A Comment on Resonance Raman Spectroscopic Investigations of Axial Ligation of the Heme Iron Atom in Soybean Leghemoglobin *a*

R. S. ARMSTRONG, M. J. IRWIN,
J. E. WELLINGTON and P. E. WRIGHT

Department of Inorganic Chemistry, University
of Sydney, N.S.W. 2006, Australia

We wish to comment on a recent paper by Österlund and Sievers purporting to identify the axial ligands of the heme iron atom in soybean leghemoglobin *a* using resonance Raman spectroscopy.¹ These workers suggested coordina-

tion of the distal histidine to the iron in the low spin component of ferric leghemoglobin and its acetate complex as well as in a previously unreported low spin component of deoxyferrous leghemoglobin. However, our own studies show that their interpretation is incorrect and indicate that the low spin components observed by Österlund and Sievers¹ in fact arise from residual coordinated nicotinate in their leghemoglobin preparations.

Fig. 1(a) shows the 400 MHz ¹H NMR spectrum of ferric soybean leghemoglobin *a* prepared according to the method of Appleby *et al.*² The protein was oxidized with $\text{Fe}(\text{CN})_6^{3-}$ and stripped of exogenous nicotinate³ by chromatography on Sephadex G-15 at high pH (0.01 M Tris buffer, pH 9.2) before separation of leghemoglobin *a* by anion exchange chromatography using DE-52 cellulose eluted with acetate buffer (0.01 M, pH 5.2). Fig. 1(b)

Fig. 1. 400 MHz ¹H NMR spectra at 25 °C and pH 6.2 of (a) soybean ferric leghemoglobin *a* stripped of nicotinate by the method of Appleby *et al.*,² (b) ferric leghemoglobin nicotinate obtained by addition of nicotinic acid to the sample in (a), and (c) ferric leghemoglobin *a* prepared by the method of Ellfolk⁵ as modified by Sievers and Rönnerberg.⁶

shows the characteristic ^1H NMR spectrum of the nicotinate complex of ferric soybean leghemoglobin *a*. Resonances from the heme and nicotinate ligand are evident at fields lower than 10 ppm and higher than 2 ppm.⁴ These resonances of the nicotinate complex are also present in spectra of ferric soybean leghemoglobin prepared according to the method of Ellfolk⁵ as modified by Sievers and Rönnberg⁶ [Fig. 1(c)]. In this preparation the high pH gel filtration which serves to strip off the nicotinate is

Fig. 2. Resonance Raman spectra of (a) deoxyleghemoglobin (pH 7.0) purified by the method of Appleby *et al.*,² (b) ferrous leghemoglobin nicotinate (pH 5.9) obtained by addition of nicotinic acid to the sample in (a), and (c) deoxyleghemoglobin (pH 7.0) purified by the method of Ellfolk⁵ as modified by Sievers and Rönnberg.⁶ Samples were 0.5 mM in heme and in 100 mM phosphate buffer. Spectral conditions were 514.5 nm laser irradiation; 5 cm^{-1} resolution; 0.5 $\text{cm}^{-1} \text{ s}^{-1}$ scan; 1 s time constant. The spectrometer was calibrated with indene and band positions are accurate to $\pm 1 \text{ cm}^{-1}$.

omitted. The NMR spectrum indicates conclusively that residual nicotinate remains bound to the heme.

The presence of this residual nicotinate in the leghemoglobin preparations used for resonance Raman studies by Österlund and Sievers¹ clearly gives rise to additional low spin bands in their spectra. Fig. 2 compares the resonance Raman spectra of samples of deoxyferrous leghemoglobin prepared by the two methods described above with that of ferrous leghemoglobin nicotinate. Additional Raman bands at 1584 and 1496 cm^{-1} due to residual nicotinate complex are evident (Fig. 2c) in the sample prepared by the methods of Ellfolk⁵ and Sievers and Rönnberg.⁶ At lower pH, more favourable for nicotinate binding, the Raman bands of the nicotinate complex are even more pronounced. The Raman spectra of deoxyferrous leghemoglobin prepared from ferric leghemoglobin stripped of nicotinate by the method of Appleby *et al.*² (Fig. 2a) indicate a pure high spin ferrous compound. The low spin bands seen by Österlund and Sievers at 1497, 1586 and 1628 cm^{-1} in the spectrum of deoxyferrous leghemoglobin were attributed by them to coordination of the imidazole of the distal histidine or residual nicotinate.¹ Our spectra clearly indicate that these bands arise from the nicotinate complex alone. The low spin Raman bands in spectra of ferric leghemoglobin and its complex with acetate¹ also arise from residual nicotinate.

The resonance Raman spectra of ferric leghemoglobin acetate measured by us⁷ differ in some features from those obtained by Österlund and Sievers¹ with excitation at 514.5 nm. These workers report an anomalously polarized band at 1584 cm^{-1} , presumably arising from residual leghemoglobin nicotinate. Our spectra of leghemoglobin acetate⁷ contain a polarized band at 1584

Table 1. Frequency^a of the strong polarized Raman band near 1375 cm^{-1} in the spectra of ferric leghemoglobin complexes.

Complex	488 nm excitation	413 nm excitation
Imidazole	Not measured	1375
Nicotinate	1375	1374
CN^-	1375	1373
OH^-	1378	1377
H_2O	1375	1373
Formate	1375	1375
F^-	1373	1373
Acetate	1374	1373

^a Frequencies are in cm^{-1} and are accurate to $\pm 1 \text{ cm}^{-1}$.

cm^{-1} . Österlund and Sievers also report bands at 1365 and 1300 cm^{-1} which are absent from Raman spectra of leghemoglobin acetate obtained by us with excitation at 413.8, 488.0 and 514.5 nm. The absence of the 1300 cm^{-1} band from our spectra is of particular importance since it has been suggested by Österlund and Sievers to be specific to a carboxylate ligand.¹ We have also measured the Raman spectrum of leghemoglobin formate⁷ and find no evidence of a band in that region.

Table 1 lists the position of the strong polarized Raman band near 1375 cm^{-1} for a number of ferric leghemoglobin complexes. It is clearly not possible to distinguish the type of axial ligand from the position of this band.

Ferric soybean leghemoglobin *a* which has been completely stripped of nicotinate still exists as an equilibrium mixture of high and low spin states.^{8,9} The rate of spin state exchange is unusually slow.¹⁰ Although coordination of the distal histidine to the iron atom in the low spin form of ferric leghemoglobin has been suggested,⁹⁻¹¹ definitive evidence for this is still lacking. Our own resonance Raman studies (Ref. 7 and unpublished experiments) have so far failed to identify unequivocally the nature of the sixth ligand.

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