Resonance Raman Spectra of the Heme in Leghemoglobin

EVIDENCE FOR THE ABSENCE OF RUFFLING AND THE INFLUENCE OF THE VINYL GROUPS*

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Resonance Raman spectra of deoxy and carbonmonoxy leghemoglobin (Lb) are compared to the corresponding forms of human adult hemoglobin (HbA). It is found that the heme "core size" indicator line has nearly the same frequency for the two deoxyhemoglobins and the \(\tau\)-electron density-sensitive line also falls at the same frequency. However, several other modes occur at very different frequencies in the spectra of the two proteins. From an examination of the spectrum of an HbA derivative in which the \(\beta\)-carbon atoms of the heme vinyl groups were deuterated, it appears that the major differences between deoxy-HbA and -Lb may result from conformational changes in the vinyl groups. No evidence for the suggested ruffling (Irwin, M. J., Armstrong, R. S., and Wright, P. E. (1981) FEBS Lett. 133, 239-243) in deoxy-Lb was found. The spectra of carbonmonoxy-Lb and -HbA were also found to be very different. As in the deoxy case, some of these frequency differences could be attributed to vinyl group conformational differences. However, from the large difference in the \(\tau\)-electron density-sensitive line, it appears that the vinyl \(\tau\)-conjugation into the porphyrin in Lb(CO) may be different than it is in HbA(CO). The vinyl conformational differences may be a consequence of the looser heme pocket in Lb than in HbA. The difference in \(\tau\)-conjugation could make a significant contribution to the difference in ligand binding affinity for these two globins.

The oxygen binding affinities of the heme prosthetic group of various hemoglobins may assume a wide range of values dependent upon the structure of the globin (Baldwin, 1975). Large affinity differences (\(\times 200\)) may be induced in tetrameric Hbs by changing the protein quaternary structure. The range of affinity differences among various monomeric and tetrameric hemoglobins is even greater. However, the exact molecular basis for the oxygen binding affinity remains to be determined.

Leghemoglobin, a monomeric oxygen binding hemeprotein isolated from root nodules of legumes, is unique among Hbs in its \(O_2\) binding characteristics (Appleby, 1962). It has one of the highest oxygen binding constants and apparently achieves this high affinity via a large increase in oxygen on-rate (Wittenberg et al., 1972; Imamura et al. 1972). This contrasts with the quaternary structure-induced modulation of oxygen affinity in animal Hbs which presumably derives from a change in off-rate (Moffat et al., 1979). Thus, a careful examination which contrasts the molecular structure at the heme prosthetic groups in Lb with those of the cooperative Hbs may be used to help discriminate between interactions that affect oxygen on-rates and those that modulate its release from the heme.

Resonance Raman scattering has been applied to the study of many hemeproteins and has been found to be a valuable technique in probing the bonds of the heme and the bonds between the iron atom and the axial ligands (for a recent review see Rousseau and Ondrias, 1983). The porphyrin modes detected in the Raman spectrum have been shown to be sensitive to the heme electron density, the spin state of the central iron atom, and the size of the porphyrin core (for a recent review, see Asher, 1981). Normal coordinate analyses of symmetric porphyrins have also been reported and they reveal that the normal modes have contributions from several symmetry coordinates (Abe et al., 1978). However, these calculations have not included contributions from the protoporphyrin-IX peripheral substituents. The importance of these substituents to the normal coordinates awaits detailed isotopic substitution studies which are currently ongoing in several laboratories, including ours.

Although the molecular basis for the high affinity of Lb is not understood, Irwin et al. (1981) proposed recently that a high ligand field strength, induced by strong Fe-N (pyrrole) bonds due to a ruffled deoxyheme, may contribute to the high oxygen affinity. They cited resonance Raman data as evidence for a ruffled porphyrin. This proposal is important since if ruffling does lead to an affinity difference it would be a case of electronic control of affinity and would have significant implications on the mechanism of ligand binding in heme proteins. Since the appraisal of binding affinities requires an assessment of both initial (deoxy) and final (liganded) structures (Ondrias et al., 1982), we have used Raman difference spectroscopy to compare the resonance Raman spectrum of deoxy and carbonmonoxy leghemoglobin to the spectra of analogous forms of HbA. Although our data from the deoxy proteins generally agree with those of Irwin et al. (1981) and...
Armstrong et al. (1980), there are some important discrepancies. From careful depolarization measurements we find that the core size marker line is at nearly the same frequency in Lb as in HbA (or myoglobin). This is in contrast to the results of Armstrong et al. (1980) who reported a large difference in this mode.

We also report the spectra of modified hemoglobins in which the protons on the terminal carbon (β) of the vinyl groups have been isotopically substituted. This substitution results in extensive frequency shifts in modes of both the deoxy and the liganded Hbs. We find that the modes which show vinyl group isotopic substitution effects also differ in the comparison between Lb and HbA for both the deoxy and the carbonmonoxy preparations. From these data, it may be inferred that the conformation of the vinyl groups or their interactions with the protein are different in Lb than they are in HbA. Energetic importance has already been associated with the presence of the vinyl groups (Yonetani et al., 1974; Sono and Asakura, 1975) and thus their conformation could affect the extraordinarily high affinity of Lb. Striking differences between Lb and HbA are also detected in the low frequency spectra of both the deoxy and the carbonmonoxy proteins. In addition, there is a 2 cm⁻¹ difference in ρυ, the electron density marker line, between Lb(CO) and Hb(CO), but no differences in this mode in the deoxy proteins.

**EXPERIMENTAL PROCEDURES**

Lb (isozyme a) was extracted from soybean root nodules purified as described by Appleby et al. (1975). Frozen lyophilized samples were reconstituted with appropriate buffers immediately before use. The preparation of hemoglobin reconstituted with β-vinyl-D₃-hematin was also previously reported (Budd et al., 1978; LaMar et al., 1978). Spectra were obtained with Raman difference instrumentation (Rousseau, 1981) using 441.6 nm (He-Cd) or 528.7 nm (argon ion) excitation and are displayed without any smoothing of the raw data. Raman difference spectra were obtained by placing samples in a two-compartment cell so that data from both samples were accumulated simultaneously and subsequently compared. Raman polarization difference spectra were gathered in a similar way by using a single compartment cell, half of which transmits the polarized scattered light and half of which transmits the depolarized scattered light (Rousseau, 1981). Deoxy samples were prepared by incubation in an N₂ atmosphere followed by addition of a few grains of sodium dithionate and by insertion into the gas-tight Raman scattering cell. Carbonmonoxy samples were flushed with CO gas after the addition of a trace amount of dithionite. Data were accumulated over a period of several hours with no evidence of sample degradation.

**RESULTS**

The Raman line originally determined (Spaulding et al., 1975) to be sensitive to the size of the porphyrin core is located in the 1550–1600 cm⁻¹ region and is anomalously polarized. In deoxyhemoglobin, there is also a depolarized Raman line at 1547 cm⁻¹. Thus, in the depolarized spectrum, both lines are present. The depolarized scattered light contribution may be eliminated by subtracting the polarized spectrum (multiplied by 0.75) from the depolarized spectrum. A series of spectra are shown in Fig. 1 for deoxy-Lb. The top spectrum (polarized) is multiplied by 0.75 and subtracted from the spectrum below it (depolarized) resulting in the anomalously polarized spectrum (third spectrum from top). Note the complete cancellation of the depolarized line at 1604 cm⁻¹. A similar series of data was obtained for deoxy-HbA. Its anomalously polarized spectrum is shown in the bottom of Fig. 1. In deoxy-HbA, the peak frequency of the anomalously polarized line is at 1555 ± 1 cm⁻¹ and in deoxy-Lb we locate the line at 1564 ± 1 cm⁻¹. The deoxy-Lb and HbA data were obtained independently by the Raman polarization difference spectroscopy technique. Direct comparison of deoxy-HbA and -Lb were also made, confirming the differences reported in Fig. 1.

**FIG. 1. Raman polarization spectra of the porphyrin "core-size" marker line in leghemoglobin.** A, polarized scattered light. B, depolarized scattered light. C, spectrum of anomalously polarized scattering from leghemoglobin obtained by multiplying the polarized spectrum by 0.75 and subtracting it from the depolarized spectrum. D, anomalously polarized spectrum of human adult hemoglobin. The spectra were obtained with 528.7 nm excitation (~75 mWatts). The spectral slit width was about 2 cm⁻¹.

Spectra comparing deoxy-Lb and -HbA with Soret excitation (441.6 nm) are shown in Figs. 2 and 3 for the low and high frequency spectral regions, respectively. The central spectra are of HbA. Above it are the spectra of Lb and on the top are the difference spectra. Spectra of the deoxy derivative of hemoglobin in which the protons on the vinyl β-carbons are deuterated are shown below the HbA spectra in Figs. 2 and 3. The bottom-most spectra are difference spectra of native deoxy-HbA minus the isotopically substituted deoxy-HbA. In these representative spectra, not all lines in each difference spectra are balanced. When the difference spectra were utilized to obtain the accurate frequency differences, additional difference spectra were calculated in which each line of interest was balanced. It should also be noted that the data in these figures are a composite of two experimental runs—one in which a comparison of deoxy-HbA and -Lb was made and one in which a comparison of deoxy-HbA and HbA (vinyl-D₃) was made. The frequencies of all the prominent lines from these data are tabulated in Table I.

Upon examination of the differences between deoxy-HbA and the β-vinyl deuterated derivative, some observations are immediately evident. First, a very large number of modes show an effect of the deuteration. Second, many of the porphyrin modes are apparently Fermi resonance coupled to the vinyl modes as evidenced from the complex line shifts in the difference spectra. On isotopic substitution, modes split, broaden, or disappear. Third, certain modes (e.g. those at 216, 1357, and 1472 cm⁻¹) are unaffected by the isotopic substitution, while other modes show very large shifts (e.g. those at 406, 431, 585, 790, 994, and 1621 cm⁻¹). The most pronounced difference is in the mode at 1621 cm⁻¹ in HbA which has already been proposed as the carbon-carbon stretching frequency of the vinyl group (Spiro and Streekas, 1974). Upon deuteration of the vinyl β-protons, the line at 1621 cm⁻¹
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Figs. 2 and 3. Low frequency (Fig. 2, left) and high frequency (Fig. 3, right) Raman spectra and difference spectra for deoxy leghemoglobin and human deoxyhemoglobin with deuterium on the \( \beta \)-carbon atoms of the vinyl groups. In the middle is the spectrum of HbA. Above it is the spectrum of Lb and on the top is the HbA-Lb difference spectrum. Below the HbA spectrum is the spectrum of the \( \beta \)-vinyl substituted derivative [HbA(\( \nu \beta \)-D)]. On the bottom is the native HbA minus the \( \beta \)-vinyl substituted derivative. The spectra were obtained with 441.6 nm excitation (about 15 milliwatts). The spectral slit width was \( \pm 5 \) cm\(^{-1} \).

disappears but in the substituted hemoglobin it no longer occurs as a single line. Rather, it appears to mix with porphyrin skeletal modes in the 1580-1610 cm\(^{-1} \) region (see the difference spectrum).

The Raman line at 994 cm\(^{-1} \) in HbA appears only as a rather structureless feature between 950 and 980 cm\(^{-1} \) in the spectrum of the deuteron-substituted sample. We assume that the mode borrows intensity from coupling with the 979 cm\(^{-1} \) porphyrin skeletal mode in the native deoxy-HbA spectrum. Very large and well defined shifts are seen in the mode at 790 cm\(^{-1} \) which shifts to 778 cm\(^{-1} \) and the modes at 406 and 431 cm\(^{-1} \) which shift to 395 and 423 cm\(^{-1} \), respectively.

The deoxy-Lb spectra differ significantly from the deoxy-HbA spectra, most notably in the low frequency region. The iron-histidine stretching mode, assigned to the line at \( \sim 216 \) cm\(^{-1} \) in HbA shifts to 224 cm\(^{-1} \), a frequency close to that of high affinity (R-structure) HbA and higher than that of myoglobin. The other lines throughout the low frequency region have very different frequencies from those of HbA and are significantly broader. The largest frequency difference occurs in the HbA mode at 406 cm\(^{-1} \) which shifts to 421 cm\(^{-1} \) in Lb. The changes in frequency of modes above 500 cm\(^{-1} \) are much smaller than are those of the low frequency modes. Some modes show no (<1 cm\(^{-1} \)) frequency differences at all.

The spectra of carbonmonoxy-HbA and its \( \beta \)-vinyl deuterated derivative and their differences are displayed in Figs. 4 and 5 using the same format as was used in the deoxy spectra. The extensive reordering of vibrational couplings engendered by ligand binding evidently leads to a reduction in the influence of the vinyl substituents on the heme normal modes. While in the deoxy preparation there were several modes which had frequency shifts on \( \beta \)-vinyl deuteration of \( 3 \) cm\(^{-1} \) or greater, in HbA(CO) only the modes at 1620, 1000, 796, and 434 cm\(^{-1} \) had such large shifts. There were, however, many modes that had smaller shifts. (See Table II). Because of the apparent overlap of several modes in the 320-400 cm\(^{-1} \) region, it is not possible to determine the quantitative effect of \( \beta \)-vinyl deuteration on these modes. It is noteworthy that in contrast to the complex pattern of changes on deuteration seen in the 1570-1630 cm\(^{-1} \) region of deoxy-HbA, in HbA(CO) the change in frequency is quite clear (Fig. 5) and affects only a single line. This mode, which shifts from 1620 to 1600 cm\(^{-1} \) on deuteration may be assigned as the vinyl stretching mode. No shifts are evident in either the \( \pi \)-electron density-sensitive mode, \( \nu_v \), at 1373 cm\(^{-1} \) or the Fe-C axial stretching mode at 505 cm\(^{-1} \) (Tsukiji et al., et al., 1982; Armstrong, et al., 1982) as a result of \( \beta \)-vinyl deuteration.

The differences exhibited in the comparison of HbA(CO) and Lb(CO) are much more widespread. In addition to differences in the vinyl-sensitive modes mentioned above, Lb(CO)
The vinyl P-protons are deuterated are also included. The question marks in the \(\beta\)-vinyl deuterated shift column designate that we are unable to identify the frequency of the line. The dashes in the \(\beta\)-spectrum are for lines that are too weak to be located.

In the previous section we have shown that there are many Raman detectable modes which have vinyl contributions. Recently, similar conclusions were drawn from \(\beta\)-vinyl deuteration studies in nickel porphyrins and from \(\alpha\)-vinyl deuteration studies in (2Melm) FePP, deoxy-Mb, and Mb\(\cdot\)Fe\(^{2+}\) by Choi et al. (1982, a and b). A complete analysis of the coupling between the vinyl modes and the porphyrin skeletal modes and a comparison between HbA results and the reported model compound and Mb results (Choi et al., 1982, a and b) is underway and will be published separately. In the present paper, we discuss the implications of the many changes caused by vinyl substitution on the Lb-HbA comparison.

The widespread differences in the vibrational properties of the heme chromophores of HbA and Lb offer a number of insights concerning the structural distinctions between these oxygen binding proteins. The behavior exhibited by individual modes in the Lb-HbA comparisons can be divided into two qualitatively distinct categories: differences in modes that have significant contributions from the heme vinyl substituents and differences in modes that are vibrationally independent of vinyl influences. The former case applies most readily to the comparisons of deoxy-HbA and -Lb, whereas other influences evidently also play a large role in the liganded proteins. We discuss each below in detail.

### DISCUSSION

In deoxy-Hbs, two modes are especially sensitive to \(\beta\)-vinyl deuteration—the modes at 406 and 1621 cm\(^{-1}\). The large shift in the mode at 1621 cm\(^{-1}\) confirms its proposed assignment (Spiro and Strekas, 1974) as resulting from the vinyl carbon-helix stretching mode. As may be seen from Fig. 3, there is a clear cut difference in this mode between Lb and HbA. Moreover, the 406 cm\(^{-1}\) line shifted to 395 cm\(^{-1}\) on \(\beta\)-vinyl deuteration. This is not surprising in view of reported data (Desbois et al., 1981) comparing Fe\(^{2+}\) PP (2Melm) to Fe\(^{2+}\)EtioP(2Melm). The former has a line at 406 cm\(^{-1}\) while the latter has no line at all in this region. This is additional evidence that the line in the 400-425 cm\(^{-1}\) region has a large vinyl component. In the recent study by Choi et al. (1982a), this line was assigned as a vinyl bending mode. Further evidence for other peripheral contributions to this mode comes from the \(\beta\)-methyl group deuteriation study of Nagai et al. (1980) in which a large shift was also found. In deoxy-Lb, the mode in this region is shifted from 406 to 421 cm\(^{-1}\), suggesting that the conformation of the vinyl groups, and possibly other peripheral groups as well, in deoxy-Lb differ from those in HbA. At present, the specific conformational differences cannot be assessed.

For the remaining lines which differ in the Lb-HbA comparison but show no \(\beta\)-vinyl deuteration effect (341, 366, 1174, and 1214 cm\(^{-1}\) in HbA), we offer no explanation. However, we suggest that these differences result either from vinyl effects which are insensitive to the mass at the \(\beta\)-vinyl positions or from other peripheral substituents. This proposal is confirmed in part by 1, 3-methyl group deuteriation studies of Nagai et al. (1980) and these lines have methyl group isotopic sensitivity and the 366 cm\(^{-1}\) line also has \(\alpha\)-vinyl deuteration sensitivity. The line at 790 cm\(^{-1}\) which has isotopic sensitivity but displays no difference between HbA and Lb we presume is simply a mode which is not sensitive to the conformational differences between Lb and HbA.

The data reported here do not confirm previous conclusions (Irwin et al., 1981; Armstrong et al., 1980) that the major differences between deoxy-HbA and -Lb arise from a more ruffled heme group in the latter. These conclusions were reached primarily by examining the behavior of modes at 400-425 cm\(^{-1}\) and at 1555 cm\(^{-1}\). As reported here, Irwin et al. (1981) found a large frequency difference between myoglobin and leghemoglobin in the line in the 400-425 cm\(^{-1}\) region. This result was cited as evidence for ruffling based on a normal mode study of Ni(OEP) by Abe et al. (1978). A mode...
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Figs. 4 and 5. Low frequency (Fig. 4, left) and high frequency (Fig. 5, right) Raman spectra and difference spectra for carbonmonoxy leghemoglobin and a derivative of human adult hemoglobin in which the \( \beta \)-carbons on the vinyl groups of the heme have been deuterated. In the middle is the spectrum of HbA(CO). Above it is the spectrum of Lb(CO) and the HbA(CO)-Lb(CO) difference spectrum is on the top. Below the HbA(CO) spectrum is the spectrum of the \( \beta \)-vinyl deuterated derivative and on the bottom is the difference spectrum between HbA(CO) and the derivative. In Fig. 4, the asterisks correspond to the position of a laser fluorescence line. In Fig. 5, the asterisks correspond to frequencies at which there occurred a laser failure. The spectra were obtained with 406.7 nm excitation (~50 milliwatts). The spectral slit width was ~5 cm⁻¹.

<table>
<thead>
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<th>Table I</th>
<th>Raman frequencies (in cm⁻¹) of HbA(CO) and Lb(CO)</th>
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<td>HbA(CO)</td>
<td>( \beta )-Vinyl Shift</td>
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<tr>
<td>240</td>
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<td>313</td>
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The frequency differences between HbA(CO) and a derivative in which the \( \beta \)-protons are deuterated are also included. The question marks and dashes have the same designation as in Table I.

in this region in Ni(OEP) was assigned as \( \nu_{24} + \nu_{30} \) and was shown to have some \( ^{15} \)N isotopic sensitivity, presumably resulting from a Ni-N (pyrrole) contribution from \( \nu_{24} \) However, the \( ^{15} \)N frequency shift was only ~2 cm⁻¹ compared to much larger frequency shifts reported here on deuteration of the \( \beta \)-positions in the vinyl groups. Thus, in the ferrous iron heme, the peripheral groups appear to dominate the behavior of this mode and the large difference in the frequency of this mode between Lb (421 cm⁻¹) and either HbA (406 cm⁻¹) or Mb (409 cm⁻¹) is more likely to result from conformational differences in peripheral groups than from variations in the Fe-N force constant due to changes in heme planarity.

Armstrong et al. (1980) have reported the high frequency Raman spectrum of Lb. By using 514.5 nm excitation, they were able to measure the frequency of the anomalously polarized line \( (\nu_{19}) \) in the 1550-1600 cm⁻¹ region. They found a low frequency for this mode \( (1551 \text{ cm}^{-1}) \) compared to deoxymyoglobin \( (1555 \text{ cm}^{-1}) \) from which they concluded, based on a correlation of model compound data, that the heme core was either expanded compared to myoglobin or that it was domed or ruffled. In the data reported here, we find only a 1 cm⁻¹ difference in the core size marker line, \( \nu_{19} \), between Lb and HbA. (There is no difference in the frequency of \( \nu_{19} \) between HbA and Mb.) As further evidence of the lack of an expanded core, indicative of a domed or ruffled heme in Lb, we have examined \( \nu_{28} \), a polarized mode in the 1470-1510 cm⁻¹ region which has also been shown to be sensitive to the core size and nonplanarity (Spiro et al., 1979; Choi et al., 1982b). We find
that in comparing Lb to HbA there is no difference in the \( \nu_1 \) frequency. From these data, there appears no basis to conclude that the deoxy-Lb heme is ruffled or domed in comparison to Lb (or Mb). Instead, our data demonstrate that the core sizes of the hemes are remarkably similar. (A 1 cm\(^{-1}\) difference in \( \nu_{380} \) corresponds to a pyrrole-nitrogen to heme-center difference of only 0.002 Å.) Similar conclusions concerning the similarity of the heme conformation in Lb and in Mb have been drawn from epr studies (Ikeda-Saito et al., 1981).

Since the heme of Lb has the same axial ligand as HbA and Mb and the iron-histidine stretching frequencies are similar, there does not appear to be any unusual strain in the iron-histidine bond of Lb which could cause an anomalous heme distortion. Furthermore, the structural and spectroscopic data indicate that the heme pocket is more open in Lb than in HbA (Appleby et al., 1982; Fuchsman and Appleby, 1971; Johnson et al., 1978; Wright and Appleby, 1977). Thus, unusual constraints in Lb would not be expected to originate from interactions between the periphery of the heme and amino acid residues of the protein. On the other hand, our finding that the modes which are sensitive to the vinyl groups have different frequencies in Lb than in HbA, is not surprising since the looseness of Lb may allow the vinyl groups to have more orientational freedom and thereby substantially different frequencies. These conclusions are born out by preliminary \(^1\)H NMR experiments using the nuclear Overhauser effect between adjacent 1-CH\(_3\) and 2-vinyl groups.\(^3\) The vinyl is oriented with \( H_2 \) closer to the methyl in Met-Lb(CN) while in Met-Mb(CN), the vinyl is oriented so that the \( H_2 \) atoms are closer to this methyl.

Carboxymonoxy-Lb.—The differences evident in the comparison of liganded HbA and Lb are more complex and cannot be as readily ascribed to a single influence on the heme. Sensitivity to the vinyl substituents is retained (particularly in the modes at 1620, 1000, 796, and 434 cm\(^{-1}\)). However, neither the shift in \( \nu_1 \) (at 1373 cm\(^{-1}\)) nor the behavior of the low frequency (<500 cm\(^{-1}\)) modes can be rationalized as resulting from direct vibrational coupling of heme and vinyl modes. In Table II, the results of \( \beta \)-vinyl deuteration on the frequencies of Hb(CO) are listed along with a comparison of the Lb(CO) data. On \( \beta \)-vinyl deuteration, there are fewer large shifts in the carboxymonoxy spectra than there are in the deoxy spectra. Thus, it appears that ligand binding and the concomitant heme geometry changes alter the coupling between the heme and the vinyl groups such that the influence of the vinyl modes on the porphyrin vibrations are diminished. Alternatively, changes in the optical properties of the heme upon binding CO could result in lowered intensities for the vinyl sensitive modes. Excitation profile studies are needed to resolve this point. In comparing HbA(CO) to Lb(CO), as in the deoxy comparison, nearly all the modes which have \( \beta \)-vinyl sensitivity also differ. On this basis, we conclude that the conformation of the vinyl groups is different in Lb(CO) than it is in HbA(CO).

Inspection of the modes of Lb(CO) in the 100-600 cm\(^{-1}\) region reveals very striking differences relative to the HbA(CO) modes. It is interesting to compare these spectra to that of Mb(CO). The latter spectrum looks qualitatively similar to the Lb(CO) spectrum with sharp lines at 252, 318, 346, 378, 410, 435, and 510 cm\(^{-1}\). The major difference between Lb(CO) and Mb(CO) is that the line at 425 cm\(^{-1}\) in Lb(CO) is split into a doublet at 410 and 435 cm\(^{-1}\) in Mb(CO). Raman spectra of six coordinate ferrous model compounds (Desbois et al., 1981) reveal that a significant contribution to the low frequency modes comes from the peripheral substituents. For example, the spectrum of Fe\(^{1+}\)BinP(Im)\(_2\), has modes only at 257, 343, and 408 cm\(^{-1}\). On the other hand, Fe\(^{1+}\)PP(Im)\(_2\), has modes at 237, 265, 310, 344, 382, and 419 cm\(^{-1}\). We conclude that peripheral substituents may very significantly influence the low frequency modes and that the differences between Lb(CO) and HbA(CO) in that region may result from differences in peripheral group orientation and interaction with the protein. Evidently, Lb(CO) and Mb(CO) are more similar to each other than to HbA in these regards.

Recently, Irwin et al. (1981) reported the spectrum of Lb(O) and found that within experimental error the Fe-O stretching mode at 576 cm\(^{-1}\) is at the same frequency as in HbA(O) (567-572 cm\(^{-1}\)) and in Mb(O) (572-577 cm\(^{-1}\)). It is interesting that in the data reported here the Fe-C stretching modes is at the same frequency in HbA(CO) and in Lb(CO) (505 ± 1 cm\(^{-1}\)) but different from the value we find in Mb(CO) (510 cm\(^{-1}\)). These findings are consistent with conclusions previously reached from oxygenated complexes (Irwin et al., 1981), namely that the Fe-O or Fe-C bond energies are unrelated to the ligand affinities. Since the distal heme pocket would be expected to influence these frequencies, it is quite surprising that the frequencies are so close in such unrelated globins, especially since Lb apparently has a looser heme pocket. Evidently, the specific distal interactions that affect the Fe-C bond are identical in HbA(CO) and Lb(CO). It has also been argued that any significant changes on the proximal side of the heme, i.e. in the iron-histidine bond, should influence the Fe-CO or Fe-O bonds (Tusbaki et al., 1982). Thus, the data suggest similar proximal environments as well.

The electron density marker line, \( \nu_1 \), is about 2 cm\(^{-1}\) higher in Lb(CO) than it is in HbA(CO) (or in Mb(CO) which is nearly the same as in HbA(CO)). This is a very large difference relative to the 0.5 cm\(^{-1}\) difference induced in this mode by the quaternary structure change in carp Hb(CO) and Hb Kansas (CO).\(^4\) It is indicative of a significant alteration in the electron density in the porphyrin \( \pi \) system. As previously discussed, it is unlikely that this difference results from distal influences at the iron since the Fe-C bond is the same for Lb(CO) and HbA(CO). The 5 cm\(^{-1}\) difference between the Fe-C stretching modes of HbA(CO) (505 cm\(^{-1}\)) and Mbc(CO) (510 cm\(^{-1}\) indicate that changes in the Fe-C bond do not affect the porphyrin electron density marker line. As noted above, by inference, we assume that the proximal environment is also the same in Lb(CO) and HbA(CO). Thus, it appears most likely that the changes in the electron density marker line result from differences in the porphyrin protein environment. Such differences could result from direct interactions (e.g. charge transfer) between the porphyrin and amino acid residues or arise from inductive effects of the vinyl groups on the \( \pi \)-orbitals of the porphyrin. Since changes in vinyl orientation are clearly indicated, as discussed above, it is not unreasonable to assume that these orientational changes could also affect the \( \pi \)-orbital delocalization. More experiments are necessary to isolate these possible contributions.

CONCLUSIONS

The evidence presented here for both deoxy-Lb and Lb(CO) indicates that the orientation of the vinyl groups and possibly other peripheral groups, differ from those of Hb or Mb. This difference in orientation presumably results from differences in interactions within the heme pocket. How this difference in heme pocket interactions affects the oxygen binding affinity is unclear. However, it seems reasonable to assume that a

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\(^3\) G. N. LaMar and S. Ramaprasad, unpublished results.

loose heme pocket would favor a high binding affinity since the conformational changes in the heme resulting from ligand binding could be accommodated without extensive protein reorganization. On the other hand, it is quite surprising that in spite of the differences in the heme pocket the Fe-C stretching frequency has the same value in HbA(CO) as it does in Lb(CO) indicating that the environment on the distal side of the iron is very similar for these two proteins.

The Raman mode sensitive to electron density, νe, is the same in deoxy-HbA and deoxy-Lb in spite of the apparent difference in the conformation of the vinyl groups. However, in the HbA(CO)-Lb(CO) comparison, a large change in νe was detected. We may consider these results in the light of the behavior of vinyl substitutions in other systems. For example, many structural and spectroscopic differences in hemoglobin and cobalt substituted hemoglobin have been detected upon replacement of the protoheme by a meso heme (saturated vinyls) (Sono and Asakura, 1975, Hori, et al., 1982). These differences could originate from the increase in π-conjugation due to saturation of the vinyl groups in the meso-hemes or from stereochemical changes resulting from the different structural interactions between the protein and the vinyl groups than between the protein and the ethyl groups.

Our data indicate the absence of any difference in the π-conjugation in the deoxy proteins, but a large difference in the liganded proteins. Thus, any inductive effects on the electronic properties of the heme are evident in only the liganded case. The importance of the change in the electron density may be seen from the equilibrium binding properties of small ligands to reconstituted hemoglobin and myoglobin with formyl substituted hemes (Sono and Asakura, 1975). A clear alteration in both the on-rates and off-rates for ligand binding was detected and these rates were found to be proportional to the electron withdrawing power of the substituted side chain. Thus, the apparent difference in π-conjugation in the carbonmonoxy proteins could have a significant effect on the ligand affinity.

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