\nu(Fe-O_2)$ stretching mode in the met-Mb spectrum.

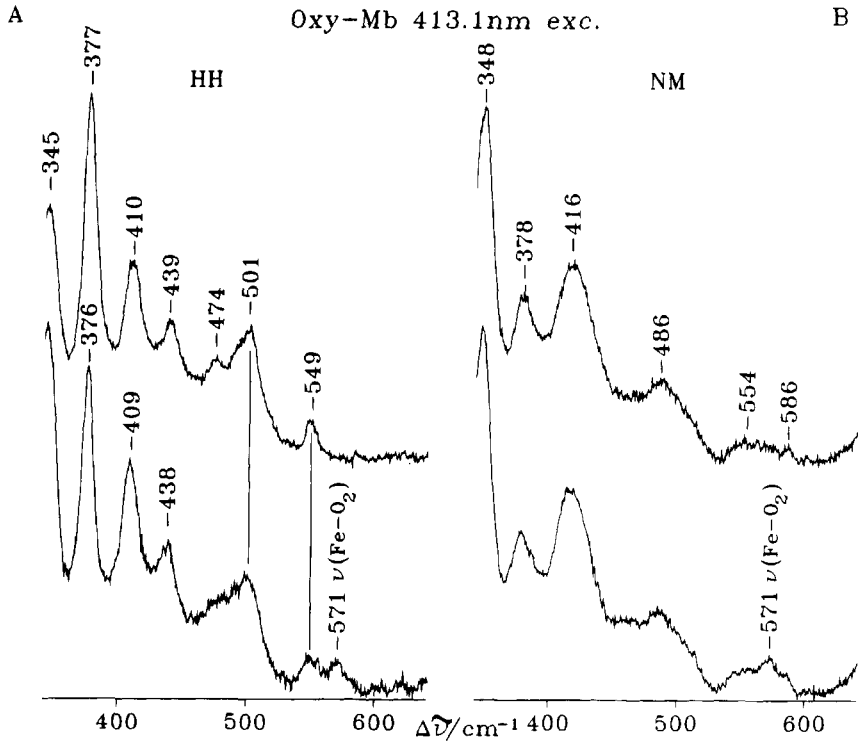

FIGURE 4: Resonance Raman spectra of the oxy forms of *Nassa mutabilis* (NM) and horse heart (HH) myoglobins in the low-frequency region, obtained with 413.1 nm excitation. Experimental conditions: 5 cm^{-1} resolution; (A) bottom spectrum obtained using a cylindrical lens, 8 mW laser power at the sample, and 24 s/cm^{-1} collection interval; top spectrum, 30 mW laser power at the sample and 6 s/cm^{-1} collection interval; (B) bottom spectrum obtained using a cylindrical lens, 8 mW laser power at the sample, and 24 s/cm^{-1} collection interval; top spectrum, 20 mW laser power at the sample, and 10 s/cm^{-1} collection interval.

Carbon Monoxide Derivative. Figure 7 shows the RR spectra, in the low-frequency region, of the $Fe^{II}-CO$ complexes of HH (a) and NM (b, c) Mbs. As in the oxy and deoxy forms, in the high-frequency region the spectra of the two proteins are quite similar and differ only by the appearance of a second band due to a $\nu(C=C)$ vinyl stretching mode at 1630 cm^{-1} in the NM Mb spectrum (data not shown). However, in the low-frequency region, the two globins differ for the frequency of the strongest band. The band at 509 cm^{-1} of HH Mb corresponds to that observed in sperm whale SW Mb at 507 cm^{-1} and assigned to the $\nu(Fe-CO)$ stretching mode, on the basis of its ^{13}CO isotopic shift (Ramsden & Spiro, 1989). Accordingly, the band at 517 cm^{-1} observed for NM Mb−CO complex, which shifts down to 513 cm^{-1} with ^{13}CO isotopic substitution, is ascribed to the same vibration. In addition, the weak band at 580 cm^{-1}, which shifts to 560 cm^{-1} upon isotopic substitution, is assigned to the $\delta(Fe-CO)$ bending mode and corresponds to the band observed at 575 cm^{-1} in the CO complex of HH Mb (and of SW Mb; Ramsden & Spiro, 1989). The $\nu(CO)$ stretching modes were found in the IR spectrum at 1947 and 1944 cm^{-1} for NM Mb and HH Mb, respectively (Figure 7, insert). Actually, NM Mb−CO, depending on the sample preparation, gave rise to a shoulder at 1965 cm^{-1} in the IR spectrum and a shoulder at 496 cm^{-1} in the RR spectrum, both modes being sensitive to the ^{13}CO isotopic substitution. Upon complete denaturation of the protein (i.e., production of a met form which had lost its capability to bind the oxygen ligand upon reduction), these two latter bands were the only ones detected in the CO adduct. Therefore, they were assigned to $\nu(CO)$ and $\nu(Fe-CO)$ stretching modes, respectively, of a protein which may contain a small percentage of heme bound in a different manner.

DISCUSSION

The homodimeric protein spectra are characterized by the presence of two $\nu(C=C)$ stretching modes, one at about 1620 cm^{-1} (as in HH Mb) and the other 10 cm^{-1} higher in frequency. Splitting of the vinyl modes due to the interaction with the protein has been reported previously for CTT hemoglobin, and the two $\nu(C=C)$ at 1624 and 1630 cm^{-1} have been shown to be uncoupled and inequivalent (Gersonde et al., 1989). Accordingly, the observation of two vinyl modes in the NM Mb, which are observed in all the forms so far studied, indicates that the 2- and 4- vinyls are unequivalent. It likely results from the close proximity of the two hemes, which induces a distortion from coplanarity, and therefore less conjugation with the heme, of the vinyl corresponding to the band at 1630 cm^{-1}. However, it must be pointed out that such an asymmetric arrangement is not observed in the Hb from *Scapharca inaequivalvis* (Song et al., 1993; Boffi et al., 1994), suggesting that the subunit assembly might be somewhat different in the two homodimers.

Comparing the high-frequency region RR spectra of the oxy- and deoxy-Mb from *Nassa mutabilis* with the corresponding forms of HH Mb, it appears that the frequencies of the core size marker bands are about 3 cm^{-1} downshifted in the NM Mb, indicating a slightly more expanded heme core size. The only exception is the ν_2 mode of deoxy NM Mb which is 3 cm^{-1} higher than the corresponding mode observed in deoxy HH Mb. Considering that the ν_2 mode is coupled to the vinyl $\nu(C=C)$ mode in protohemes, since saturation of the vinyl groups raises the ν_2 frequency by about 10 cm^{-1} (Choi et al., 1982b), the observed upshift of the ν_2 frequency implies a lower vibrational coupling between this

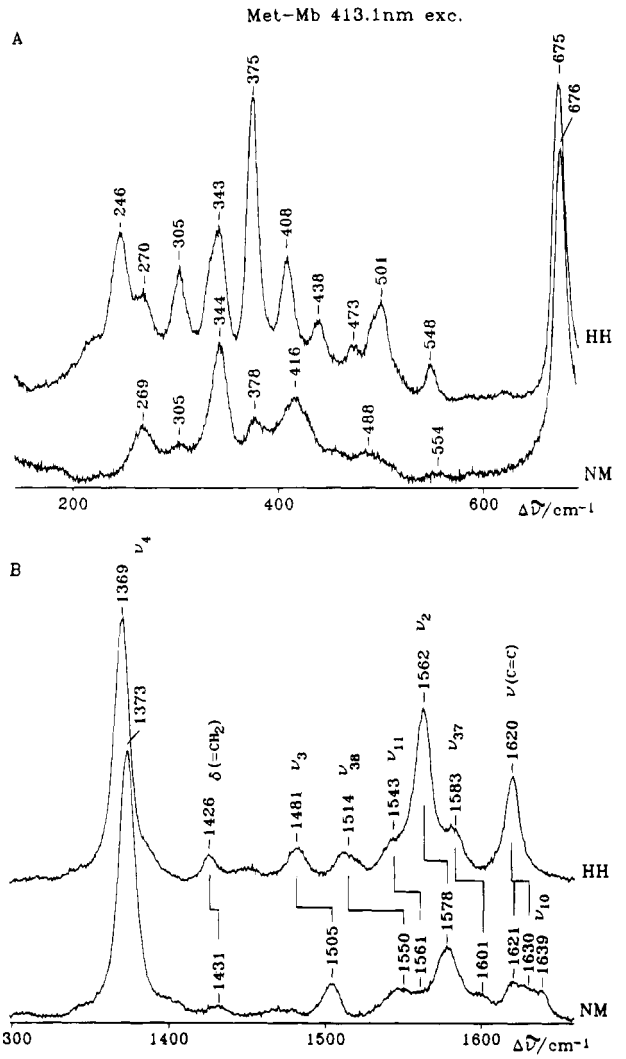

FIGURE 5: Resonance Raman spectra of met forms of *Nassa mutabilis* (NM) and horse heart (HH) myoglobins, obtained with 413.1 nm excitation wavelength. Panel A shows the low-frequency region, and panel B shows the high-frequency region. Experimental conditions: 30 mW laser power at the sample, 5 cm^{-1} resolution, and 3 s/cm^{-1} collection interval.

mode and the vinyl stretching modes, as a consequence of the upshifting of one of these modes. On the other hand, the oxy form does not show any different degree of coupling with respect to the corresponding form in HH Mb. Structural alterations may be involved in this different behavior, since the deoxy form has a 5-c hs heme, while the oxy form has a 6-c ls heme.

In the low-frequency region, substantial differences in the intensities and frequencies of the bands involving the bending modes of the peripheral substituents of the heme are observed. In particular, the band assigned to the propionyl group is much weaker in NM Mb than in HH Mb.

These data may indicate that the interactions of the vinyl and propionyl groups with the protein are quite different between the monomeric and the homodimeric protein.

Deoxy-Mb. The frequency of the vibration involving the bonding of the central Fe atom with the proximal imidazole is markedly different between the monomeric and dimeric proteins. The ν(Fe−Im) stretching mode in NM deoxy-Mb shifts down by 4 cm^{-1} with respect to the corresponding band observed in HH Mb. The Raman frequency of the Fe−ligand stretching mode gives an indication of the effective bond strength: the lower the frequency, the weaker is the bond. Accordingly, the downshift of the ν(Fe−Im) stretching mode has been interpreted as due to a weakening of the bond between the Fe atom and the proximal ligand with respect to the HH deoxy-Mb, in agreement with the increased pK_a value observed for the protonation of the proximal bond in the deoxygenated derivative of this protein, as derived from the pH dependence of CO binding kinetics (Coletta et al., 1992), and with the slightly expanded heme core size with respect to that of HH Mb.

It has been proposed that the ν(Fe−Im) frequency of Hbs and Mbs reflects the protein-induced proximal strain which causes a tilting of the proximal histidine from the heme normal [see Friedman et al. (1990) and references cited therein]. Therefore, the lower frequency observed in NM Mb might be due to a protein-induced proximal ligand strain as a result of heme−heme interaction due to the close proximity of the two hemes in the dimer. The tilting of the proximal ligand is expected to cause a lower affinity for oxygen binding, by analogy with the R and T quaternary states in Hb which are charaterized by ν(Fe−Im) at 223 and 215 cm^{-1}, respectively. Actually, the oxygen partial pressure at half-saturation for NM Mb is 4.7 mmHg (Geraci et al., 1976, 1977), much higher than in monomeric Mb (Antonini & Brunori, 1971). On the basis of the Hoard−Perutz theory, which explains the cooperativity in Hb as due to a lower oxygen affinity in the T state as a consequence of the lower mobility of the proximal histidine (Hoard & Scheidt, 1973; Perutz, 1970), imidazole picket fence porphyrins iron(II) *meso*-tetrakis[a,a,a,a-[(*o*-pivaloyl)amido]phenyl]porphyrin (TpivPP) and their oxygen adducts have been studied to model the R and T conformations in Hb. The 1-MeIm adduct shows a ν(Fe−Im) at 225 cm^{-1} (Hori & Kitagawa, 1980), with a Fe−Im bond length of 2.07 Å (Collman et al., 1974; Jameson et al., 1978a). A substantially longer Fe−Im (2.107 Å) bond has been determined for the 2-MeIm adduct where the 2-methyl group hinders the approach of the ligand to the porphyrin plane (Jameson et al., 1978b; 1980). Accordingly, the RR frequency of the ν(Fe−Im) has been found at lower frequency (209 cm^{-1}) (Hori & Kitagawa, 1980). The steric hindrance of the bound imidazole has a dramatic effect on the observed O_2 affinities. Thus, the complex with 1-MeIm shows a P_{50} = 0.49 mmHg, whereas for the 2-MeIm complex the P_{50} = 620 mmHg (Walters at al., 1980). Therefore, the trend of the ν(Fe−Im) frequencies is consistent with the P_{50} variation; i.e., the 1-MeIm adduct gives an O_2 affinity similar to that of Mb or R-state Hb, while the 2-MeIm adduct mimics the T-state of Hb (Collman et al., 1978, 1980). Therefore, the relation between the Fe−His stretching frequency and the oxygen affinity, which applies to the tetrameric Hb and model compounds, seems to be valid also for NM Mb, suggesting a critical role for the Fe−His bond. It is interesting to note that recently Song et al. (1993) showed that for the homodimeric SI Hb the frequency of the Fe−His stretching mode, observed at 203 cm^{-1}, does not correlate with the dissociation constant of the last oxygen molecule, proposing that in this case the Fe−His bond does not play the same role in heme−heme communication in SI Hb as it does in vertebrate hemoglobins. However, it must be pointed out that the frequency and intensity of the Fe−His stretching mode have been reported to depend on the geometrical parameters of the orientation between the heme plane and the proximal histidine, namely,

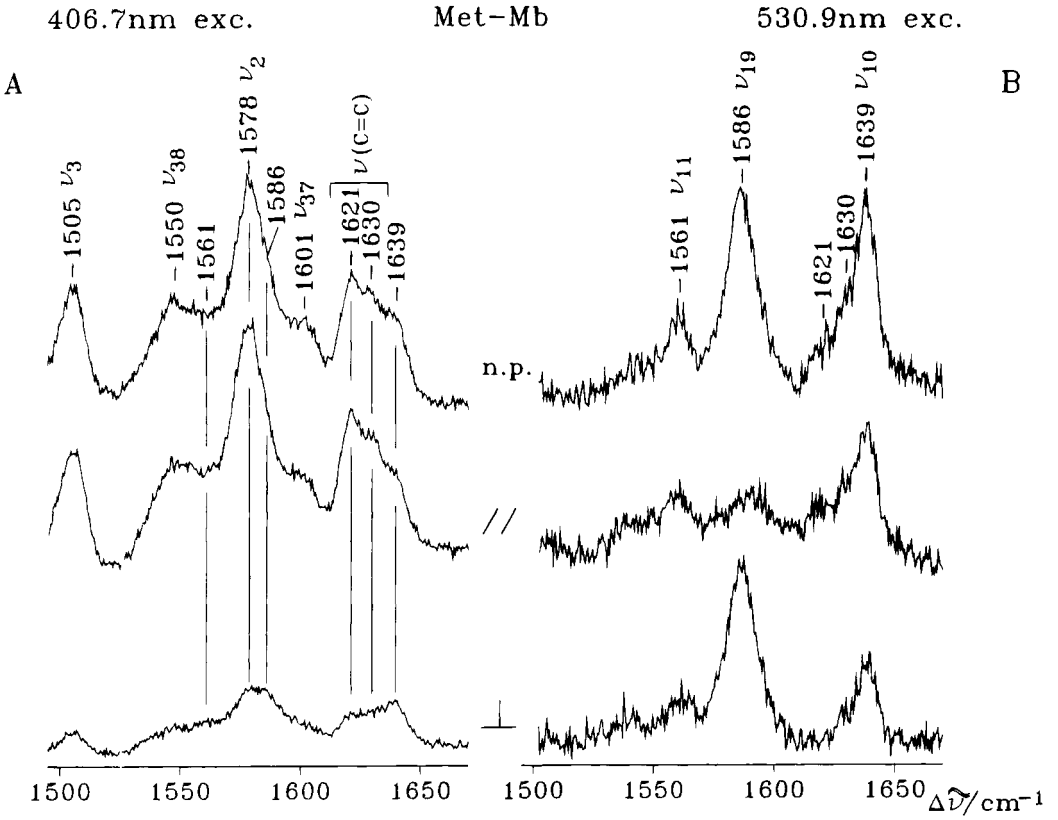

FIGURE 6: Resonance Raman spectra in polarized light of metmyoglobin from *Nassa mutabilis* (NM), obtained with different excitation wavelengths. Experimental conditions: 5 cm^{-1} resolution; (A) 406.7 nm excition, 30 mW laser power at the sample, 2 s/cm^{-1} collection interval for the nonpolarized spectrum (n.p.), and 9 and 12 s/cm^{-1} collection intervals for the spectra taken in parallel (||) and perpendicular (⊥) polarized light, respectively; (B) 530.9 nm excitation, 80 mW laser power at the sample, 9 s/cm^{-1} collection interval for the n.p. spectrum, and 9 and 6 s/cm^{-1} collection intervals for the spectra taken in parallel (||) and perpendicular (⊥) polarized light, respectively.

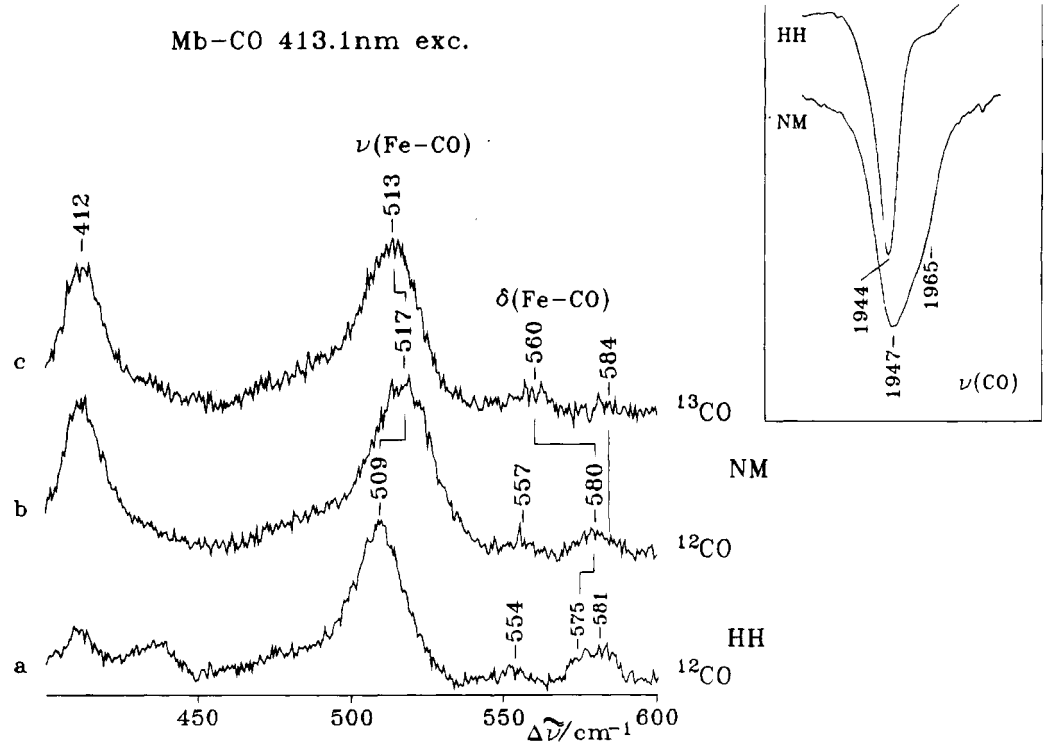

FIGURE 7: Resonance Raman spectra of CO complexes of *Nassa mutabilis* (NM) and horse heart (HH) myoglobins, obtained with 413.1 nm excitation wavelength. Experimental conditions: 5 mW laser power at the sample; 5 cm^{-1} resolution; (a) 10 s/cm^{-1} collection interval, (b) 60 s/cm^{-1} collection interval, and (c) 50 s/cm^{-1} collection interval. The insert shows the IR spectra of the CO complexes of NM and HH myoglobins; a total of 600 transients were accumulated; the spectral resolution was 4 cm^{-1}.

on the tilt angle (ϑ) between the heme normal and the Fe−Im vector, and on the azimuthal angle (ϕ) which denotes the rotation of the projection of the His plane on the porphyrin plane with respect to the N(1)−Fe−N(3) axis (Bangcharoenpaurpong et al., 1984; Friedman et al., 1990). Such parameters are not independent of each other, and their variation brings about alterations of the frequency and of the intensity for the RR bands. Therefore, since SI Hb and NM Mb display closely similar ligand binding thermodynamic parameters (Geraci et al., 1977; Chiancone et al., 1981), and likely the same mode of heme−heme interaction (Coletta et al., 1990, 1992), the dramatic difference observed between the two proteins for the ν(Fe−Im) stretching mode (Song et al., 1993) might be ascribable to differences in the tilt and azimuthal angles. As a matter of fact, a recent NMR investigation of the CN-met form of the SI Hb indicates that the orientation of the imidazole plane of the proximal histidine is not eclipsed with the N(1)−Fe−N(3) axis, as in the vertebrate hemoproteins, but is rotated by about 40° (McGourty et al., 1989). Therefore, on the basis of these considerations, NM Mb seems to show a Fe−His proximal bond with a conformation similar to that observed for the other vertebrate hemoproteins, even though with a larger strain on the tilt angle θ, as indicated by the frequency shift and the altered bond energy (Coletta et al., 1992). On the other hand, SI Hb appears to display a more marked and extended alteration of the geometrical interrelationships of the proximal bond, giving rise to a changed relationship between O_2 affinity and the frequency of the ν(Fe−Im) stretching mode, which does not necessarily imply a different role for the proximal bond between the two hemoproteins, as for the close similarity of the heme−heme communication system (Coletta et al., 1990). However, such a possible difference of the azimuthal angle ϕ between NM Mb and SI Hb is likely referrable to variations of the conformation on the proximal side of the heme pocket, and the occurrence of a variety of structural organizations for a cooperative homodimer can be conceived.

The intensity and frequency variations of the bands assigned to the bending modes of the peripheral substituents in the region between 290 and 420 cm^{-1} in NM Mb, with respect to HH Mb, indicate that the interactions of the vinyl and the propionyl groups of the heme with the protein and, as a consequence, their orientations are different in the two globins.

Oxy-Mb. The ν(Fe−O_2) of NM Mb appears at the same frequency (571 cm^{-1}) as that observed for HH Mb, and is very similar to that observed for human Hb (Nagai et al., 1980), homodimeric SI Hb (Song et al., 1993), and cytochrome *c* oxidase (Han et al., 1990a,b). This is not surprising, considering that the frequency of the Fe−O_2 stretching mode does not appear to be very sensitive to proximal and distal interactions (Song et al., 1993). The variations observed in the low-frequency region lead to the same conclusions drawn for the deoxy derivative; i.e., the orientation and interactions of the vinyl and propionyl groups are different in the two proteins.

Met-Mb. The met form gives rise to RR spectra which are similar to those of the oxy form, except for the expected absence of the ν(Fe−O_2) stretching mode. In addition, the absorption spectrum is characteristic of a ferric 6-c low-spin heme (Figure 8), distinctly different from that of oxy-Mb. The similarity of the RR spectra between the two forms is due to the fact that the binding of π acid molecules such as CO and O_2 to the Fe^{II} form causes a frequency increase of the core size marker bands as a consequence of back-donation of the d_π electron orbitals of the Fe atom to the lowest energy π^* orbitals of the axial ligand (Spiro & Li, 1988). Two vinyl bands are still observed in ferric met-Mb with the former at 1621 cm^{-1}, slightly more enhanced with respect to oxy-Mb, behavior which possibly is due to the interaction of one vinyl group with the internal sixth ligand which binds the Fe atom.

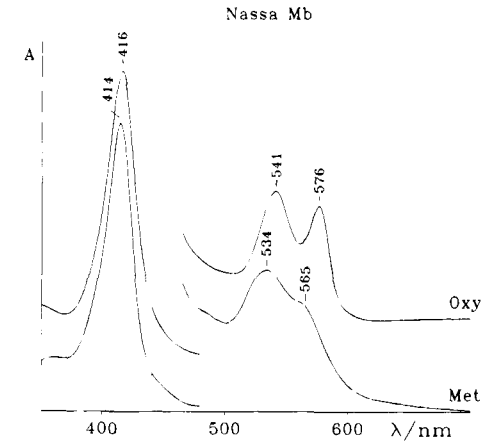

FIGURE 8: Electronic absorption spectra of oxy- and met-Mb from *Nassa mutabilis*.

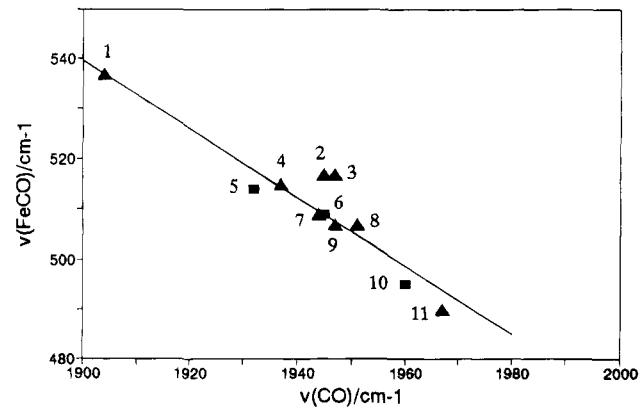

FIGURE 9: Plot of observed ν(FeCO) vs ν(CO) of heme protein−CO (triangles) and model compound−CO (squares) adducts. The numbers correspond to the entries in Table 2.

No final conclusion can be drawn on the nature of the internal sixth ligand bound in the met form. The presence of a bisimidazole heme is likely, considering that the amino acid sequence predicts the presence of a histidine on the distal side of the heme (Geraci et al., 1993), but other possibilities cannot be ruled out at this stage.

Carbon Monoxide Derivative. The RR and IR spectra of the CO adduct give information on the characteristics of the distal side. The frequencies of the ν(Fe−CO) stretching, δ(Fe−CO) bending, and ν(CO) stretching modes indicate that the CO molecule is tilted from the normal to the heme plane. The frequencies are very similar to those observed for HH Mb, SW Mb (Ramsden & Spiro, 1989), HbA (Tsubaki et al., 1982), and elephant Mb (Kerr et al., 1985) and almost identical to those reported for SI Hb (Song et al., 1993). Figure 9 illustrates a plot of the ν(Fe−CO) stretching frequency against the ν(CO) stretching frequency for different heme proteins and model compounds (Table

Table 2: Vibrational Frequencies (cm^{-1}) of FeCO Linkage in Carbonyl Complexes of Metalloporphyrins and Heme Proteins

molecule[a]	ν(Fe−CO)	ν(CO)	reference
(1) HRP, pH 7	537	1904	Evangelista-Kirkup et al. (1986)
(2) NM Mb	517	1947	this work
(3) Si Hb	517	1945	Song et al. (1993)
(4) elephant Mb, pH 7	515	1937	Kerr et al. (1985)
(5) FeSP-13	514	1932	Yu et al. (1983)
(6) FeSP-15	509	1945	Yu et al. (1983)
(7) HH Mb, pH 7	509	1944	this work
(8) HbA	507	1951	Tsubaki et al. (1982)
(9) SW Mb, pH 7	507	1947	Ramsden & Spiro (1989)
(10) PPDME(ImH)	495	1960	Evangelista-Kirkup et al. (1986)
(11) SW Mb, pH 4	490	1967	Ramsden & Spiro (1989)

[a] HRP, horseradish peroxidase; NM, *Nassa mutabilis*; Mb, myoglobin; SI Hb, hemoglobin from *Scapharca inaequivalvis*; FeSP-13, -15, strapped hemes with 13 and 15 atom hydrocarbon chains across one face of the heme and with *N*-methylimidazole as fifth ligand; HH, horse heart; HbA, human hemoglobin; SW, sperm whale; PPDME(ImH), protoporphyrin IX dimethyl ester with imidazole as fifth ligand.

2), all having imidazole as a fifth ligand. The solid line is a least-squares fit to a large body of data from CO adducts of iron porphyrin complexes and heme proteins (Uno et al., 1987; Kerr & Yu, 1988; Li & Spiro, 1988). The distribution of the points along the line depends on the extent of electron back-donation from the Fe d_π to the CO π^* orbitals (Paul et al., 1985; Tsubaki et al., 1986; Uno et al., 1987; Li & Spiro, 1988). The ν(Fe−CO) increases, while ν(CO) decreases, as back-bonding increases. Interactions of the bound CO with polar and/or H-bond donating groups on the distal side of the heme enhance back-donation. The points for the NM Mb−CO and SI Hb−CO adducts fall very close to one another. For the latter protein, the frequencies of the three modes involving the Fe−CO group led Song and co-workers (Song et al., 1993) to estimate a Fe−C−O bond angle of 171 ± 5°, in the limit of the three-body oscillator approximation (Yu et al., 1983). A similar conclusion can be drawn for the NM Mb−CO complex.

CONCLUSIONS

In the low-frequency region, NM and HH Mbs show different patterns of intensity of the RR bands due to modes which have contribution from the vinyl and propionate groups of the heme. These data, together with the splitting of the vinyl stretching modes into two bands in the high-frequency region, observed in all the derivatives of NM Mb, indicate that the substituents have a different interaction with the side chains which can result in a different orientation compared with the monomeric Mb.

The downshifted frequency of the ν(Fe−Im) stretching mode, with respect to that of HH Mb, indicates a weaker bond between the proximal ligand and the Fe atom, an observation which is consistent with its lower oxygen affinity and a slight relative expansion of the heme core. The effect is interpreted to be due to a protein-induced proximal ligand strain which results from protein-mediated heme−heme interactions made possible by the close proximity of the two hemes in the dimer. These interactions are also responsible for the cooperative ligand binding mechanism.

The met derivative which can be reduced back to the deoxy form, regenerating the capability to bind both O_2 and CO, contains a 6-coordinate low-spin heme. A distal histidine residue is a plausible candidate for the sixth coordinate ligand.

The oxy form gives rise to a ν(Fe−O_2) at the same frequency as other globins, a finding which is in agreement with the documented knowledge that its frequency is not very sensitive to proximal- and distal-side interactions. The CO adduct gives rise to modes of the FeCO fragment whose frequencies indicate a tilted Fe−CO unit (with respect to the heme plane) with an estimated angle of about 170°.

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