

Axial Ligands of the Heme Iron in Soybean Leghemoglobin *a* as Investigated Using Resonance Raman Spectroscopy

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Resonance Raman spectra of ferric and ferrous soybean (*Glycine max*) leghemoglobin *a*, their nicotinic acid and imidazole derivatives as well as the spectrum of the acetate compound of ferric leghemoglobin have been measured. The results support the previous view that the sixth ligand of the high-spin form of ferric leghemoglobin is water and that of the low-spin form is imidazole, *i.e.* the distal histidine. Upon reduction leghemoglobin becomes pentacoordinated. The spectra of the different derivatives show that the oxidation-sensitive polarized band at about 1370 cm^{-1} is also ligand sensitive. A polarized band occurring at about 1560 cm^{-1} in the spectra of the ferric forms lies at about 1540 cm^{-1} in the spectra of the reduced derivatives, showing it to be oxidation sensitive. The acetate derivative has a depolarized band at 1300 cm^{-1} , which is not seen in the spectra of any other derivatives. The ligand-sensitive bands are also at wavenumbers well separated from those of other compounds. This complex can, therefore, be used as a model compound for hemoproteins with a carboxylic group ligated to the heme iron.

Leghemoglobins, monomeric hemoproteins occurring in the root nodules of leguminous plants, are associated with symbiotic nitrogen fixation when plants are infected with *Rhizobium* bacteria.^{1,2} The prosthetic group of leghemoglobin is protoheme³ and the primary structure of several leghemoglobins has also been determined. The amino acid sequence of soybean (*Glycine max*) leghemoglobin *a*^{4,5} shows that the peptide chain contains only two histidines, which correspond to the proximal and distal histidines of other hemoproteins.¹ The tertiary structure of soybean leghemoglobin *a* has not yet been elucidated, but that of the related yellow lupin has been determined by X-ray diffraction at a resolution of 2.8 \AA ⁶ and refined to a resolution of 2.0

\AA .⁷ The tertiary structure is similar to that of myoglobin, but has a more open heme pocket. The distal histidine is mobile and easily detachable when bulky ligands bind to heme iron.⁷

Purified ferric soybean leghemoglobin *a* exists at neutral or acid pH as a thermal equilibrium mixture of high-spin and low-spin species as shown by optical and magnetic measurements,⁸ but in crude root nodule extracts leghemoglobin is comprised almost totally of the low-spin type.⁹ It has been suggested that this might depend on a hemochrome formation with the distal histidine as the sixth ligand of heme iron.⁹ Another explanation for the hemochrome formation is that leghemoglobin occurs as a nicotinic acid complex, because nicotinic acid is naturally present in legume root nodules.¹⁰

Resonance Raman spectroscopy is sensitive to the coordination and the axial ligands of heme iron.^{11,12} In this study we have tried to elucidate the ligation of the heme iron in soybean leghemoglobin *a* using different strong-field ligand derivatives as comparison. The resonance Raman spectra of the acetic acid derivative of leghemoglobin is also measured, because leghemoglobin is unique in binding carboxylic acid. It can, therefore, be used as a model compound for hemoproteins with a carboxylic group ligated to the heme iron. A preliminary report dealing with ferric leghemoglobin *a* and its nicotinic acid compound has been published.¹³

EXPERIMENTAL

Materials

Leghemoglobin *a* was prepared from soybean root nodules by the modified method of Ellfolk as

described previously.¹⁴ The preparation contained some fluorescent material, which was removed by gel chromatography on a Sephadex G-50 fine (Pharmacia, Uppsala, Sweden) column (2.5 × 80 cm) in 50 mM sodium phosphate buffer, pH 6.0. The fractions were pooled and concentrated by ultrafiltration (UM-10, Diaflo; Amicon, N.Y., Oosterhout (NB), Holland). The concentration of the leghemoglobin solution was calculated from the heme content.¹⁵

The nicotinate and imidazole derivatives of leghemoglobin *a* were prepared by adding some crystals of nicotinic acid or imidazole, respectively,

to 150 μ M leghemoglobin *a* in 50 mM sodium phosphate, pH 6. The reduced derivatives were obtained after addition of solid sodium dithionite to the nicotinate and imidazole compounds.

The acetate compound was prepared by adding 0.1 ml 1.5 mM ferric leghemoglobin to 0.9 ml 150 mM sodium acetate buffer, pH 5.6.

Ferrous leghemoglobin *a* was prepared by adding solid sodium dithionite to 150 μ M leghemoglobin in sodium phosphate buffer, pH 6.0.

The complete formation of different derivatives was checked spectrophotometrically. Oxygen was removed from all solutions by bubbling with argon.

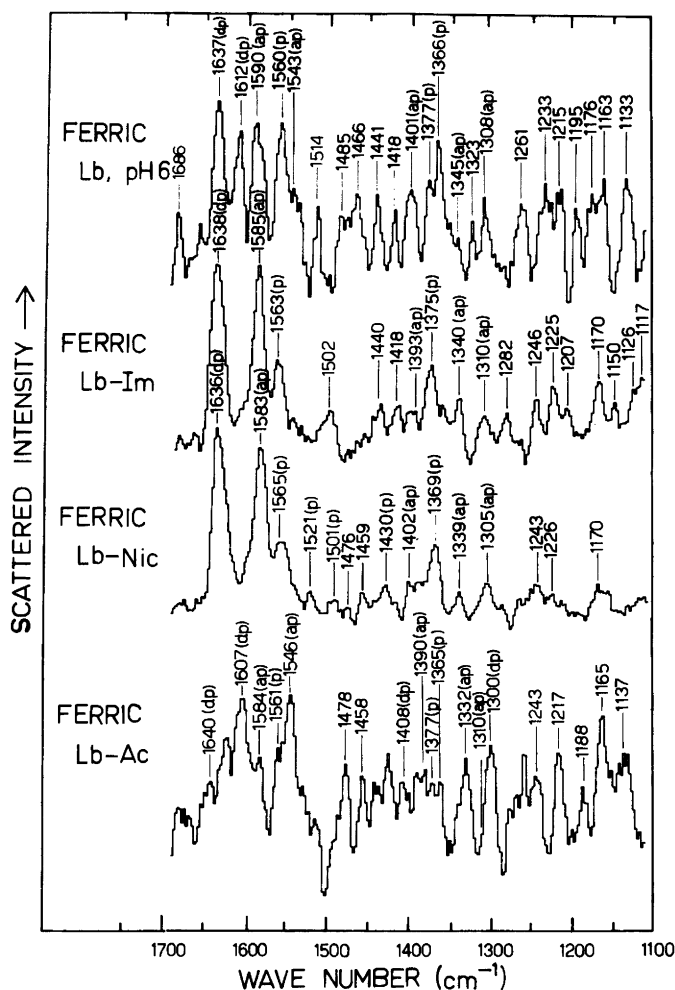


Fig. 1. The unpolarized resonance Raman spectra of ferric leghemoglobin *a* and its nicotinate and imidazole derivatives at pH 6, and its acetate compound at pH 5.6. The hemoprotein concentration on heme basis was 150 μ M. The spectra were measured with Ar⁺-ion laser excitation at 514.5 nm (incident power 60–100 mW, slit width 250 μ m, scan rate 50 $\text{cm}^{-1}/\text{min}$, integrating time 3 s).

For resonance Raman measurements the solutions were filtered through a membrane filter (Millipore, S.A., Molsheim, France) in the Raman cell and measured under argon.

All reagents were of analytical grade.

Methods

Resonance Raman spectra were excited using the 514.5 nm line from the Spectra Physics model 164 Ar⁺-ion laser, with the incident beam polarized perpendicularly to the scattering plane. A ground-glass stoppered spinning cell at a constant temperature of +10 °C was used to avoid photodecomposition of the thermally unstable hemoprotein.¹⁶ Light scattered at 90° was collected through a polarization scrambler in a Jarrel-Ash 25–300 double monochromator equipped with a thermoelectrically cooled ITT FW 130 photomultiplier tube and a photon-counting circuitry. Polarized spectra were obtained with the aid of a polaroid disc inserted between the sample and the scrambler. The resultant data were treated as described previously.¹²

The polarization ratio for the linearly polarized incident light, $\rho_1 = I_{\parallel}/I_{\perp}$, is defined as follows: p, polarized, $\rho_1 < 3/4$; dp, depolarized, $\rho_1 = 3/4$; ap, anomalously polarized, $\rho_1 > 3/4$.

RESULTS

Unpolarized resonance Raman spectra of ferric leghemoglobin *a* and its imidazole, nicotinate and acetate compounds in the wavenumber region 1100–1700 cm⁻¹ are presented in Fig. 1. The spectra are generally complex due to the presence of high-spin and low-spin forms. Oxidation and/or spin marker bands are compiled in Table 1, together with those of some previously published hemoproteins. In Fig. 2 the polarized spectra of ferrous leghemoglobin *a* are given together with the unpolarized spectra of imidazole and nicotinate derivatives in the wavenumber region 1300–1700 cm⁻¹. The shorter region was chosen to avoid prolonged presence of the oxidizable ferrous compounds in the cell. Selected bands are given in Table 1. The polarization states of the bands are based on polarized spectra.

DISCUSSION

The heme ligands of ferric leghemoglobin. Native ferric leghemoglobin differs from other hemoglobins in its spin state. In contrast to purely high-spin ferric myoglobin and hemoglobin, leghemoglobin is a

thermal mixture of high- and low-spin species at acid pH, as shown which optical and magnetic measurements⁸ and EPR.^{22,23} At room temperature ferric leghemoglobin contains about 60% of the high-spin form,⁸ whereas at the temperature of liquid helium the low-spin form dominates to about 95% of the total leghemoglobin.²² Because of spectral titrations, it was assumed that the sixth ligand of heme iron of soybean leghemoglobin *a* is a water molecule deprotonating with a pK of 8.34²⁴

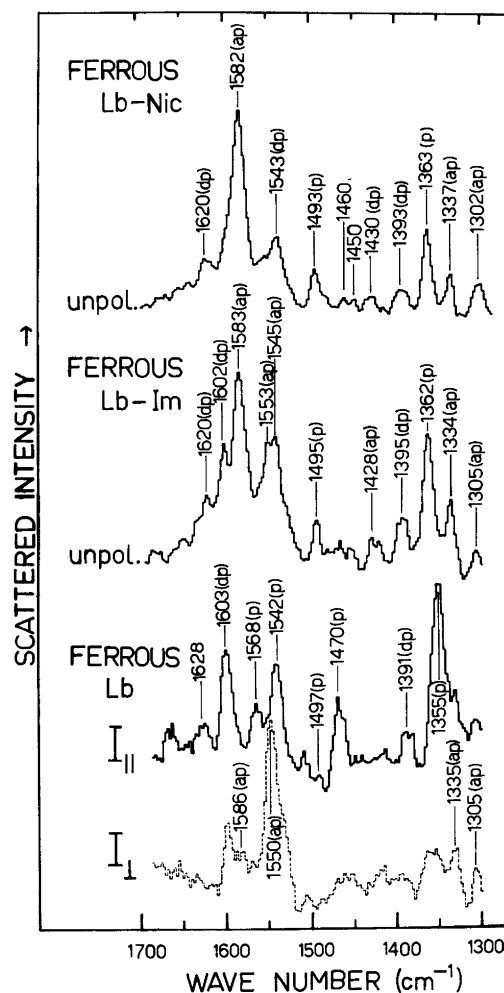


Fig. 2. The polarized resonance Raman spectra of ferrous leghemoglobin *a* at pH 6 and the unpolarized spectra of the ferrous nicotinate and imidazole compounds at the same pH. The hemoprotein concentration on heme basis was 150 μ M. The spectra were measured as in Fig. 1.

Table 1. Resonance Raman frequencies of leghemoglobin *a* and its derivatives compared to some other hemoproteins. Lb, soybean leghemoglobin *a*; Mb, horse myoglobin; YCCP, yeast cytochrome *c* peroxidase; PaCCP, *Pseudomonas aeruginosa* cytochrome *c* peroxidase; cyt. *b*₅, rabbit liver cytochrome *b*₅; His, histidine; Ac, acetate; Nic, nicotinate; Im, imidazole; (—), no ligand; F, fluoride; Met, methionine; CN, cyanide; p, polarized; dp, depolarized; ap, anomalously polarized; d, doublet; hs, high spin; ls, low spin. Value in parenthesis denotes a very weak band.

Compound	Spin	Sixth ligand	Resonance Raman frequencies (cm ⁻¹)						Ref.	
			(ap)	(ap)	(p)	(p)	(p)	(ap)		(dp)
Ferric										
Lb, pH 6	hs	H ₂ O	1308	1345	1366		1560	1543	1612	This work
	ls	His(?)			1377			1590	1637	
Lb-Ac	hs	Ac	1310	1332	1365		1561	1546	1607	This work
	ls	(?)			(1377)			1584	1640	
Lb-Nic	ls	Nic	1305	1339	1369		1565	1583	1636	This work
Lb-Im	ls	Im	1310	1340	1375		1563	1585	1638	This work
Mb, pH 7 ^a	hs	H ₂ O	1305	1340	1370			1562	1613	17
YCCP, pH 6 ^b	hs	(—)	1305	1340	1373			1567	1628	12
YCCP-F	hs	F	1300	1336	1366			1548	1607	12
PaCCP	hs	COOH	1313d	1343	1367			1553	1606	18
	hs	(—)						1572	1632	
	ls	Met			1377			1580	1640	
cyt. <i>b</i> ₅	ls	His			1375		1563	1586	1639	19
Ferrous										
Lb, pH 6	hs	(—)	1305	1335	1355	1470	1542	1550	1603	This work
	ls	(?)				(1497)		(1586)	(1628)	
Lb-Nic	ls	Nic	1302	1337	1363	1493	1543	1582	1620	This work
Lb-Im	ls	Im	1305	1334	1362	1495	1545	1583	1620	This work
	hs	(—)						(1553)	1602	
Mb, pH 7 ^a	hs	(—)	1302		1355	1472		1557	1604	20
YCCP, pH 6 ^b	hs	(—)	1303	1332	1358	1472		1547	1603	12
YCCP-CN	ls	CN	1305	1337	1365	1495	1540	1583	1620	12
PaCCP	hs	(—)	1314d		1355	1475		1555	1602	18
	ls	His or Met			1363	1490		1580	1618	
cyt. <i>b</i> ₅	ls	His	1306	1342	1361	1495		1586	1618	21

^aFrom unpolarized spectra. ^bIn the resonance Raman study of YCCP an enzymatically active preparation with measured $A_{407nm}/A_{780nm} = 1.30$ was used. According to recently published X-ray crystallography data of YCCP, the sixth ligand is water.^{20,25} Sufficient details are, however, not given about the activity and absorbance ratio of the final preparation used for crystallization. Direct comparison of the results is therefore not possible.

and it was assumed to be the weak-field ligand of the high-spin form. The two low-spin species were observed by EPR. The major component was assumed to be a bis-histidine complex and the minor one a nicotinic acid complex.^{22,23}

In an attempt to identify the ligands of native ferric soybean leghemoglobin *a*, its resonance Raman spectrum was measured together with those of the nicotinic acid and imidazole compounds. The resonance Raman spectra of the nicotinate and

imidazole compounds show typical features of ferric low-spin compounds. The polarized band at about 1370 cm⁻¹, regarded as oxidation-sensitive,²⁵ is also ligand-sensitive as seen in Table 1. The very intense spin-sensitive anomalously polarized band at 1583–1585 cm⁻¹, as well as the spin and oxidation-sensitive depolarized bands at 1636 and 1638 cm⁻¹ are also typical for low-spin compounds. The polarized band at about 1560 cm⁻¹ is evidently an oxidation marker band. As expected, native ferric

leghemoglobin *a* shows double sets of spin-sensitive bands (Fig. 1, Table 1). The high-spin compound is represented by the oxidation and ligand sensitive polarized band at 2366 cm^{-1} , the anomalously polarized band at 1543 cm^{-1} and the depolarized band at 1612 cm^{-1} . The frequency of the band at 1612 cm^{-1} is indicative of a hexacoordinated ferric heme with a weak-field ligand.^{12,25} Its frequency is close to that of myoglobin (Table 1) and hemoglobin (not shown) which are known to have a water molecule as the sixth ligand. Resonance Raman data thus support earlier work²⁴ which proposes water as the sixth ligand for leghemoglobin as well. The much weaker bands at 1377 , 1590 and 1637 cm^{-1} are typical for low-spin species. The position of the ligand-sensitive band at 1377 cm^{-1} is close to that of the imidazole complex. The resonance Raman spectrum of the low-spin species of ferric leghemoglobin is similar to that of rabbit liver cytochrome *b₅* (Table 1), which is known to have histidine as its fifth and sixth ligands.²⁶ This implies that the low-spin ligand is imidazole, *i.e.* the distal histidine at position 61 of the protein chain. From our spectra it is not possible to conclude whether there are any traces of a nicotinate complex. A schematic drawing of the imidazole, water, acetic acid and nicotinic acid compounds of ferric leghemoglobin is presented in Fig. 3. In the figure iron is assumed to be a little displaced out of the heme plane towards the distal histidine as in the acetate compound of lupin leghemoglobin, in which

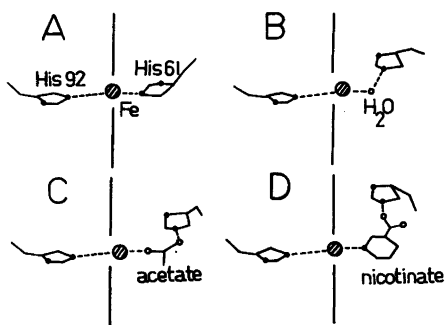


Fig. 3. The heme environment in leghemoglobin derivatives. Only the proximal (92) and the distal (61) histidines, and the sixth axial ligand of the heme iron are shown. The hatched open circle denotes heme iron, the filled circles nitrogen atoms and the open circles oxygen atoms. A, the bis-histidine compound with distal histidine as the sixth ligand; B, a water molecule as the sixth ligand; C, the acetate compound; and D, the nicotinate compound.

the iron is about 0.1 \AA towards the histidine.⁷ The distal histidine is mobile. It can either bind directly to the heme iron (Fig. 3A), or move away and allow external ligands to bind to the iron. The ligands shown in the figure are water (Fig. 3B), acetate (3C) and nicotinate (3D). A bond between imidazole N_{ϵ} -nitrogen and an oxygen atom of the ligand locks the ligand into its position.

Leghemoglobin is unique in its ability to bind straight chain carboxylic acids such as acetate and higher homologues to heme iron.²⁴ The acetate complex is easily crystallized, and the X-ray crystallography of lupin leghemoglobin acetate compound shows that one of the oxygens is ligated to the heme iron and the other to the distal histidine N_{ϵ} -nitrogen⁷ as seen in Fig. 3C. The resonance Raman spectrum of leghemoglobin acetate is consistent with a hexacoordinated high-spin compound, but has weak bands showing that small amounts of a low-spin compound are present. The low-spin species seems to represent remainders of the bis-histidine form, judged by the position of the ligand-sensitive bands.

A shift of 5 cm^{-1} is seen at 1607 cm^{-1} in the depolarized band. The high-spin hexacoordinated fluoride compounds of hemoproteins, here represented by the fluoride derivative of yeast cytochrome *c* peroxidase, also have depolarized bands at 1607 cm^{-1} or thereabouts. A strong depolarized line is detected at 1300 cm^{-1} (Fig. 1). It seems to be an indication of carboxyl ligation to heme iron, because it is not observed in other hemoproteins or leghemoglobin compounds, and can therefore not be a protein effect. Leghemoglobin acetate can thus be regarded as a model compound for hemoproteins which have a carboxylic group, originating from protein aspartic or glutamic acids or heme propionate side chain, ligated to the iron. The specific features of its resonance Raman spectrum can be used in an attempt to identify that type of ligand in cases where X-ray crystallography data are not available. A recent example is the identification of the weak-field ligand in *Pseudomonas aeruginosa* cytochrome *c* peroxidase.¹⁸

The anomalously polarized bands around 1310 and 1340 cm^{-1} (Table 1) originate from the vinyl side chains of the prosthetic group, protoheme,²¹ in congruence with previous results.³ In the spectrum of the acetate compound (Fig. 1) the band at 1310 cm^{-1} is concealed by the intense band at 1300 cm^{-1} , but in the perpendicularly polarized spectrum (not shown here) it is clearly seen.

Ferrous leghemoglobin and its derivatives. Ferrous leghemoglobin is a pure high-spin compound⁸ as can also be seen in the resonance Raman spectrum (Fig. 2). Traces of a low-spin component are also present, judged by the spin-sensitive bands at 1497, 1586 and 1628 cm⁻¹. Ferrous leghemoglobin is pentacoordinated like myoglobin, hemoglobin and yeast cytochrome *c* peroxidase (Table 1). On reduction, the imidazole of the distal histidine is released, evidently due to conformational changes. Comparison of the weak low-spin bands to the spectra of the nicotinate and imidazole compounds do not allow definite conclusions to be drawn about the sixth ligand in the small fraction of the low-spin species. It can be either a nicotinate derivative, if traces of nicotinate are left after purification of the protein, or it can be a small fraction of denatured leghemoglobin in which the distal histidine, after major conformational changes, is irreversibly bound to the heme iron, the presence of which can be detected by the very sensitive resonance Raman technique. The resonance Raman spectrum of the nicotinate compound is a pure low-spin spectrum with no traces of high-spin bands. The imidazole compound, however, apparently contains traces of high-spin form. This can depend on photodissociation of the complex.

In ferric derivatives the polarized band at about 1560 cm⁻¹ is unusually intense if compared to other hemoproteins. A similarly intense band at 1542–1545 cm⁻¹ is found in the ferrous compounds, showing it to be oxidation sensitive. There is a vinyl-induced band in this region,²⁶ and its relatively high intensity in the leghemoglobin derivatives evidently depends on the characteristic vinyl-protein interactions in the heme pocket.²⁷

Note added in proof. A resonance Raman study of soybean leghemoglobin by R. S. Armstrong, M. J. Irwin and P. E. Wright from the University of Sydney has been published in *Biochem. Biophys. Res. Commun.* 95 (1980) 682, conclusions being essentially similar.

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