Resonance Raman studies of selectively labelled hemoglobin tetramers

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Abstract

In this paper, we report the high quality low-frequency resonance Raman (RR) spectra of oxyhemoglobin (oxyHb) and its reconstituted analogs, in which protons in ferric protoporphyrin IX were substituted by deuterium atoms in meso positions (oxyHb-d\textsubscript{3}), methyl groups (oxyHb-d\textsubscript{12}), and both meso positions and methyl groups (oxyHb-d\textsubscript{16}). Analyzed collectively, the RR spectra of the low-spin dioxygen adduct species studied here reveal isotopic-sensitive modes that induce subtle differences in shape of the spectrum of oxyhemoglobins. The most significant spectral differences are observed in the region of 350–440 cm\textsuperscript{-1} which contains bending modes of the peripheral substituents, i.e. δ(C\textsubscript{13,17}C\textsubscript{14}C\textsubscript{15}) and δ(C\textsubscript{13}C\textsubscript{14}C\textsubscript{15}) + δ(CN) (structure and atom numbering scheme being given in Fig. 1). Several in-plane (ν\textsubscript{5b}, ν\textsubscript{25}, ν\textsubscript{50}, ν\textsubscript{53}, ν\textsubscript{55} and ν\textsubscript{58}) and out-of-plane (γ\textsubscript{7}, γ\textsubscript{16}, γ\textsubscript{22}, and γ\textsubscript{23}) heme vibrations have also been identified. The results presented here provide convincing evidence for the utility of selectively labelled hemoglobins in the definitive assignment of the low-frequency Raman bands. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: resonance Raman spectroscopy; Vibrational assignment; Hemoglobins

1. Introduction

The long standing intense interest in the structure and function of the tetrameric hemoglobin molecule continues, not only because of its fundamental importance in physiological oxygen transport [1–3], but also because it has come to serve as the paradigm for the large class of proteins whose functional efficiency depends on allosteric transitions [4–7]. In terms of the classical two-state model of allosteric transitions, the crystal structures of deoxy hemoglobin and various ligated forms are widely accepted to represent the low- and high-affinity quaternary states, designated T and R, with the transition between them being induced by altered intra- and inter-subunit contacts triggered by the binding of exogenous ligands at the heme active sites. The key to gaining a molecular level understanding of this mechanism lies in the structural characterisation of the important but elusive intermediates as well as the two terminal states. Thus, selective labelling of the heme group in α or/and β chains can play very important role in...
understanding structural changes in hemoglobins upon oxygen uptake.

Of all the potentially applicable spectroscopic methods, resonance Raman (RR) is among the most powerful tools providing the capability to monitor the structure and bonding of various molecular fragments throughout the tetramer [8,9], wherein deuterated protohemes are incorporated into both type of subunits.

2. Experimental

Protoporphyrin IX dimethyl ester (PPIXdME) and ferriprotoporphyrin IX chloride (Fe(III)PPIXCl) were purchased from Porphyrin Products (Logan, Utah). All porphyrins were used only after checking for purity by using thin-layer chromatography (TLC), proton NMR and electronic absorption spectroscopy [10–12]. All solvents and chemical reagents of HPLC or spectroscopic grade used in this work, such as deuterated methyl sulfoxide (DMSO-d₆), deuterated chloroform, deuterated methanol (CH₃OD), tetrabutyrammonium hydroxide (TBAOH), as a 1.0 M solution in methanol, anhydrous pyridine, anhydrous acetonitrile, anhydrous tetrahydrofuran (THF), anhydrous hexane and sodium p-(hydroxymercuiri)benzoate (PMB) were purchased from Aldrich Chemical Company and used without further purification. Bio-gel P-6DG (Bio-Rad Laboratories, Hercules, CA), CM cellulose (CM-52) and diethylaminoethyl cellu-
lose (DE-53) (Whatman, Fairfield, NJ) were used for protein column chromatography.

2.1. Preparation of selectively deuterated hemoglobins

2.1.1. Synthesis of protoheme-d₄

All procedures involving metal-free protoporphyrin and its dimethyl ester were done under subdued light since they are highly light sensitive [10]. Special care was also taken to eliminate trace water (glassware was flame-dried while purging with dry nitrogen). Deuterium substitution at the four methine carbons was accomplished according to a previously published procedure [13,14]. Proton NMR spectra (300 MHz) in CDCCl₃ revealed the meso-positions were deuterated to the extent of >95% with little loss (less than 5%) of vinyl protons [10–12,15]. Iron(III)(PPIXdME-d₄)Cl was prepared by heating at 60°C iron(II) chloride in anhydrous acetonitrile under dry nitrogen followed by addition of PPIXdME-d₄ in methylene chloride as described in detail elsewhere [16]. Hydrolysis of Fe(III)(PPIXdME-d₄)Cl ester groups was done in a solution of THF/methanol/water/potassium hydroxide at 65°C under a nitrogen atmosphere as previously reported [17]. TLC, pyridine hemochromogen electronic absorption spectra and proton NMR were done to check purity of the final samples [11,15,18,19].

2.1.2. Synthesis of protoheme-d₁₂

All procedures were conducted under a dry N₂ atmosphere in order to avoid isotopic dilution by atmospheric moisture and air oxidation of the ferrous porphyrin species. Special care was also taken to eliminate trace water (glassware was flame-dried while purging with dry nitrogen). Deuterium substitution at the four methyl groups was accomplished according to a previously published procedure [20] by gently refluxing 98 mg of the Fe(III)(PPIX)Cl in 25 ml of fresh deuterated methyl sulfoxide containing 0.75 ml of tetrabutyrammonium hydroxide, TBAOH, for 32 h. The reaction was quenched through precipitation of the iron porphyrin by the addition of 1 M aqueous HCl. Later, the iron porphyrin was separated by centrifugation (10 min at 7000 rpm) and the solid was rinsed three times with deionized water. After
further crystallization from a THF/hexane mixture, the Fe(tpPIX-d12)Cl was dried at room temperature in a vacuum desiccator. TLC, pyridine hemo-
chromogen electronic absorption spectra and proton NMR were done to check the purity of the final samples [16,17,21]. 1H NMR spectra revealed that
the methyl groups were deuterated to the extent of
>95% with little loss (less than 5%) of vinyl and
proionic protons [10–12,15].

2.1.3. Synthesis of protoheme-d16
First, ferric protoheme-d14 chloride was obtained (as
described above) followed by deuterium substitution
of the methyl groups as described in synthesis of
protoheme-d12. Obtained ferric protoheme-d16
chloride is a protoheme in which all four methine
carbons and four methyl groups were deuterated. 1H
NMR spectra showed that deuteration at desired

positions was at least 95% of exchange with little
loss of vinyl and propionic protons.

2.1.4. Protein preparation
Hemoglobin (Hb) was isolated from red blood cells
according to established procedure [21] and stored as
the CO adduct. ApoHb was prepared by the acid–
acetone method [22] and reconstitution with meso-d4,
methyl-d12, and d16-deuterated protohemes to form
Hb-d4, Hb-d12, and Hb-d16, respectively, was accom-
plished as described previously [16,21,23].

2.1.5. Sample preparation for resonance Raman
measurements
All oxy samples were prepared in 0.030 M phos-
phate buffer of pH 7.0 using the following procedure.
First, the carbonmonoxy adducts were made by
passing CO over a stirred Hb solution placed in a
small round bottomed flask. Then, dioxygen adducts
were obtained by photodissociation of CO from a
heme sample at 0°C (sample immersed in an ice-
water bath) while gently purging the hemoglobin
sample with O2.

2.1.6. Resonance Raman measurements
Resonance Raman spectra were obtained with a
Spex Model 1403 Czerny-Turner double monochro-
mator equipped with a DM1B spectroscopy labora-
tory coordinator and a Hamamatsu R928
photomultiplier. Excitation at 413.1 nm (krypton ion
laser, Coherent Model Innova (I)100-K3) was used to
measure RR spectra of the oxy forms of the Hb
samples. Power at the sample was maintained at
16 mW. Before and after measurements, the oxy
samples were checked for traces of CO adduct and
metHb (monitoring the $\nu_4$ mode around 1375 cm$^{-1}$
with the 441.6 and 413.1 nm line, respectively). All
RR measurements were done at room temperature in a
spinning standard NMR tube to lessen local heating.
Additionally, a small magnetic stirring bar was placed
inside the NMR tube and held in a fixed position by an
external magnet to provide efficient mixing of the Hb
solution at the laser illumination point. Typical
concentrations of around 0.2 mM (total heme concen-
tration) were used. Spectral band-pass was set to
4 cm$^{-1}$. The accuracy of the frequency readings was
±0.5 cm$^{-1}$.
Table 1
Allocation of the observed low-frequency out-of-plane modes and in-plane skeletal frequencies hemoglobin analogs to the local coordinates

<table>
<thead>
<tr>
<th>Mode</th>
<th>Symmetry</th>
<th>Assignment</th>
<th>Frequency</th>
<th>Hb</th>
<th>Hb-d₄</th>
<th>Hb-d₁₂</th>
<th>Hb-d₁₆</th>
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</thead>
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<tr>
<td>γ₂₃</td>
<td>E₉</td>
<td>δ(pyr.tilting)</td>
<td>231</td>
<td>231</td>
<td>231</td>
<td>231</td>
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<tr>
<td>γ₂₄</td>
<td>E₉</td>
<td>γ(C₅₋C₆₅)</td>
<td>240</td>
<td>238</td>
<td>240</td>
<td>238</td>
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<tr>
<td>νₙ</td>
<td>A₁₆</td>
<td>β(C₆₋C₆₅)</td>
<td>263</td>
<td>262</td>
<td>237</td>
<td>237</td>
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<tr>
<td>ν₅₂</td>
<td>E₆₆</td>
<td>β(C₆₋C₆₅)</td>
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<td>265</td>
<td>258</td>
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<tr>
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<td>292</td>
<td>294</td>
<td>290</td>
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<tr>
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<td>γ(pyr.tilting)</td>
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<tr>
<td>ν₈</td>
<td>A₁₆</td>
<td>ν(Fe-Npyr)</td>
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<td>346</td>
<td>337</td>
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<tr>
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<td>δ(C₆₋C₆₅)</td>
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<td>361</td>
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<td>δ(C₆₋C₆₅)</td>
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<td>375</td>
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<td></td>
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<td>δ(C₆₋C₆₅) + δ(CN)</td>
<td>409</td>
<td>404</td>
<td>399</td>
<td>395</td>
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<td></td>
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<td>δ(FeOO)</td>
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<td></td>
<td></td>
<td>δ(C₆₋C₆₅) + δ(CN)</td>
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<td>423</td>
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<td>δ(pyr.rot.)</td>
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<td>477</td>
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<td>δ(pyr.swivel)</td>
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<td>E₆</td>
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<td>A₂₆</td>
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<td>558</td>
<td>547</td>
<td>534</td>
<td></td>
<td></td>
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<tr>
<td>ν₄₈</td>
<td>E₈</td>
<td>ν(Fe-O₂)</td>
<td>570</td>
<td>570</td>
<td>570</td>
<td>570</td>
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<tr>
<td></td>
<td></td>
<td>δ(pyr.deform)</td>
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<td>579</td>
<td>574</td>
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</table>

3. Results and discussion

The spectra of the dioxygen adducts of the isotopically labelled hemoglobins studied here are shown in Fig. 2, with the observed frequencies and tentative assignments being given in Table 1 (see Fig. 1 for labelling scheme). The spectrum of native oxyHb is consistent with those reported previously by other workers [24–28], though the quality of the spectra obtained here is generally higher. The previously assigned [29–31] ν(Fe–O) stretching frequency is observed at 570 cm⁻¹ in the spectrum of the native tetramer and, as expected, does not shift significantly in the spectra of any of the isotopically labelled analogs. It is noted that the corresponding δ(FeOO) bending mode, which has also been previously identified in high quality ¹⁶O/¹⁸O difference spectra [32–34], is too weak to be definitively assigned or identified in these absolute spectra, but occurs near 425 cm⁻¹, being buried under the conglomerate of bands observed between 400 and 440 cm⁻¹. While these δ(FeOO) bending modes have been reported to shift upon ¹⁸O substitution in oxygenated heme proteins wherein the FeOO fragment is severely bent [35], giving rise to coupling with out-of-plane heme modes, inasmuch as no evidence was obtained for such coupling in the ¹⁶O/¹⁸O difference spectra for native hemoglobin [32], no deuterium sensitivity is expected for this hidden bending mode in the derivatives being studied here.

Weak heme deformation modes are observed near the ν(Fe–O), the ν₄₈ shifting to form a low intensity shoulder on the stronger 570 cm⁻¹ mode in the labelled analogs, while the ν₂₅ (558 cm⁻¹) and γ₂₁ (545 cm⁻¹) experience relatively large shifts upon both methine deuteration and methyl group deuteration, the latter, out-of-plane, mode being especially sensitive to the methine deuteration (Table 1). In the spectra of the native, methine-deuterated and methyl-d₁₂ samples two additional modes are easily identified at 509 cm⁻¹ (γ₂₂) and 482 cm⁻¹ (ν₃₃), shifting by comparable amounts upon each type of deuteration. However, the apparently nearly additive shifts of the ν₂₅ and γ₂₁ modes observed for the d₁₆ analog results in a conglomerate of unresolved bands spanning the region between about 460 and 505 cm⁻¹.

Over the years the pattern of relatively higher intensity bands between 370 and 430 cm⁻¹ have been
consistently associated with in-plane deformations of the heme peripheral substituents; i.e. the four methyl groups and the two vinyl and two propionate substituents [36–39]. Spiro and coworkers [40–42] followed by others [43–45], used heme containing specifically deuterated vinyl groups to identify two modes between 410 and 430 cm⁻¹ having significant contributions from vinyl bending, the higher frequency mode being associated with the 8-position vinyl and the lower with the 3-vinyl group (note that the older nomenclature referred to the 3- and 8-positions as the 2- and 4-positions). In the cases of monomeric proteins, such as myoglobin (Mb) and cytochrome c peroxidase (ccp), two relatively isolated bands are normally observed in this region. However, for the hemoglobin derivatives being studied here, this region apparently contains a rather diffuse packet of eight bands (in addition to the possible contributions of the weak δ(FeOO) bending modes for the dioxygen adducts), deconvolution of these being possible for very high quality spectral data [46]. While these modes undoubtedly contain contributions from vinyl group bending coordinates, it is equally clear that methyl and pyrrole deformations also contribute substantially, given the relatively large shifts (about 10 cm⁻¹) observed here for the -d₁₂ and -d₁₆ analogs. Furthermore, only the lower frequency partner which has been associated with the 3-vinyl substituent, is sensitive to methine deuteration, while the band attributed to the 8-vinyl mode component is not.

The strong band appearing at 373 cm⁻¹ and the lower intensity feature at slightly higher frequency (385 cm⁻¹) have usually been attributed to modes involving deformations of the two propionate substituents, although it is again important to note that for many monomeric proteins, including Mb and ccp, only one band is observed near 375 cm⁻¹, again, the hemoglobin samples being studied here exhibit a more complex spectrum. While the higher frequency mode shifts slightly upon methine deuteration, both modes exhibit substantial shifts in the -d₁₂ and -d₁₆ analogs, again illustrating the fact that these modes apparently contain contributions from methyl deformations as well. Efforts are underway to attempt to prepare hemes containing highly deuterated propionate groups in the absence of deuteration at other positions; such samples apparently not having been utilized for RR studies in the past.

Clearly, further insight into the nature of these apparently complex a substituent-associated modes will require acquisition of spectra for more isotopically labelled derivatives, which are planned for the near future, as well as an intensive calculational effort to support suggested assignments. This is an important issue inasmuch as the disposition of the vinyl and propionate peripheral substituents may be a factor in manipulating and fine-tuning heme reactivity in a diverse number of heme proteins and enzymes [37,42,47–50].

The strongest band observed in the low-frequency region of the RR spectra of oxyHb is the ν₈ mode occurring near 346 cm⁻¹, although it should be noted that in studies currently in progress this strong feature can usually be resolved into several components (ν₆, ν₅₆, and ν₅₁) exhibiting differing deuterium sensitivities. For example, while the major component, ν₈, exhibits no apparent shift upon methine deuteration, a second band, labelled ν₄₆, appears at lower frequency (325 cm⁻¹) in the spectrum of oxy Hb-d₁₄; assignment of this out-of-plane mode to this region being consistent with corresponding studies of well-behaved simple model systems [51–54]. It should be further noted that both of the lowest frequency totally symmetric core modes (i.e., ν₈ discussed above and ν₀, occurring at 263 cm⁻¹) are quite sensitive to methyl group deuteration, the lower frequency mode shifting by 26 cm⁻¹. The ν₈ mode especially, possessing a substantial -d₁₂ shift (9 cm⁻¹) and normally exhibiting relatively strong intensity in the spectra of hemoglobins, both in the terminal and intermediates ligation states of hemoglobin tetramers, may be particularly useful for distinguishing differences in subunit structural dynamics [9,55], by employing selectively deuterated hemoglobin hybrids (e.g., (α₉β₅)₂), as has been recently demonstrated in our laboratory [14].

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References


