Vol. 95, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 31, 1980 Pages 682-689

RESONANCE RAMAN EVIDENCE FOR CONSTRAINED HEME STRUCTURE IN SOYBEAN LEGHEMOGLOBIN AND ITS DERIVATIVES

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Received June 20,1980

SUMMARY

Resonance Raman spectra have been obtained for soybean leghemoglobin and many of its derivatives. The spectra reveal a stronger ligand field at the iron atom in leghemoglobin than in myoglobin or hemoglobin. Differences in the frequency of an anomalously polarized band near 1550 cm^{-1} indicate an expanded porphyrin core or greater ruffling or doming of the porphyrin ring in deoxyleghemoglobin than is present in deoxymyoglobin. These heme constraints are retained in the various low spin complexes. The significance of the unusual heme structure in leghemoglobin in relation to its rapid oxygen binding reaction is discussed.

Heme proteins involved in storage and transport of oxygen show great variations in oxygen affinity (1-3). It is of considerable importance to determine the molecular basis of these variations. The low oxygen affinities of T state hemoglobin (4) and model iron(II) porphyrin complexes with sterically hindered axial bases (5,6) have been attributed to constraint of the proximal histidine. This constraint is proposed to be relaxed in R state hemoglobin, isolated hemoglobin chains and vertebrate myoglobins, resulting in increased oxygen affinity. However leghemoglobin, a monomeric oxygen-binding heme protein occurring in the nitrogen-fixing root nodules of legumes, binds oxygen with even greater affinity (7), largely as a consequence of an extremely fast oxygen binding reaction (3). Previous studies of soybean and lupin leghemoglobins have revealed an open and flexible heme pocket which would clearly facilitate oxygen binding (7-13). Contributions from electronic and geometric constraints on the heme may be equally important but have as yet remained largely undetected. We have therefore undertaken resonance Raman studies of soybean leghemoglobin to obtain information on these latter effects. Direct evidence is obtained for a constrained heme structure within the protein.

0006-291X/80/140682-08$01.00/0

MATERIALS AND METHODS

Ferric leghemoglobin a was isolated by standard methods (14). The ferric derivatives were prepared by addition of excess ligand to solutions of the protein in phosphate buffer (10 mM, pH 6.0) until no further changes were observed in the optical spectrum. Spectra of leghemoglobin hydroxide were obtained in borate buffer (25 mM, pH 10.0). The ferrous derivatives were prepared by addition of a two-fold excess of dithionite to ferric leghemoglobin under N_2. The CO derivative was then obtained by passage of CO over the deoxyleghemoglobin. Oxyleghemoglobin was prepared by applying deoxyleghemoglobin to a G-15 Sephadex column to remove excess dithionite and allowing the eluent to equilibrate with air. Solutions thus prepared were stable for the duration of the experiments as evidenced by absorption spectra taken after resonance Raman spectra had been obtained. Ferric myoglobin (Fluka, horse heart) was purified on a CM 52 column using a gradient from 10 mM phosphate (pH 6.0) to 100 mM phosphate (pH 7.0). Derivatives were prepared in the same way as the corresponding leghemoglobin derivatives.

Resonance Raman spectra were obtained with excitation at 514.5 nm and 488.0 nm from a Spectra Physics 164 Ar^+ ion laser. Solutions 0.4 mM in heme were contained in a spinning cell at room temperature. Light scattered at 90° was analysed by a Spex 14018 double monochromator fitted with a Spex 1478 spatial filter, 1800 line/mm holographic gratings and a cooled RCA 31034 photomultiplier tube. The signal was recorded digitally by a PAR model 1110 digital synchronous computer. Indene was used as a wavenumber standard and all spectra were recorded at least twice on duplicate samples. Wavenumber shifts were accurate to better than 2 cm^{-1} for all bands and to 1 cm^{-1} for well resolved bands.

RESULTS AND DISCUSSION

Laser excitation within the visible absorption bands of heme-proteins gives enhanced Raman scattering from porphyrin ring vibrational modes, most of which are observed between 1100 cm^{-1} and 1700 cm^{-1} (15). Extensive studies have shown that these modes are sensitive to changes in the geometry and electronic structure of the heme (16-19). All of the derivatives of soybean leghemoglobin afforded good quality resonance Raman spectra which were free of significant fluorescence. Representative spectra are shown in figure 1. The general spectral patterns and dependence upon excitation frequency are similar to those previously observed for myoglobin and hemoglobin (15,20).

Kitagawa (21) has devised a classification of heme proteins into four groups (A-D) on the basis of the relative intensities and frequencies of four Raman bands (I-IV). Table 1 summarizes the position and polarization properties of these four bands in the spectra of leghemoglobin derivatives. The fluoride and acetate complexes are classified as high spin ferric, in accord with their

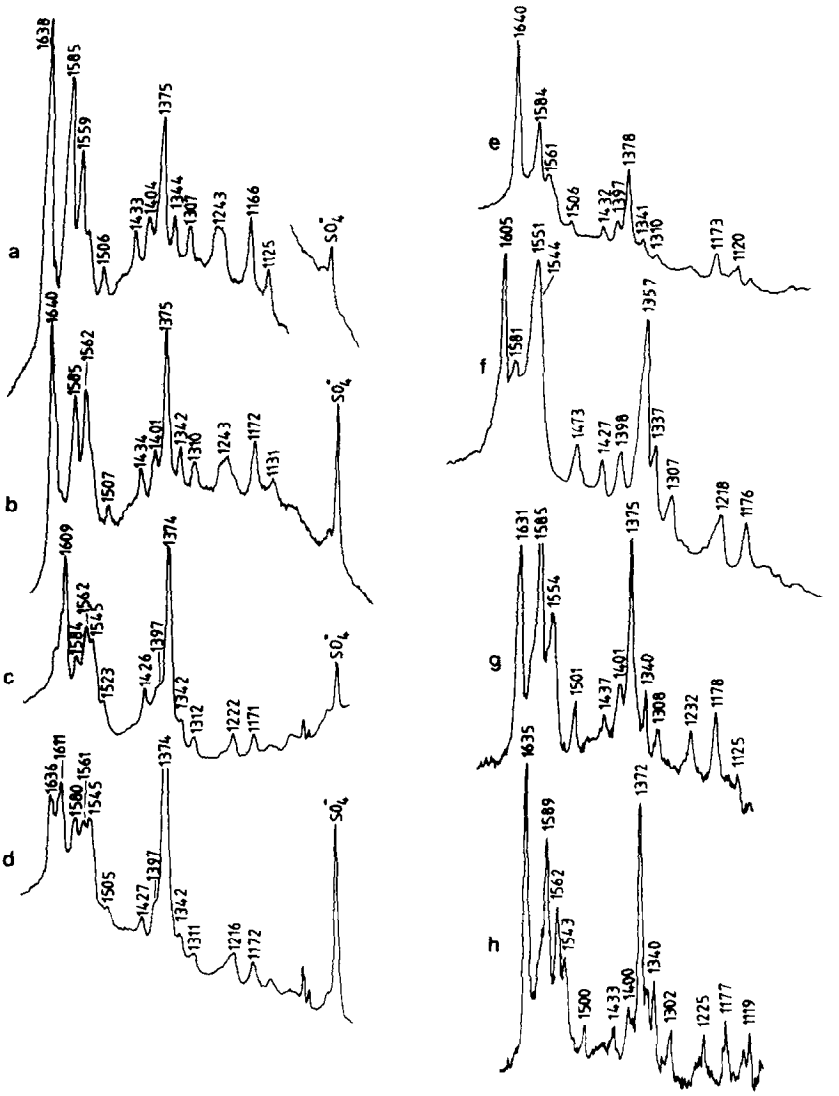

Figure 1. Resonance Raman spectra at 20°C of (a) ferric leghemoglobin nicotinate. (b) ferric leghemoglobin cyanide. (c) ferric leghemoglobin acetate. (d) ferric leghemoglobin (pH 6.0). (e) oxyleghemoglobin. (f) deoxyleghemoglobin. (g) carbon monoxy-leghemoglobin. (h) carbon monoxymyoglobin.

Laser excitation wavelength was 514.5 nm for spectra (a), (b), (e), (f), (g) and (h); and 488.0 nm for spectra (c) and (d).

Typical conditions were 5 cm^{-1} resolution, 5 s time constant, scan 12 $cm^{-1}min^{-1}$, 250 mW laser power at sample (except for carbon monoxy complexes where the laser power was 12 mW).

known spectral and magnetic properties (22). The low spin ferric derivatives yield similar Raman spectra to the corresponding myoglobin and hemoglobin derivatives (15,20). Ferric leghemoglobin azide is classified as a low spin

Table 1. The frequencies and polarization properties of bands I-IV (21) and of the anomalously polarized C_t-N marker band (labelled C(ap) (28)) for leghemoglobin (Lb) and myoglobin (Mb) derivatives. Spectra were obtained with 514.5 nm laser excitation.

COMPOUND	BAND					GROUP
	I	II	III	IV	C(ap)	
Lb(II) nicotinate	1622(dp)	1540(dp)	1495(p)	1364(p)	1584(ap)	A
deoxy Lb(II)	1605(dp)	1544(dp)	1473(p)	1357(p)	1551(ap)	B
deoxy Mb(II)	1604(dp)	1546(dp)	1472(p)	1355(p)	1556(ap)	
LbO_2	1640(dp)	1561(dp)	1506(p)	1378(p)	1584(ap)	
LbCO	1631(dp)	1554(dp)	1501(p)	1375(p)	1585(ap)	
MbO_2	1641(dp)	1553(dp)	1508(p)	1377(p)	1589(ap)	
MbCO	1635(dp)	1562(dp)	1500(p)	1372(p)	1589(ap)	
Lb(III) 3-chloropyridine	1640(dp)	1564(dp)	1510(p)	1375(p)	1589(ap)	C
Lb(III) pH10.0†	1638(dp)	1569(dp)	1507(p)	1378(p)	1588(ap)	
Lb(III) pyridine	1639(dp)	1559(dp)	1507(p)	1376(p)	1587(ap)	
Lb(III) azide	1642(dp)	1563(dp)	1506(p)	1377(p)	1586(ap)	
Lb(III) nicotinate	1638(dp)	1559(dp)	1506(p)	1375(p)	1585(ap)	
Lb(III) cyanide	1640(dp)	1562(dp)	1507(p)	1375(p)	1585(ap)	
Lb(III) fluoride	1606(dp)	1563(dp)	1482(p)	1373(p)	1555(ap)	D
Lb(III) acetate	1611(dp)	[1563(dp) 1545(dp)]	—	1374(p)	—	
Lb(III) pH6.0	[1638(dp) 1612(dp)]	[1561(dp) 1547(dp)]	1502(p)	1375(p)	1584(ap)	spin state mixtures
Lb(III) formate	[1641(dp) 1607(dp)]	[1562(dp) 1545(dp)]	1507(p)	1375(p)	1584(ap)	

† Classifies as low spin using irradiation at 514.5 nm. With excitation at 488.0 nm a weak band at 1480 cm^{-1} indicates a small contribution from a high spin component.

p (polarized), dp (depolarized), ap (anomalously polarized).

complex. No bands characteristic of a high spin ferric component were present. This is in marked contrast to the azide complexes of myoglobin and hemoglobin, both of which exist as equilibrium mixtures of spin states (23-25). The only significant differences between the spectra of the low spin ferric leghemoglobin complexes are in the position of the anomalously polarized band near 1585 cm^{-1}. The frequency of this band appears to decrease with increasing ligand field strength from 1588-1589 cm^{-1} in the mixed spin hydroxide and 3-chloropyridine complexes to 1585 cm^{-1} in the fully low spin nicotinate or cyanide complexes (table 1)[a].

a Ferric leghemoglobin nicotinate is a fully low spin complex whilst the pyridine and 3-chloropyridine complexes have increasing high spin character corresponding to a weakening of the axial ligand field (Mabbutt, B.M. and Wright, P.E., manuscript in preparation).

The spectrum of ferric leghemoglobin at pH 10.0 (with excitation at 488.0 nm) contains a band at 1505 cm^{-1} characteristic of low spin heme and a much smaller one at 1480 cm^{-1} indicative of approximately 20% of a high spin component. In contrast the hydroxide complexes of myoglobin and hemoglobin are 70% and 45% high spin respectively (23). Ferric leghemoglobin at pH 6.0 and the formate complex are also characterised as mixed spin in table 1 since their spectra contain bands characteristic of both high and low spin ferric complexes[b]. The Raman results are thus in accord with other spectroscopic measurements (7,22,26).

Leghemoglobin *in vivo* functions in the ferrous state. In studying the ferrous derivatives we were particularly interested in comparing the resonance Raman spectra of leghemoglobin with those of myoglobin and hemoglobin. However, a review of the literature revealed large discrepancies in the positions of many Raman bands for any given derivative (17,21,27,28). We therefore recorded spectra of the ferrous derivatives of myoglobin under the same conditions used to obtain the leghemoglobin spectra. Of note is the ease with which spectra were obtained, particularly that of carbon monoxymyoglobin. Photodissociation was insignificant at powers well above those used by other workers (17).

The spin state and oxidation state marker bands (I-IV) classify the ferrous leghemoglobin derivatives as expected. The frequencies of bands III and IV are the same within experimental errors for corresponding leghemoglobin and myoglobin complexes. Great variation is seen in band II, even amongst the low spin ferric derivatives of leghemoglobin, and no conclusions can be drawn from it. Most important are the differences in the frequency of the anomalously polarized band between corresponding leghemoglobin and myoglobin derivatives. This is consistently 4-5 cm^{-1} lower in frequency in the

b A preliminary report of the resonance Raman spectrum of ferric Lb has appeared recently (34) which also shows the presence of bands due to high and low spin forms of aquoferric Lb. However these spectra differ from those obtained by us in the position of many of the bands.

leghemoglobin complexes. The frequency of this band has been shown by several workers (16,18,19,29) to correlate with the core size of the porphyrin ring. Using the parameters of Huong and Pommier (29) we calculate an increase in the porphyrin centre - pyrrole nitrogen atom (C_t-N) distance of 0.01Å in deoxyleghemoglobin relative to deoxymyoglobin. Of great importance, this expanded core is retained in the low spin oxygen and carbon monoxide complexes. It has been proposed that bands I and III (in our nomenclature) also correlate with the porphyrin core size (19) but this is clearly not the case for many of the derivatives in table 1, e.g., compare the O_2, CO and nicotinate complexes of ferrous leghemoglobin, all of which are low spin and should have comparable core size. This is clearly a consequence of the sensitivity of these bands to the nature of the bond to the axial ligand (17,30). An alternative explanation of the frequency lowering of the anomalously polarized band has been given by Spiro et al. (19). Their calculations show that doming or ruffling of the porphyrin ring will have similar effects to core expansion. A shift of 5 cm^{-1} is consistent with tilting of the pyrrole rings out of the mean porphyrin plane by about 6°. We are unable to distinguish between doming or ruffling and porphyrin core expansion in leghemoglobin at the present time.

The resonance Raman spectra thus reveal two unique features of the heme in leghemoglobin compared to those in myoglobin or hemoglobin. First, the spectra indicate a greater degree of core expansion or ruffling or doming of the heme in leghemoglobin. Second, the occurrence of a fully low spin azide complex, 80% low spin hydroxide complex and mixed spin formate complex indicates a stronger ligand field in leghemoglobin than in myoglobin or T state hemoglobin. The corresponding derivatives of these latter proteins are either mixed spin, with a greater proportion of high spin component (23-25) or fully high spin (myoglobin formate (31)). NMR studies of the azide and cyanide complexes of leghemoglobin have also revealed a strong axial ligand field (32).

Finally it is important to consider the implications of the unusual heme structure in leghemoglobin in relation to its very rapid oxygen binding reaction and high oxygen affinity. Whilst we have detected constraints on the heme (core expansion, ruffling or doming) in deoxyleghemoglobin, these are *retained* in the various low spin complexes. Thus heme distortion in the deoxy form does not appear to be the primary factor leading to rapid reaction with oxygen. If it were, relaxation to a normal unconstrained oxy-complex would then be expected. The strong ligand field may well facilitate reaction with O_2 by lowering the energy required for spin pairing in the transition from the high spin deoxy to the low spin oxyleghemoglobin and/or stabilize the complex by decreasing the dissociation rate constant. In this regard we note the increase in ligand field strength (as reflected in changes in the position of spin state equilibria) associated with transitions between the low affinity tense state and the high affinity relaxed state of tetrameric hemoglobin (4,25,33). It is hoped that studies of metal-ligand vibrations in progress will provide direct information on the origins of the heme distortion and the strong ligand field. The relative contributions of the unique heme structure and the open and flexible heme pocket to the high oxygen affinity and large association rate constant of leghemoglobin have yet to be evaluated.

ACKNOWLEDGEMENTS

We thank Dr. C. A. Appleby for supply of leghemoglobin and for helpful discussions, Dr. D. W. James for technical advice and the Australian Research Grants Committee for financial support.

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