RESONANCE RAMAN STUDIES OF SOYBEAN LEGHEMOGLOBIN AND MYOGLOBIN

Origin of the differences in O_2 dissociation rate constants

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1. Introduction

Despite extensive investigations by a wide variety of techniques, the molecular mechanisms by which oxygen affinities are controlled in hemeproteins are only poorly understood. Amongst the monomeric hemoglobins and myoglobins, leghemoglobin, from *Rhizobium*-infected nitrogen-fixing legume root nodules, is notable for its very high oxygen affinity [1]. This arises from a combination of an extremely large oxygen association rate constant and a moderately small oxygen dissociation rate constant [2]. Previous studies of soybean and lupin leghemoglobins have revealed an open and flexible heme pocket which would facilitate reaction with oxygen [1,3–7]. We have found evidence from resonance Raman [8] and NMR [9] spectroscopy for a constrained heme structure and stronger ligand field at the iron atom in soybean leghemoglobin (Lb) compared to myoglobin (Mb) or hemoglobin (Hb). Here, we report resonance enhancement of low frequency vibrational modes in deoxy- and oxy-leghemoglobin and myoglobin. The spectra indicate no significant differences between the strength of the Fe–O_2 bond in oxy-leghemoglobin and oxy-myoglobin nor of the Fe–N_ϵ(proximal histidine) bond in the deoxy-proteins. However, differences in the strength of the in-plane Fe–N(pyrrole) bonds are indicated. It is proposed that heme ruffling in leghemoglobin, rather than changes in Fe-histidine bonding, leads to strenghtening of the ligand field and stabilization of the oxygen complex.

2. Materials and methods

Soybean ferric leghemoglobin a was purified as in [10]. Horse heart myoglobin (Fluka or Sigma type III) was purified by chromatography on a Sephadex G-25 column. No differences were found between the proteins from different sources. Sperm whale myoglobin (Sigma type II) was purified on a CM 52 column using a gradient from 10 mM phosphate (pH 6.0) to 100 mM phosphate (pH 7.0).

The deoxy-derivative of each of these proteins was prepared under argon in a spinning sample cell, sealed with a septum, by injection of a 2-fold excess of sodium dithionite to ferric Lb or Mb in phosphate buffer (10 mM, pH 7.0) containing EDTA (0.1 mM). The oxy-derivatives were prepared from the deoxy-proteins by introduction of $^{16}O_2$ or $^{18}O_2$ by syringe such that the samples were under a 1:1 mixture of argon and oxygen at atmospheric pressure. All samples were filtered with 0.22 μm Millipore filters and their absorption spectra were taken before and after resonance Raman experiments.

Spectra were obtained with excitation at 413.1 nm using a Coherent CR-2000K supergraphite Kr^+ laser and a Spex 1401 double monochromator. At other wavelengths a Spectra Physics 164 Ar^+ laser and a Spex 14018 double monochromator fitted with a spatial filter and floating lens adaptor were employed. In both cases photon counting was used. Signal averaging of multiple scans was performed with the aid of a Hewlett-Packard 9835A desktop computer and a Hewlett-Packard 6948B multiprogrammer to interface to the photon counter. The spectrometer was calibrated with carbon tetrachloride and individual spectra were checked against plasma lines. All peaks are accurate to ± 1 cm^{-1}.

3. Results and discussion

Figure 1 shows the low wavenumber region of the

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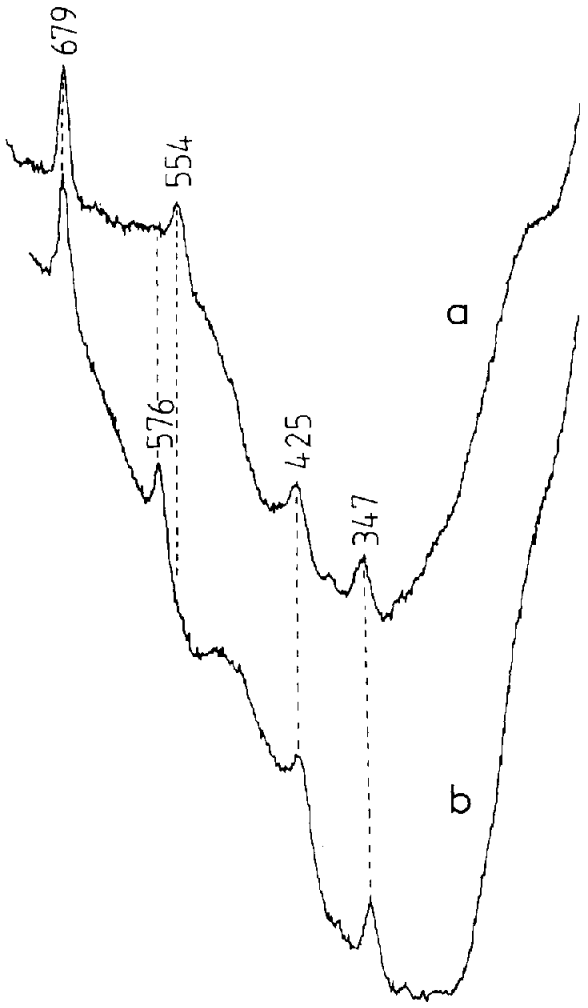

Fig.1. Resonance Raman spectra of: (a) soybean $^{18}O_2$-leghemoglobin; (b) soybean $^{16}O_2$-leghemoglobin; laser excitation at 488.0 nm; 200 mW at sample; 3 s time constant; 12 cm^{-1} min^{-1} scan; 8 cm^{-1} resolution.

Raman spectra of $Lb^{16}O_2$ and $Lb^{18}O_2$ obtained with excitation at 488.0 nm. The band at 576 cm^{-1} in the spectrum of $Lb^{16}O_2$ shifts 22 cm^{-1} on substitution of $^{18}O_2$ and clearly arises from the Fe–O_2 vibration. No other bands are sensitive to the isotopic substitution. Fe–O_2 stretching frequencies have also been measured for a series of hemoglobins [11,12] and myoglobins [13,14]. The Fe–O_2 stretching band was observed at 567–572 cm^{-1} in oxy-hemoglobins while values of 572 cm^{-1} and 577 cm^{-1} have been reported for oxy-myoglobin. Thus there is no significant difference in the Fe–O_2 bond strength in oxy-myoglobin and oxy-leghemoglobin.

Figure 2 and 3 show the low wavenumber region of the resonance Raman spectra of sperm whale and horse heart deoxy-myoglobins and of soybean deoxy-leghemoglobin obtained with excitation at 457.9 and 413.1 nm. Assignment of the Fe–N_ϵ(proximal histidine) stretch has been somewhat controversial to date. Most workers [11,15–17] have assigned this vibration to a band around 220 cm^{-1}. Our work provides further support for this assignment.

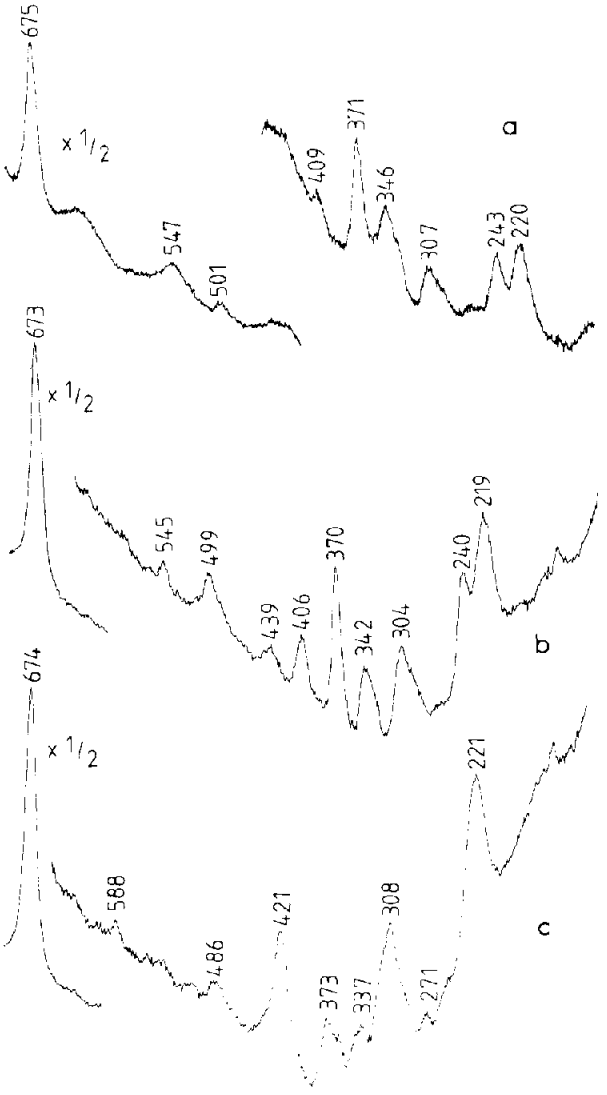

Fig.2. Resonance Raman spectra of: (a) horse heart deoxy-myoglobin; (b) sperm whale deoxymyoglobin; (c) soybean deoxyleghemoglobin; 4 scans were averaged for deoxyleghemoglobin. Typical conditions were: laser excitation at 457.9 nm; 70 mW at sample; 5 s time constant; 12 cm^{-1} min^{-1} scan; 6 cm^{-1} resolution.

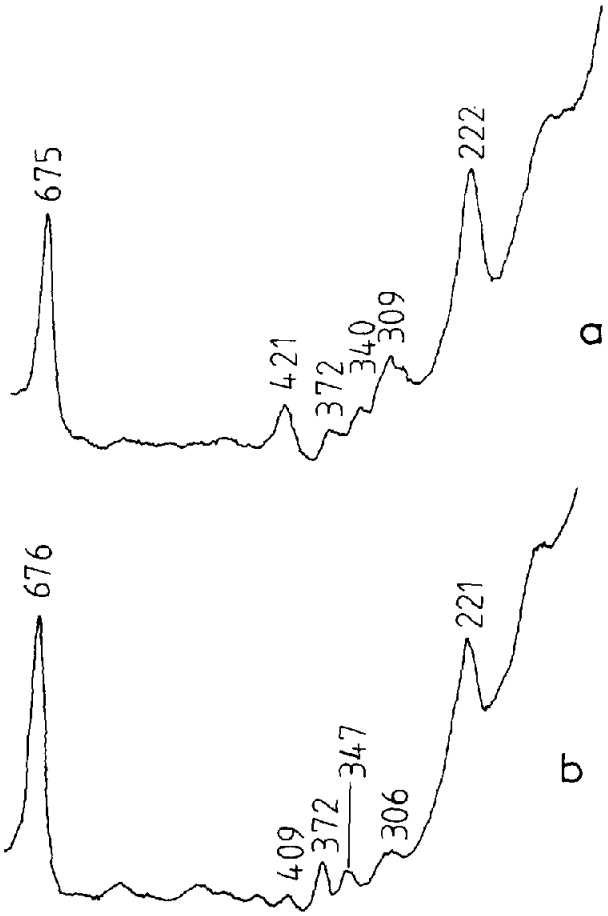

Fig.3. Resonance Raman spectra of: (a) soybean deoxyleghemoglobin; (b) horse heart deoxymyoglobin; laser excitation at 413.1 nm; 450 mW at sample; 1 s time constant; 1 cm^{-1} s^{-1} scan; 4.5 cm^{-1} resolution.

With irradiation at 457.9 nm (and also 441.6 nm [14]) all bands in the low wavenumber region show appreciable resonance enhancement (fig.2). However, with excitation at 413.1 nm there is a general decrease in intensity of the Raman bands relative to that at 220 cm^{-1} (fig.3). Since the Soret absorption band is near 430 nm in deoxy-leghemoglobin and deoxymyoglobin the porphyrin in-plane vibrations would be exptected to decrease in intensity as the exciting wavelength is decreased to 413.1 nm. Since substantial resonance enhancement of the band at 220 cm^{-1} is retained on post-Soret excitation, this vibration is clearly not in resonance with the Soret absorption but probably with a charge-transfer transition in this region. We note that an absorption band at slightly shorter wavelength than the Soret has been found in magnetic circular dichroism spectra of deoxymyoglobin [18]. The 220 cm^{-1} band must thus correspond to an out-of-plane vibrational mode. This is in accord with its assignment as an Fe–N_ϵ(proximal histidine) stretch [11,15–17] but not as an Fe–N(pyrrole) vibration [14]. The Fe–N_ϵ(proximal histidine) stretching band occurs at 219–221 cm^{-1} for deoxymyoglobin and 221–222 cm^{-1} for deoxy-leghemoglobin (fig.2,3), i.e., there is no significant difference in bond strength. This finding contradicts the suggestion made on the basis of X-ray diffraction studies of lupin leghemoglobin acetate that the Fe–N_ϵ bond is weaker in leghemoglobin than in other hemeproteins [7]. An increase in bond length of the magnitude suggested in [7] would result in a large decrease in ν_{Fe-N_ϵ}. The resonance Raman spectra thus indicate conclusively that in the functionally-important deoxyforms, there is no difference in Fe–N_ϵ bond strength between Lb and Mb. Furthermore, it now appears that the stronger ligand field in leghemoglobin [8,9] arises from the equatorial influence of the porphyrin.

In [8] we reported differences in the high frequency porphyrin ring vibrational modes of leghemoglobin and myoglobin and many of their derivatives which indicate a greater degree of core expansion or ruffling or doming of the heme in leghemoglobin. It was not possible to distinguish between these different heme constraints on the basis of the resonance Raman data alone. However, refinement of the 2.0 Å resolution X-ray structure of lupin leghemoglobin acetate has revealed pronounced ruffling of the heme [9] and it is proposed that it is this heme ruffling which influences the resonance Raman spectra. It is well known that heme ruffling leads to shortening of the equatorial Fe–N(pyrrole) bonds and this should be manifest in the low frequency region of the resonance Raman spectrum.

A band appears at 406 cm^{-1} and 408 cm^{-1} in the spectra of sperm whale and horse heart deoxy-myoglobins, respectively, but occurs at 421 cm^{-1} for deoxy-leghemoglobin (fig.2). The loss of intensity of this band on post-Soret excitation (413.1 nm, fig.3) shows that it is associated with porphyrin in-plane vibrational modes. The band shifts 12–15 cm^{-1} to higher energy on going from myoglobin to leghemoglobin, as would be expected for an increase in Fe–N(pyrrole) bond strength due to heme ruffling. We note that a band remains at this high frequency (425 cm^{-1}) in the spectra of LbO_2. According to the

normal coordinate analysis of octaethylporphinatonickel(II) [Ni(OEP)] no pure M–N(pyrrole) vibration is expected [20,21]. However, the analysis predicts a band near 425 cm^{-1} which has considerable contribution from M–N(pyrrole) bending. In the Raman spectrum of Ni(OEP) this band actually occurs at 415 cm^{-1} and is sensitive to ^{15}N(pyrrole) substitution [20,21]. We thus suggest that the band that appears in the 406–421 cm^{-1} region in the spectra of myoglobin and leghemoglobin has contributions from Fe–N(pyrrole) modes.

The present resonance Raman studies have provided detailed structural information on the iron coordination sphere in oxy- and deoxy-leghemoglobin and myoglobin. In seeking to relate the heme structures of these proteins to differences in their reaction with oxygen, it is important to consider not merely their oxygen affinities but rather their association and dissociation rate constants since these can be influenced independently. The resonance Raman spectra reveal no difference in the Fe–O_2 bond strength in oxymyoglobin and oxyleghemoglobin which might explain the higher dissociation rate of the former (k_{off} is 11 s^{-1} and 4.4 s^{-1} for MbO_2 and LbO_2, respectively [2]). This is in accord with the other Raman studies of the oxygen complexes of picket-fence porphyrins [16,22], hemoglobin [11] and myoglobins substituted with formyl hemes [13], all of which show that the Fe–O_2 bond energy is unrelated to oxygen affinity. Of great importance, the strength of the Fe–N_ϵ(proximal histidine) bond in deoxyleghemoglobin is identical to that in deoxymyoglobin. Thus the difference in free energy of oxygen binding between myoglobin and leghemoglobin is not localized in either of the Fe–O_2 or Fe–N_ϵ(proximal histidine) bonds. This is in contrast to the *R* and *T* forms of hemoglobin for which differences in the strength of the Fe–N_ϵ bond have been observed [11].

The principal difference in heme structure between leghemoglobin and myoglobin detected by resonance Raman spectroscopy is in the strength of the Fe–N(pyrrole) bonds. These bonds are stronger in leghemoglobin as a result of heme ruffling and we suggest this to be the origin of the stronger ligand field. It is well known that dissociation rates of octahedral low spin d^6 complexes are greatly influenced by ligand field effects [23]. We thus propose that the decreased oxygen dissociation rate of LbO_2 relative to MbO_2 arises, at least in part, from increased ligand field stabilization in the former complex. We shall report elsewhere on high resolution NMR and kinetic studies which show that interactions between the bound O_2 and the distal histidine also influence the dissociation rate (J. B. Wittenberg, B. A. Wittenberg, R. J. Morris, C. A. Appleby, J. H. Bradbury and P. E. Wright, manuscripts in preparation). It has been suggested that electrostatic [24] and electronic [25] interactions between the protein and heme may be important in regulating the oxygen affinity of tetrameric hemoglobins. As will be discussed in detail elsewhere, on the basis of changes in the frequency of the oxidation state marker band near 1375 cm^{-1}, these factors do not appear to contribute significantly to differences in the oxygen affinity of leghemoglobin and myoglobin. However, differences in ligand field strength in Lb and Mb have been established unequivocally and are expected to influence significantly the oxygen dissociation rates.

Acknowledgements

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