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Resonance Raman study of deoxy and ligated (O_2 and CO) mesoheme IX-reconstituted myoglobin,hemoglobin and its α and β subunits

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Abstract

In this work, we corrected the resonance Raman (RR) results presented earlier for deoxy mesoheme IX-reconstituted hemoglobin (mesoHb) α and β subunits implied that mesohemes in these subunits undergo substantial structural changes upon formation of a hemoglobin tetramer (Biochemistry 29 (1990) 5087). We show that these data were probably due to the improper handling of the deoxy mesoheme subunit preparation. Additionally, we discuss the RR spectra of deoxy, oxy, and CO species of mesoheme IX-reconstituted myoglobin (mesoMb) and α and β deoxy meso hemoglobin subunits, including their analogues with deuterium-substituted mesoheme IX in all methyl groups (d_{12}). Based on the obtained data, we propose a complete RR band assignment for all of the investigated molecules. The most pronounced changes are observed for the γ_7 mode (out-of-plane movement of methane carbon atoms) associated with the interaction of the ethyl groups with the globin. We also show that in mesoheme IX-reconstituted proteins, the O_2 molecule binds stronger than in the case of native species. This is manifested by the up-shift of ν(Fe–O_2).

Keywords: Resonance Raman spectroscopy; Mesohemoglobin; Mesomyoglobin; Hemoglobin; Myoglobin

1. Introduction

One of the concepts developed to understand the differences in exogenous ligand binding by α and β hemoglobin subunits and the roles they play in the cooperativity mechanism consisted in substituting the vinyl groups in the protoheme molecule. Many of the studies have been done on the so-called mesoheme, in which both vinyls located at positions 3 and 8 according to the labeling scheme given in Fig. 1 (2 and 4 in old nomenclature) were replaced by the ethyl groups [1–6], since mesoheme IX is a good analogue of the prosthetic group *c*-type cytochromes, in which a terminal hydrogen atom of each of the two ethyl groups is replaced by sulfur atoms of thioether links to the polypeptide chain. On the other hand, the substitution of mesoheme in *b*-type cytochromes, myoglobin (Mb), hemoglobin (Hb), catalases, and horseradish peroxidases has been proven to constitute a sensitive monitor for testing the prosthetic group–protein interaction in the heme pocket [7,8]. It has been demonstrated that Hb reconstituted with this non-natural heme shows higher oxygen affinity and lower cooperativity in oxygen binding [9–11]. However, the basic porphyrin vibrational pattern remains practically unaltered.

Resonance Raman spectroscopy (RR) offers an impressive amount of information on the heme molecule and its environment. However, in order to understand the structural changes in detail, one has to establish reliable mode assignments. In the last decades, special attention has been devoted to the low-frequency region, i.e. 200–600 cm^{-1}, where bands appear associated

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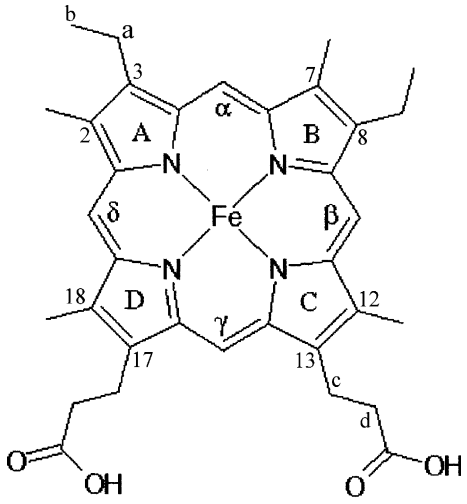

Fig. 1. Structure and labeling scheme for mesohemin IX.

mainly with the in-plane and out-of-plane deformation modes of the macrocycle and its peripheral substituents as well as the metal–ligand stretching and bending vibrations [12–14]. The studies on model compounds such as nickel(II) complexes of octaethylporphyrin (NiOEP) [15–17], etioporphyrin-I (NiETP-I) [18], or 1,5-dihydroxy-1,5-dimethyloctaethylbacteriochlorin (Ni(HOEBC)) [19] laid out a reliable framework for the interpretation of the low-frequency modes observed in the RR spectra of heme proteins. In addition, the $^{14}N/^{15}N$, $^{1}H/^{2}H$, $^{54}Fe/^{57}Fe$, and $^{32}S/^{34}S$ isotope labeling of hemes in Mb [20,21], HbA and its isolated subunits and mixed hybrids [10,22,23], cytochrome *c* [24], cytochrome *c* peroxidase [25], and cytochrome P*450* [26] allowed one to confirm the reliability of the RR band assignment. The approach of specifically isotope-substituted hemes is especially useful in the case of Hb hybrids, where α and β subunits can be monitored simultaneously using the same excitation line, as clearly demonstrated previously [22,23]. However, it has to be emphasized that the definitive assignment of the RR bands of most natural heme proteins or those substituted with chemically modified hemes has not been available yet.

Recent studies also show that despite slight differences in the RR spectra between the α and β subunits, which arise from small structural differences, the spectra of Hb tetramers can be treated as a "sum" of the RR spectra of both subunits [21,22,27–29], with the exclusion of the *ν*(Fe–His) mode of deoxyHb, since the isolated subunits have properties close to the R state, while deoxyHb exists in the T state [30,31]. Surprisingly, a different behaviour, in which the RR spectra of deoxy α and β subunits of mesoHb differ markedly from the RR spectrum of deoxy mesoHb tetramer, has been reported in the literature [32]. This suggests that the heme structures of deoxy meso subunits undergo substantial changes upon tetramer formation. Since such changes have not been observed in the case of other heme proteins, we have decided to reinvestigate this case.

Thus, we measured the RR spectra of deoxy mesoHb and its α and β subunits and their CO and O_2 adducts, and compared these data to the RR spectra of respective mesoMb. In addition, we prepared and measured their analogues with deuterium-substituted mesoheme IX in all methyl groups (d_{12}). It has to be emphasized that this is the first such detailed RR study on mesoheme proteins allowing one to assign all low-frequency RR features of these species. A comparison between the data obtained for mesoheme IX-substituted Mb and Hb and their parent compounds offers additional insight into the structure of non-natural hemes, thus leading to a better understanding of the RR spectra of non-naturally occurring heme proteins.

2. Experimental

Mesoheme IX (Fe^{III}(MPIX)Cl) was prepared from mesoporphyrin IX (MPIX) [33]. MPIX was purchased from the Sigma–Aldrich Chemical Company (Poznań, Poland) and used only after checking for purity, using thin-layer chromatography (TLC), proton nuclear magnetic resonance (^{1}H NMR), and electronic absorption spectroscopy [34]. All solvents and chemical reagents, such as DMSO-d_6 (= deuterated dimethyl sulfoxide), TBAOH (= tetrabutylammonium hydroxide) (as 1.0 M solution in methanol), anhydrous THF (= tetrahydrofuran), anhydrous hexane, anhydrous acetonitrile, iron(II) chloride, and PMB (= sodium *p*-(hydroxymercuri)benzoate), were also purchased from the Sigma–Aldrich Chemical Company (Poznań, Poland), and used without further purification. Bio-gel P-6DG (Bio-Rad Laboratories, Hercules, CA) and CM cellulose (CM-52) were used for protein column chromatography.

2.1. Synthesis of mesoheme-d_{12}

The procedure was conducted under N_2 atmosphere. Deuterium in the four methyl groups was substituted according to the previously published procedure [35] by gently refluxing 98 mg of the Fe^{III}(MPIX)Cl in 25 ml of DMSO-d_6 containing 0.75 ml of TBAOH, for 32 h. The reaction was quenched through precipitation of iron porphyrin by adding 1 M of 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 the THF/hexane mixture, the Fe^{III}(MPIX-d_{12})Cl was dried at room temperature in a vacuum desiccator. TLC, pyridine hemochromogen electronic absorption spectra

and proton NMR were performed to check the purity of the final samples [36]. The NMR spectra (500 MHz, Brucker AMX-500) in DMSO-d_6/2 M KCN in D_2O (1:6) revealed that the methyl groups were deuterated to the extent of >95% with little loss (less than 5%) of the vinyl and propionate protons [34,35].

2.2. Protein preparation

Horse-heart myoglobin (Mb) from the Sigma–Aldrich Chemical Company (Poznań, Poland) and human HbA from a local blood centre were isolated from red blood cells according to a well-established procedure [22]. ApoMb and apoHb were prepared by the acid–acetone method [37]. Reconstitution with mesoheme IX and mesoheme-d_{12} IX was accomplished as described previously [22], by adding dropwise a 1.2 molar excess of mesoheme IX dissolved in minimum volume of 0.1 M KOH into a stirred globin solution at 0 °C, while maintaining pH at 6.0. Then, the iron(III) sample was reduced with sodium dithionite in CO/Ar_2 atmosphere. MesoHb and mesoHb-d_{12} were dissociated into their α and β subunits by a standard reaction with PMB [38], and then purified as reported in the literature [32].

2.3. Sample preparation for Raman measurements

For RR measurements, all samples of about 0.2 mM of total mesoheme concentration were prepared in 0.03 mM phosphate buffer of pH 7.0. First, the carbon monoxide adducts were made by passing CO over a stirred protein solution placed in a small round-bottom flask. Then, dioxygen adducts and deoxy forms were obtained by photodissociation of CO from a heme sample immersed in an ice-water bath, with the sample gently purged with O_2 and N_2, respectively. Each of the deoxy mesoHb samples was transferred to a glass ampoule topped with a rubber stop-cork, and evacuated.

2.4. Resonance Raman measurements

The RR spectra were obtained with a triple-grating Jobin Yvon Spectrometer (model T64000) equipped with a CCD detector (Jobin Yvon Inc.). Excitation at 413.1 nm from a Coherent krypton-ion laser (model Innova 90) was used at 90° scattering with power at the sample of 3 and 15 mW for CO and O_2 adducts, respectively. Excitation at 441.6 nm from a Liconix He:Cd laser (model LGN-512) was used at 90° scattering, with power at the sample of 10 mW. All measurements were taken at room temperature. In order, to avoid local heating, the sample (placed in a glass ampoule topped with a rubber stop-cork) was spun vertically by a 6 V electrical motor. The spectral band-pass was set at 4 cm^{-1}. Before and after the measurements, the deoxy samples were checked for possible formation of either dioxygen adducts or met species by monitoring the ν_4 mode range (1350–1380 cm^{-1}) [20].

3. Results and discussion

3.1. Deoxy form

Fig. 2 shows the RR spectra, excited at 441.6 nm, of deoxy mesoMb and mesoMb-d_{12} in the range of 200–450 cm^{-1}, while Fig. 3 presents the RR spectra of deoxy mesoHb, mesoHb-d_{12}, and their isolated α and β subunits in the same range as in Fig. 2. It is noted that the data published previously [32] were obtained with a 406.7 nm laser line. However, the maximum absorption of the mesoheme IX-reconstituted deoxy hemoglobin has the Soret band at 421 nm [32]. Thus, there are no virtual differences in the RR enhancement of the bands when using 406.7 or 441.6 nm lines. In order, to prove this point, in the upper-left corner of Fig. 3 we inserted the RR spectrum of mesoheme IX-substituted α subunit excited at 406.7 nm. The suggested band assignment to the in- and out-of-plane local coordinates in the RR spectra of these proteins is listed in Table 1. It is evident from Fig. 3 that the presented RR spectra of deoxy mesoHb and its subunits differ markedly from those reported previously [32]. As discussed further in the paper, the previously published RR data on deoxy mesoheme IX-substituted α and β subunits resemble the RR spectra of O_2 adducts (see Fig. 6). It is known that mesoheme IX binds O_2 stronger than native species.

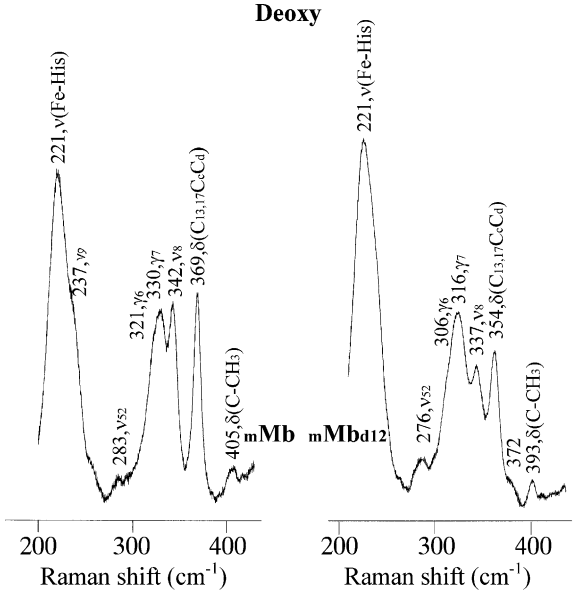

Fig. 2. Resonance Raman spectra of deoxy mesoMb and mesoMb-d_{12}. Experimental conditions – sample concentration: 0.2 mM per heme; 0.03 mM phosphate buffer of pH 7.0; excitation line: 441.6 nm; power at the sample: 10 mW.

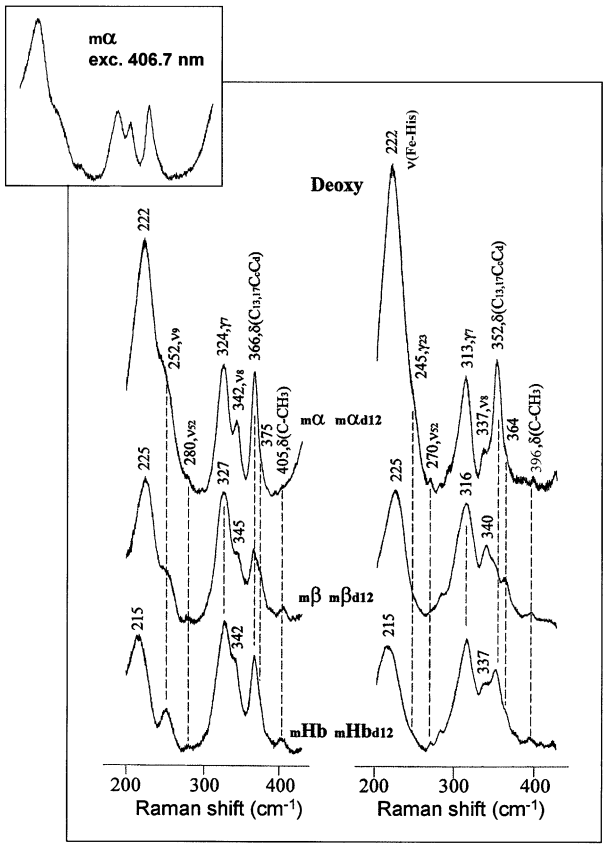

Fig. 3. Resonance Raman spectra of deoxy mesoHb, mesoHb-d_{12} and their isolated α-meso and β-meso subunits. Measurement conditions as in Fig. 2.

Thus, in our opinion, too low vacuum was applied to remove O_2 from the sample. As a result, O_2 did not dissociate completely from the complex, and thus the spectra presented are mixtures of deoxy and O_2 adduct, with a predominant abundance of the dioxygen adduct.

In the low-frequency RR spectra of five-coordinate heme proteins, in which histidine serves as the axial ligand, the most characteristic band is due to the Fe–His stretching vibration. This band is observed in the range of 200–258 cm^{-1}, depending upon the nature of heme protein, solution conditions (such as pH, ionic strength, temperature), or possible vibrational coupling with the adjacent porphyrin modes [13,14,30,38]. The definitive assignment of this mode was made by the $^{54}Fe/^{57}Fe$ and $^{14}N/^{15}N$ isotopic substitution [39,40]. Thus, the *ν*(Fe–His) mode of deoxy mesoMb presented in Fig. 2 is located at 221 cm^{-1}. It has to be noted that this frequency is not influenced by Mb reconstitution at positions 3 and/or 8 or by pH conditions, thus being insensitive to the peripheral substitution of the protoheme [5,13,20,41,42]. That is why its position is unchanged in the deoxyMb-d_{12} spectrum. This invariant RR behaviour of *ν*(Fe–His) is in agreement with the NMR and

Table 1
Frequencies, shifts upon d_{12} substitution, and band assignment to in- and out-of-plane local coordinates in RR spectra of deoxy proto- and mesoheme IX-reconstituted Mb and Hb and its subunits

Mode	Assignment	Frequency (cm^{-1})							
		$mesoMb^{deoxy}(\Delta d_{12})$	$Mb^{deoxy}(\Delta d_{12})$	$meso\text{-}\alpha^{deoxy}(\Delta d_{12})$	$\alpha^{deoxy}(\Delta d_{12})$	$meso\text{-}\beta^{deoxy}(\Delta d_{12})$	$\beta^{deoxy}(\Delta d_{12})$	$mesoHb^{deoxy}(\Delta d_{12})$	$Hb^{deoxy}(\Delta d_{12})$
	ν(Fe–His)	221 (0)	220 (0)	222 (0)	219 (0)	225 (0)	223 (0)	215 (0)	214 (0)
ν_9	$\delta(C_\beta C_1)_{sym}$	237 (?)	240 (7)	252 (?)	262 (15)	252 (?)	262 (15)	252 (?)	262 (15)
ν_{52}	$\delta(C_\beta C_1)_{sym}$	283 (7)	274 (4)	280 (10)	278 (6)	280 (10)	278 (6)	280 (10)	283 (?)
γ_6	pyr. tilting	321 (15)	332 (7)		337 (7)		336 (7)		
γ_7	$\gamma(C_\alpha C_m)$	330 (14)	304 (5)	324 (11)	302 (5)	327 (11)	304 (6)	327 (11)	303 (6)
ν_8	*ν*(Fe–N)	342 (5)	342 (4)	342 (5)	343 (4)	345 (5)	346 (4)	342 (5)	343 (4)
	$\delta(C_{13,17}C_cC_d)$	369 (15)	370 (11)	366 (14)	364 (12)	366 (14)	364 (12)	366 (14)	364 (12)
	$\delta(C_{13,17}C_cC_d)$			375 (11)	377 (10)	375 (11)	377 (10)	375 (11)	377 (10)
	$\delta(C\text{–}CH_3)$	405 (12)		405 (9)		405 (9)		405 (9)	

EPR (electron paramagnetic resonance spectroscopy) data [31,43].

The *ν*(Fe–His) mode of the natural abundance and d_{12}-labeled deoxy meso-α and -β subunits is observed at 222 and 225 cm^{-1}, respectively, i.e. very close to that of deoxy mesoMb. On the other hand, *ν*(Fe–His) of deoxy mesoHb and mesoHb-d_{12} is seen at 215 cm^{-1}. These frequencies are very close to those published previously [32]; however, the intensities reported there are much weaker than those usually expected for deoxy species [5,13]. Our data are in agreement with those published previously, which showed that the *ν*(Fe–His) frequency for the α subunit is lower by a few wavenumbers than that for the isolated β subunit, suggesting the presence of some strain in the Fe–His bond that leads to the heterogeneity of the subunits [13,14,31]. It has to be emphasized that the deoxy α and β subunits have properties closer to the R state, while deoxyHb exists in the T state. This includes all chemically modified hemes at positions 3 and/or 8 and most of the natural mutant Hbs [13,30,31,44].

A characteristic feature of Mb is the ν_9 band [20]. This vibration, observed at 240 cm^{-1} for deoxyMb [20] and its mutants [45], and around 260–270 cm^{-1} for nickel(II) model compounds [15,17–19], is mainly composed of the bending modes: C_β–C_a and C_β–C_c coupled to C_β–C_{methyl}. Similar frequencies were reported for deoxyMbs reconstituted with spirographis, isospirographis, and 3,8-diformyloprotoporphyrin IX [41]. The frequency of this band is apparently strongly correlated with the position of the $\delta(C_\beta$–$C_{propionate})$ band. In other words, the ν_9 band appears as a shoulder at 236–240 cm^{-1} on the *ν*(Fe–His) band whenever $\delta(C_\beta$–$C_{propionate})$ is above 370 cm^{-1}. When the "propionate" mode appears at a frequency lower than 370 cm^{-1}, the ν_9 band is missing from the spectrum (probably moving into the *ν*(Fe–His) envelope) [45]. This band is known to shift by 19 cm^{-1} in the RR spectrum of NiETP-d_{12} [41]. In deoxy mesoMb (Fig. 2), this band is observed at 237 cm^{-1} as a shoulder on *ν*(Fe–His), i.e. 3 cm^{-1} lower than in the native deoxy Mb. As expected, it moves down in its d_{12} analogue, thus disappearing into the complex *ν*(Fe–His) envelope.

So far, the ν_9 band has not been clearly assigned in the RR spectra of deoxy protoHb and its α and β subunits [27,28,32]. On the other hand, based on careful examination of the RR spectra, we proposed that this mode be located at 262 cm^{-1} in the RR spectra of deoxyHb, its α and β subunits, as well as deoxyHb (Hb-d_4) and deoxyHb hybrids deuterated at meso positions, in which only one subunit has methine-deuterated protoheme [10,22]. Thus, this frequency implies that this mode is up-shifted in comparison to the deoxyMb frequency and is very close to that reported for metMb or carbonmonoxyMb [1]. A similar frequency (250 cm^{-1}) has been reported recently for deoxy meso homodimeric *Scapharca inaequivalvis* hemoglobin [46]. Thus, in Fig. 3 this mode is observed as a shoulder at 252 cm^{-1} in deoxy mesoHb and its α subunit. Surprisingly, it is strongly enhanced in the β subunit. Upon d_{12} substitution, this band moves towards lower frequency and is buried under the *ν*(Fe–His) band.

The bands centred at 330 and 316 cm^{-1} in deoxy mesoMb and mesoMb-d_{12} (Fig. 2), respectively, are assigned to the γ_7 mode associated with an out-of-plane movement of the methine carbons [20,16]. It is known that in deoxyMb, this band moves from 302 to 298 cm^{-1} upon d_{12} substitution [21]. It has been shown that its intensity depends upon out-of-plane distortion of the heme caused by the Fe ion deviating from the mean porphyrin plane [45]. A larger distance of Fe(II) from the porphyrin produces higher intensity of the γ_7 mode. This mode is observed at 324 and 327 cm^{-1} in deoxy meso-α and -β subunits (Fig. 3), respectively, i.e. very close to that of mesoMb. Upon d_{12} substitution, these bands shift to 313 and 316 cm^{-1}, respectively, i.e. by 11 cm^{-1}. This difference in γ_7 frequency for the α and β subunits supports the evidence for their heterogeneity [47], as recently postulated for protoheme subunits, where γ_7 of α was observed at 299 cm^{-1} versus 302 cm^{-1} for γ_7 of β [22]. In deoxy mesoHb and mesoHb-d_{12}, this mode is observed at 327 and 316 cm^{-1}, respectively, i.e. virtually at the same frequency as for the β subunit. It is noted that this mode is slightly up-shifted in comparison to our studies on deoxyHb, its d_4 analogue, and their subunits [22]. Apparently, this up-shift in mesohemes is caused by the influence of the ethyl groups on the heme distortion, in addition to their different interaction with the heme environment.

At the low frequency side of the γ_7 band in the RR spectra of deoxy mesoMb, there is a weak shoulder at around 321 cm^{-1} that shifts to 306 cm^{-1} upon the methyl group deuteration (Fig. 2). Two modes are expected around this frequency, i.e. γ_6 and ν_{51} [20,24]. γ_6 is a pyrrole-tilting mode which gains RR intensity in the high-spin heme complexes in which the Fe ion is out of the heme plane. On the other hand, ν_{51} is due to the C_β–$C_{substituent}$ asymmetric deformation, and its RR intensity is expected to be weaker than that for ν_{52}. Since ν_{52} is usually observed as a very weak band (280–283 cm^{-1}), it is reasonable to assign the 321 cm^{-1} band to the γ_6 vibration (Fig. 2). The rather significant shift (15 cm^{-1}) upon the methyl group's deuteration is consistent with the data acquired for other deoxy heme proteins and their model compounds [15, E. Podstawka, P. Mak, J.R. Kincaid, L.M. Proniewicz, to be submitted].

The band at 342 cm^{-1} in the RR spectra of deoxy mesoMb, mesoHb, meso-α subunit is assigned to ν_8(Fe–$N_{pyrrole}$ stretching), and shifts to 337 cm^{-1} upon the d_{12} substitution. However, for the meso-β subunit and its d_{12} analogue, this mode is observed at 345 and 340 cm^{-1}, respectively. Again, the 3 cm^{-1} difference in the

band frequencies of the deoxy meso-α and -β subunits (Fig. 3) confirms their heterogeneity. It is known that this frequency is fairly invariant in high-spin hemes [13,20,22,24,42]. For example, this band is observed at 344 cm^{-1} in deoxy mesoHb of monomeric hemoglobin from insect *Chironomus thumi thumi* (CTT) [5].

The band at 369 cm^{-1} in the RR spectrum of deoxy mesoMb that shifts to 354 cm^{-1}, i.e. by 15 cm^{-1}, upon the d_{12} substitution (Fig. 2) is assigned to a C_β-propionate bending mode [20,14,25,43], one of the most characteristic heme modes. Almost the same frequency of this mode is observed in the deoxy mesoHb of CTT [5]. This band was observed at 362 cm^{-1} in deoxyMb with α-structure [42], showing that the local heme structure influences the orientation or conformational changes of the propionate groups. As a matter of fact, this bending mode in metcyanoMb and carbonmonoxyMb is upshifted by 6 and 10 cm^{-1}, respectively, in comparison to deoxyMb [20].

In the deoxy meso-α subunits (Fig. 3), there are two porphyrin-propionate bending modes seen in the RR spectra at 366 cm^{-1} (strong) and 375 cm^{-1} (shoulder). In the meso-β subunit, the 366 cm^{-1} band is more pronounced than that at 375 cm^{-1}. In the deoxy mesoHb, the 366 cm^{-1} band is much stronger than the other one, which is barely seen as a weak shoulder. These two bands reveal sensitivity to the methyl group deuteration and shift to lower frequency by 14 and 10 cm^{-1} for the 366 and 375 cm^{-1} bands, respectively. The appearance of two "propionate" frequencies is characteristic for deoxy Hbs, their mutants, as well as some other heme proteins [13,25,30,38,47–50], while only one propionate band has been reported for Mb.

It is well established that despite the two propionate groups per heme molecule in Mbs, Hbs, their subunits, cytochrome *c*, and cytochrome perxidase, only one RR band is observed in the spectra of various myoglobins, while usually two bands with intensity variations are observed for the other heme proteins discussed above [10,23]. There is an overall agreement that observation of only one "propionate" bending vibration in the RR spectra of Mbs means that there are still two vibrations with coinciding frequencies [51].

It has been proposed that such behaviour of the "propionate" bands is based on the presence of two propionate groups with different spherical arrangements with respect to the porphyrin plane as well as to the different distortions of the heme ring [41,44,46]. Moreover, different sizes of the heme pockets in two Hb subunits were taken into account [11], while other authors preferred a varying number of hydrogen bonds formed by the propionate groups with surrounding proteins [14]. Nevertheless, the frequency of the bending porphyrin-propionate mode and its intensity variations have to be related to the conformational changes of the propionate substituents.

As expected, the bands due to the vinyl group vibrations are missing in all RR spectra presented in Figs. 2 and 3. Thus, the mode observed at 405 cm^{-1} in the RR spectra of deoxy mesoMb and mesoHb and its subunits can be assigned to a bending C_β–C_{methyl} motion coupled to the C_β–C_α–C_m deformation vibration [17–19,24]. In the RR spectrum of deoxy mesoMb-d_{12} (Fig. 2), this band shifts by 12 cm^{-1}, while in the case of deoxy mesoHb-d_{12} and its subunits, a shift of 9 cm^{-1} is observed. It has to be pointed out that the 405 cm^{-1} frequency coincides with the "vinyl" mode frequency of deoxy Mb (405 cm^{-1}) and deoxy Hb (404 cm^{-1}) and its subunits [20,22].

In their elegant work using Mb reconstituted with the hemes with a deuterated vinyl group at position 8 only and with both vinyl groups (positions 3 and 8), Harada and co-workers [52] showed that the vinyl modes are involved in strong mixing with the other porphyrin modes. Thus, such mixing has to influence the frequency shifts upon deuteration of the vinyl groups involved. That is why oxidation, spin, and ligation states have to influence the observed shift of the vinyl modes. In other words, the frequency shifts on vinyl deuteration may be used as a probe of the orientation of the vinyl groups. Our data support these findings in the sense that we show proper porphyrin modes responsible for mixing with the vinyl modes in native Mb and Hb, i.e. the bending C–CH_3 mode. This conclusion is very important for understanding the "vinyl" band behaviour upon the selective substitution of the methyl groups in native heme proteins.

3.2. Ligated (O_2 and CO) forms

Fig. 4 presents the RR spectra of ligated species of mesoMb and mesoMb-d_{12}, while Figs. 5 and 6 show the RR spectra of mesoHb, its subunits, and d_{12} analogues, such as carbonmonoxide and dioxygen adducts in the range of 200–600 cm^{-1}, respectively. The proposed band assignments to the in- and out-of-plane local coordinates in the RR spectra of carbonmonoxide and dioxygen adducts of these proteins are listed in Tables 2 and 3, respectively. As is evident from these figures, the CO and O_2 adducts of meso-reconstituted Mb and Hb RR spectra show many spectral similarities. However, the overall RR pattern for ligated species is very different from that for deoxy species discussed earlier in this paper. The attachment of the sixth ligand in the *trans* position to the proximal histidine causes that the heme molecule becomes more planar. Thus, the enhancement pattern of the modes is different than that for deoxy forms. As expected for the six-coordinate hemes with a histidine ligand, the *ν*(Fe–His) vibration is not resonance-enhanced, thus it does not appear in the presented spectra. In addition, the *ν*(Fe–O), *ν*(Fe–CO), and *δ*(Fe–C–O) modes are expected to appear in the spectra

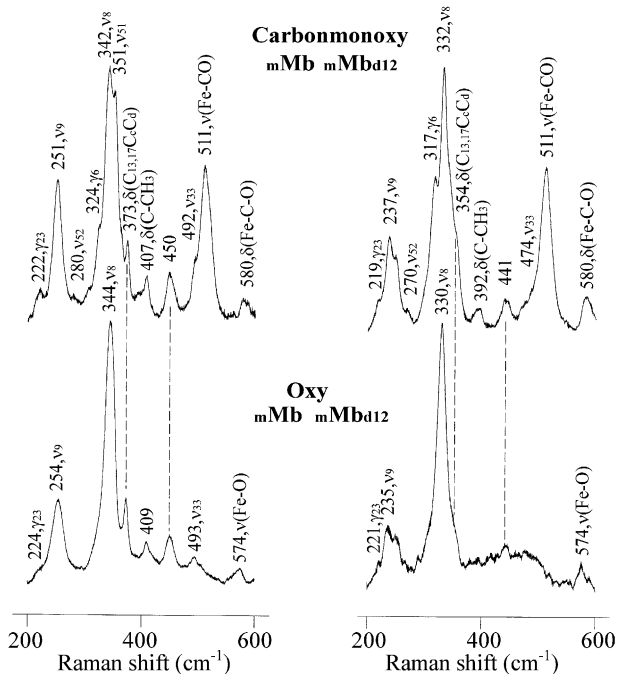

Fig. 4. Resonance Raman spectra of carbonmonoxide and dioxy adducts of mesoMb and mesoMb-d_{12}. Experimental conditions – sample concentration: 0.2 mM per heme; 0.03 mM phosphate buffer of pH 7.0; excitation line: 413.1 nm; power at the sample: 3 and 15 mW for CO and O_2 adduct, respectively.

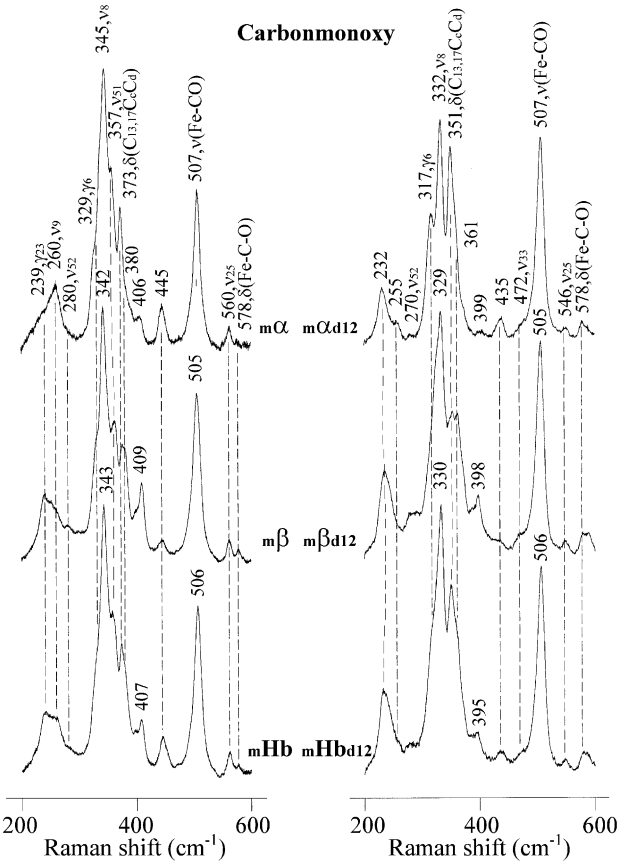

Fig. 5. Resonance Raman spectra of carbonmonoxide complex of mesoHb, mesoHb-d_{12} and their isolated α-meso and β-meso subunits. Experimental conditions – sample concentration: 0.2 mM per heme; 0.03 mM phosphate buffer of pH 7.0; excitation line: 413.1 nm; power at the sample: 3 mW.

[53,54]. As shown previously, the δ(Fe–O–O) mode is not enhanced in the case of Mb^{O2} [29,54] but can be extracted from the RR spectra of Hb^{O2} [30,55] or dioxygen adducts of cytochrome P*450*$_{cam}$ [55].

Based on the shifts observed upon the isotopic substitution of the methyl groups and the similarities among meso-, protohemes, and some model compounds, we propose band assignments of low-frequency modes in the RR spectra of the species under study. Thus, there are three modes below 300 cm^{-1} in the RR spectra of meso hemes that can be assigned. By analogy to the RR spectra of the carbonmonoxide and dioxide adduct of native Mb, we are able to assign the low-frequency mode of $mesoMb^{CO}$ and $mesoMb^{O2}$, i.e. ν_9. The relatively intense bands at 251 and 254 cm^{-1} in the RR spectra of CO and O_2 adducts (Fig. 4), respectively, are assigned to the ν_9 mode (A_{1g} in D_{4h} symmetry), which, as mentioned earlier, is due to the strongly mixed Fe-pyrrole and CC_cC_d bending motions [15,17–20]. As seen in Fig. 4, it moves towards lower frequency by 14 and 19 cm^{-1} for the CO and O_2 adducts of mesoMb-d_{12}, respectively. This prominent shift is expected since ν_9 is known to shift by 20 cm^{-1} in the RR spectrum of NiEPI-d_{16}.

The bands in the 320–360 cm^{-1} spectral region dominate the RR spectra of the low-spin CO and O_2 adducts of mesoMb, mesoHb and its isolated subunits. This frequency region is rather congested, and includes three overlapping γ_6 (324–329 cm^{-1}), ν_8 (343–345 cm^{-1}), and ν_{51} (351–362 cm^{-1}) bands due to the skeletal tetrapyrrole ring vibrations [56]. The ν_{51} mode is clearly observed only for CO complexes of the proteins discussed.

In the RR spectra of carbonmonoxide complexes of the mesoMb-d_{12} and meso-α-d_{12} chain, the γ_6 and ν_8 bands move down to 317 and 332 cm^{-1}, respectively. Similarly, for the mesoHb-d_{12} and meso-β-d_{12} subunit, those modes shift to 317 cm^{-1} and 330 or 329 cm^{-1}, respectively. The ν_{51} band shifts also down and disappears under the strong ν_8 band. A comparable frequency shift is observed for dioxygen adducts. As discussed earlier in the paper, the appearance of the γ_6 band is associated with the doming of the heme ring. The crystal structure of HbO_2 shows that the iron ion is out of the heme plane in α subunits [57]. This is supported by the observation of the γ_6 mode in the RR spectra. This band is hidden under the strong ν_8 band; however, it moves down with the d_4 substitution (deuterium substitution in the *meso* position) and is clearly observed in the RR spectrum of α^{O2}-d_4 at 324 cm^{-1} (E. Podstawka, P. Mak, J.R. Kincaid, L.M. Proniewicz, to be submitted). The same situation is observed for the meso-α subunit. It has to be emphasized that this mode is not observed in the β

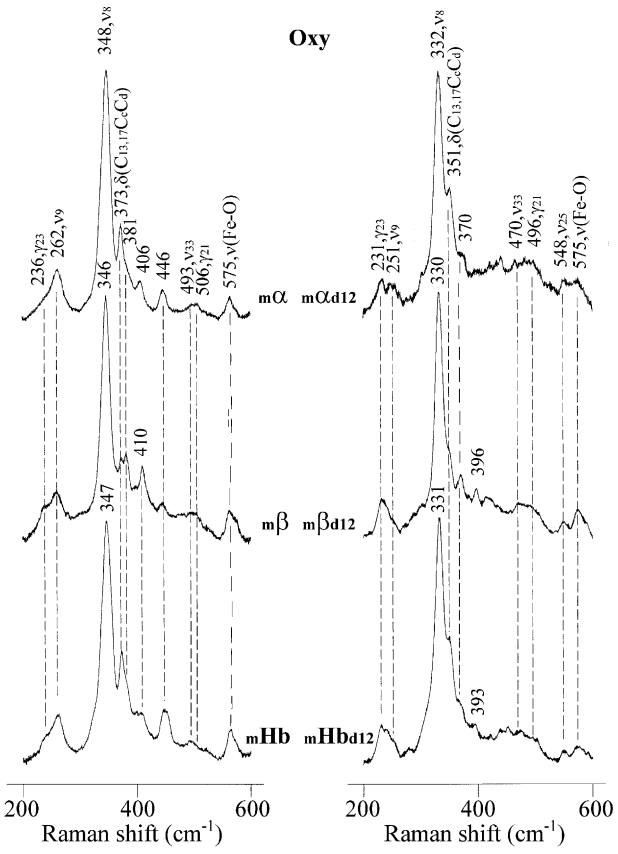

Fig. 6. Resonance Raman spectra of dioxy adduct of mesoHb, mesoHb-d_{12} and their isolated α-meso and β-meso subunits. Experimental conditions – sample concentration: 0.2 mM per heme; 0.03 mM phosphate buffer of pH 7.0; excitation line: 413.1 nm; power at the sample: 15 mW.

subunits of native HbO_2. On the other hand, the crystal structure of Hb^{CO} shows that the binding of CO to the iron ion causes its displacement only in β subunits [58]. This is why the γ_6 mode appears very weakly in the RR spectra of Hb^{CO} and β^{CO} only when the native heme is replaced by the heme-d_4 (E. Podstawka, P. Mak, J.R. Kincaid, L.M. Proniewicz, to be submitted). In the RR spectra of the dioxygen adducts of mesoHb and its subunits, the γ_6 mode is not clearly visible. However, in the case of the CO complexes (Fig. 5), a weak shoulder at 329 cm^{-1} that shifts by 12 cm^{-1} (to 317 cm^{-1}) with the d_{12} substitution can be assigned to this mode. If this is true, it suggests that both $mesoHb^{CO}$ subunits have a distorted porphyrin ring with the iron ion out of plane.

The 373 cm^{-1} band in the RR spectra of the investigated meso proteins is one of the most characteristic bands. It is due to the bending porphyrin-propionate mode (see above), and it is fairly invariant in frequency in the CO and O_2 adducts of mesoMb and mesoHb. This band appears in the RR spectra of CO and O_2 adducts at a slightly higher frequency than that for the deoxy form of mesoMb (369 cm^{-1}) and mesoHb (366 cm^{-1}). On the other hand, it is observed at a lower frequency than in the case of native Mb^{O2} (376 cm^{-1}) or

Table 2
Frequencies, shifts upon d_{12} substitution, and band assignment to in- and out-of-plane local coordinates in RR spectra of carbonmonoxide complexes of proto- and mesoheme IX-reconstituted Mb and Hb and its subunits

Mode	Assignment	Frequency (cm^{-1})							
		$mesoMb^{CO}(\Delta d_{12})$	$Mb^{CO}(\Delta d_{12})$	meso-$\alpha^{CO}(\Delta d_{12})$	$\alpha^{CO}(\Delta d_{12})$	meso-$\beta^{CO}(\Delta d_{12})$	$\beta^{CO}(\Delta d_{12})$	$mesoHb^{CO}(\Delta d_{12})$	$Hb^{CO}(\Delta d_{12})$
γ_{23}	pyr. tilting	222 (3)		239 (?)	232 (0)	239 (?)	232 (0)	239 (?)	232 (09)
ν_9	$\delta(C_\beta C_1)_{sym}$	251 (14)	254 (9)	260 (?)	264 (19)	260 (?)	264 (19)	260 (?)	264 (19)
ν_{52}	$\delta(C_\beta C_1)_{sym}$	280 (10)	270 (?)	280 (?)	275 (9)	280 (?)	275 (9)	280 (?)	275 (9)
γ_6	pyr. tilting	324 (7)	350 (?)	329 (12)		329 (12)		329 (12)	
ν_8	ν(Fe–N)	342 (10)	347 (10)	345 (13)	342 (7)	342 (13)	342 (7)	343 (13)	342 (7)
ν_{51}	$\delta(C_\beta C_1)_{asym}$	351 (?)		357 (?)	363 (7)	357 (?)	363 (7)	357 (?)	363 (7)
	$\delta(C_{13,17}C_cC_d)$	373 (19)	379 (17)	373 (22)	375 (11)	373 (22)	375 (11)	373 (22)	375 (11)
	$\delta(C_{13,17}C_cC_d)$			380 (19)	387 (11)	380 (19)	387 (11)	386 (12)	387 (11)
	δ(C–CH_3)	407 (15)		406 (7)		409 (11)		407 (12)	
	pyr swiv.	450 (9)		445 (10)		445 (10)		445 (10)	
ν_{33}	pyr. rot.	492 (18)	479 (17)		480 (17)		480 (17)		480 (17)
	ν(Fe–CO)	511 (0)	509 (0)	507 (0)	505 (0)	505 (0)	503 (0)	506 (0)	504 (0)
ν_{25}	pyr. rot.		554 (17)	560 (14)	558 (21)	560 (14)	558 (21)	560 (14)	558 (21)
	δ(Fe–C–O)	580 (0)	575 (0)	578 (0)	577 (0)	578 (0)	577 (0)	578 (0)	577 (0)

Table 3
Frequencies, shifts upon d_{12} substitution, and band assignment to in- and out-of-plane local coordinates in RR spectra of dioxygen adducts of proto- and mesoheme IX-reconstituted Mb and Hb and its subunits

Mode	Assignment	Frequency (cm^{-1})							
		$mesoMb^{O2}(\Delta d_{12})$	$Mb^{O2}(\Delta d_{12})$	meso-$\alpha^{O2}(\Delta d_{12})$	$\alpha^{O2}(\Delta d_{12})$	meso-$\beta^{O2}(\Delta d_{12})$	$\beta^{O2}(\Delta d_{12})$	$mesoHb^{O2}(\Delta d_{12})$	$Hb^{O2}(\Delta d_{12})$
γ_{23}	pyr. tilting	224 (3)		236 (5)	230 (0)	236 (5)	230 (0)	236 (5)	230 (0)
ν_9	$\delta(C_\beta C_1)_{sym}$	254 (19)	254 (12)	262 (11)	263 (18)	262 (11)	263 (18)	262 (11)	263 (18)
ν_{52}	$\delta(C_\beta C_1)_{sym}$		270 (?)		274 (11)		274 (11)		274 (11)
ν_8	ν(Fe–N)	344 (14)	345 (7)	348 (16)	339 (8)	346 (16)	339 (8)	347 (16)	339 (8)
	$\delta(C_{13,17}C_cC_d)$	373 (19)	377 (17)	373 (22)	373 (12)	373 (22)	373 (12)	373 (22)	373 (12)
	$\delta(C_{13,17}C_cC_d)$			381 (11)	386 (11)	381(11)	386 (11)	381 (11)	386 (11)
	δ(C–CH_3)	409		406 (?)		410 (14)		410 (17)	
		450 (9)		446 (?)		446 (?)		446 (?)	
ν_{33}	pyr. rot.	493 (?)		493 (23)	493 (16)	493 (23)	493 (16)	493 (23)	493 (16)
	ν(Fe–O_2)	574 (0)	572 (0)	575 (0)	570 (0)	575 (0)	570 (0)	575 (0)	570 (0)

Mb^{CO} (379 cm^{-1}). This band shifts by 19 cm^{-1} in both mesoMb-d_{12} adducts and is observed as a high-frequency shoulder at 354 cm^{-1} on the ν_8 band.

As expected, the second "propionate" vibration appears at 380 cm^{-1} for the CO (Fig. 5) and at 381 cm^{-1} for the O_2 adduct of mesoHb and its subunits (Fig. 6). In the case of the CO adducts of mesoHb-d_{12}, the lower frequency "propionate" band appears clearly at 351 cm^{-1} ($\Delta\nu = 22$ cm^{-1}), while the other expected band is observed as a shoulder at 361 cm^{-1} ($\Delta\nu = 19$ cm^{-1}) (Fig. 5). Both "propionate" bands are clearly observed in the CO adduct of meso-β-d_{12} subunit at the same frequency as for mesoHb-d_{12}. However, in the case of the α-subunit, only the 351 cm^{-1} band is observed as a strong, fairly symmetrical band, while the other expected propionate bending mode is too weak to be seen. A similar situation is noticed for the O_2 adducts (Fig. 6), where the propionate-porphyrin bending at 373 cm^{-1} moves towards lower frequency by 22 cm^{-1} (to 351 cm^{-1}), while the 381 cm^{-1} band shifts to 370 cm^{-1} ($\Delta\nu = 11$ cm^{-1}). This small difference in frequency of both "propionate" modes suggests that these two groups vibrate independently but with similar frequencies and different Raman activity (band intensity). When both methyl groups at positions 12 and 18 of the heme ring are deuterated (as a matter of fact, all methyl groups are deuterated, but those at positions 12 and 18 have the greatest impact on the propionate group behaviour), the bending mode at the lower frequency is more affected than that at the higher frequency ($\Delta\nu$ of 22 vs 19 cm^{-1}). What is interesting, in the meso-β subunit a higher frequency band gains in intensity at the expense of the other one in comparison to the meso-α subunit. This postulates that there is a considerable coupling between the propionate groups and the pyrrole ring vibrations. In principle, pyrrole rings should have their own different frequencies, even if they are expected to be similar. The appearance of the 405 and 450 cm^{-1} bands (Figs. 5 and 6) is due to δ(C–CH_3) and the pyrrole swivel (γ_{22}). This shows clearly that the so-called "vinyl" modes in protoheme strongly couple with these two modes. Their observed isotopic shift (d_{12}) supports the suggested assignment.

The ν(Fe–CO) of mesoHb and its α and β subunits is observed at 506, 507, and 505 cm^{-1}, respectively (Fig. 5), as a strong, symmetric RR band. As in the case of mesoMb^{CO} (511 cm^{-1}) (see above), this mode is insignificantly higher (but the shift is real) than in the native proteins pointing out a very small influence of the ethyl substitution on the vibrational properties of the Fe–CO moiety. This is supported by the observation of the δ(Fe–C–O) mode at 578 cm^{-1}, i.e. at virtually the same frequency as for human Hb^{CO}. Obviously, these modes do not move upon d_{12} substitution. It has to be noted as well that this 2 cm^{-1} shift in ν(Fe–CO) observed for the subunits has been also reported for human α^{CO} and β^{CO}

chains at 505 and 503 cm^{-1}, respectively [59]. The intensity of the δ(Fe–C–O) mode relative to that of ν(Fe–CO) is much weaker for Hb subunits and a tetramer than for Mb.

The ν(Fe–CO) and δ(Fe–C–O) frequencies of the isolated chains of mesoHb are different than those of mesoMb, thus in this regard they are not similar as postulated previously for the native ones [60]. However, it has been demonstrated very recently that the replacement of a F1–G5 segment in myoglobin (the so-called heme binding module) with that of α subunits converts the proximal heme Mb site into the Hb α-subunit type [42]. It turned out that a similar replacement in the hemoglobin β subunit does not cause any measurable changes in the environmental heme structure of the synthesized heterotetramer [61]. These experiments showed that the binding of an axial ligand, such as CO by Mb, is determined by the amino acid sequence of the proximal site, i.e. the proximal structure of the monomeric species (Mb and Hb subunits). This can explain a different behaviour of the CO binding to the discussed proteins that can be simply monitored by RR spectroscopy using the ν(Fe–CO) and δ(Fe–C–O) modes (also, of course, if possible, the ν(C–O) mode).

The binding of O_2 to mesoMb and mesoMb-d_{12} is characterized by the ν(Fe–O_2) mode observed as a weakly resonance-enhanced band at 574 cm^{-1} (Fig. 4). Its frequency directly reflects the strength of the Fe–O_2 bond. It is noted that this frequency is higher than that reported for the O_2 adduct of horse heart Mb at 571–572 cm^{-1}, thus the substitution of native meso heme causes strengthening of the Fe–O_2 bond [29,54]. This observation is supported by the fact that in vacuum O_2 dissociates easier from native Mb than from mesoMb. It has to be emphasized that ν(Fe–O_2) at 574 cm^{-1} in mesoMb^{O2} is the highest among oxyMbs. The same frequency is observed only for the dioxygen adduct of the Leu29-Trp (L29W) mutant of sperm whale Mb [62]. However, in this case, such high frequency is caused by the Fe–O_2 distortion by the indole chain steric hindrance that forces the Fe–O–O angle to be 111°. This is not the case of mesoMb^{O2}, where the Fe–O–O angle is expected to be around 120° as for native Mb^{O2} [63].

The ν(Fe–O_2) mode for mesoHb^{O2} and its isolated chains is observed at 575 cm^{-1} (Fig. 6), i.e. much higher than reported for human Hb^{O2} at 568 cm^{-1} [29,54,64]. Obviously, this difference in the ν(Fe–O_2) frequency between both proteins indicates structural differences in the heme. It is probable that the iron-imidazole linkage to proximal histidine and/or hydrogen bonding interaction between the bound O_2 molecule and a distal histidine are responsible for such an effect. Again, as in the case of mesoMb^{O2} (see above), molecular oxygen binds much stronger to meso than to native heme, thus it is more difficult to remove it from the adduct under vacuum conditions in order to obtain deoxy species.

The ν(Fe–O_2) band at 575 cm^{-1}in Fig. 6 overlaps with the ν_{25} band that is due to the pyrrole out-of-plane deformation mode [10,20]. However, ν_{25} moves to 548 cm^{-1} upon d_{12} substitution, while ν(Fe–O_2) does not shift. It has to be noted that in the case of human Hb^{O2}, its ν(Fe–O_2) is lower by 3 and 4 cm^{-1} than reported for α and β subunits, respectively [29]. These RR data do not agree with the X-ray study results [57], where the Fe–O_2 bond lengths for α and β chains were reported to be 1.66 and 1.87 Å, respectively, since the difference in ν(Fe–O_2) is insignificant between both subunits. Such difference in frequency between meso subunits and mesoHb has not been observed in this work (Fig. 6).

4. Conclusions

In conclusion, the results presented in this work correct the RR spectra of deoxy meso-α and -β subunits reported previously [32]. The results obtained before present the RR spectra of a mixture of deoxy (around 10%) and the most probable O_2 adducts. We show clearly that deoxy meso subunits do not change their structure upon formation of deoxy mesoHb. We also show that the RR patterns of the present meso species (both deoxy and ligated) are very similar to those of native proteins. Somewhat surprisingly, the replacement of vinyl with ethyl groups does not change substantially the frequencies of γ_6, ν_8, ν_9, and $\delta(C_\beta–C_{propionate})$ that involve out-of-plane motions of the heme molecule and the bending modes of the peripheral groups. The most pronounced change is observed for the γ_7 mode due to the out-of-plane movement of methine carbon atoms. This mode is elevated when compared to protoheme, which may be due to the different interaction of the ethyl versus vinyl groups of meso heme with the globin. It is well established that this mode is very sensitive to disorder in the heme pocket.

No stronger binding of the CO molecule by meso heme is clearly observed in the RR spectra where ν(Fe–CO) of mesoMb and mesoHb is insignificantly higher (by 1 cm^{-1}) in comparison to the native species. On the other hand, a stronger binding of O_2 is reflected in the ν(Fe–O_2) mode observed at 574 and 575 cm^{-1} for mesoMb^{O2} and mesoHb^{O2}, respectively (572 and 568 cm^{-1} for native Mb^{O2} and native Hb^{O2}).

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References

[1] Y. Mie, K. Sonoda, S. Neya, N. Funsaki, I. Taniguchi, Bioelectron. Bioenerg. 46 (1998) 175–184.
[2] K. Kawabe, K. Imaizumi, K. Imai, I. Tyuma, H. Ogoshi, T. Iwahara, Z.-I. Yoshida, J. Biochem. 92 (1982) 1703–1712.
[3] K. Kawabe, K. Imaizumi, Z.-I. Yoshida, K. Imai, I. Tyuma, J. Biochem. 92 (1982) 1713–1722.
[4] H. Hori, M. Ikeda-Saito, T. Yonetani, J. Biol. Chem. 257 (1982) 3636–3642.
[5] E.A. Kerr, N.-T. Yu, K. Gersonde, D.W. Parish, K.M. Smith, J. Biol. Chem. 260 (1985) 12665–12669.
[6] W.A. Kalsbeck, A. Ghosh, R.K. Pandey, K.M. Smith, D.F. Bocian, J. Am. Chem. Soc. 117 (1995) 10959–10968.
[7] J.R. Kincaid, Y. Zheng, J. Al-Mustafa, K. Czarnecki, J. Biol. Chem. 271 (1996) 28805–28811.
[8] L. Herenyi, A. Suisalu, K. Mauring, K. Kis-Petik, J. Fidy, J. Kikas, J. Phys. Chem. B 102 (1998) 5932–5940.
[9] E. Peterson, J.M. Friedman, Biochemistry 37 (1998) 4346–4357.
[10] E. Podstawka, J.R. Kincaid, L.M. Proniewicz, J. Mol. Struct. 596 (2001) 157–162.
[11] D.W. Seybert, K. Moffat, J. Mol. Biol. 113 (1977) 419–430.
[12] E. Kerr, N.-T. Yu, in: T.G. Spiro (Ed.), Biological Application of Raman Spectroscopy: Resonance Raman Spectra of Heme and Metalloproteins, vol. 3, Wiley/Interscience, New York, 1988 (Chapter 2).
[13] T. Kitagawa, in: T.G. Spiro (Ed.), Biological Applications of Raman Spectroscopy, vol. 3, Wiley, New York, 1988.
[14] J.R. Kincaid, in: K.M. Kodish, K.M. Smith, R. Guilard (Eds.), The Porphyrin Handbook, vol. 7, Academic Press, New York, 2000 (Chapter 51).
[15] X.-Y. Li, R.S. Czernuszewicz, J.R. Kincaid, P. Stein, T.G. Spiro, J. Phys. Chem. 94 (1990) 47–61.
[16] X.-Y. Li, R.S. Czernuszewicz, J.R. Kincaid, T.G. Spiro, J. Am. Chem. Soc. 111 (1989) 7012–7023.
[17] X.-Y. Li, R.S. Czernuszewicz, J.R. Kincaid, Y.O. Su, T.G. Spiro, J. Phys. Chem. 94 (1990) 31–47.
[18] S. Hu, A. Mukherjee, Ch. Piffat, R.S.W. Mak, X.-Y. Li, T.G. Spiro, Biospectroscopy 1 (1995) 395–412.
[19] S. Hu, A. Mukherjee, T.G. Spiro, J. Am. Chem. Soc. 115 (1993) 12366–12377.
[20] S. Hu, K.M. Smith, T.G. Spiro, J. Am. Chem. Soc. 118 (1996) 12638–12646.
[21] P. Mak, E. Podstawka, J.R. Kincaid, L.M. Proniewicz, Biopolymers (Biospectroscopy) (2004), in press.
[22] E. Podstawka, C. Rajani, J.R. Kincaid, L.M. Proniewicz, Biopolymers (Biospectroscopy) 57 (2000) 201–207.
[23] J.R. Kincaid, C. Rajani, L.M. Proniewicz, K. Maruszewski, J. Am. Chem. Soc. 119 (1997) 9073–9074.
[24] T. Hayashi, T. Matsuo, Y. Hitomi, K. Okawa, A. Suzuki, Y. Shiro, T. Iizuka, Y. Hisaeda, H. Ogoshi, J. Inorg. Biochem. 91 (2002) 94–100.
[25] G. Smulevich, S. Hu, K.R. Rodgers, D.B. Goodwin, K.M. Smith, T.G. Spiro, Biospectroscopy 2 (1996) 365–376.
[26] H. Oshio, T. Ama, T. Watanabe, K. Nakamoto, Inorg. Chim. Acta 96 (1985) 61–66.
[27] K. Nagai, T. Kitagawa, H. Morimato, J. Mol. Biol. 136 (1980) 271–289.
[28] M.R. Ondrias, D.L. Rousseau, T. Kitagawa, M. Ikeda-Saito, T. Inubushi, T. Yonetani, J. Biol. Chem. 257 (1982) 8766–8770.
[29] S. Jeyarajah, L.M. Proniewicz, H. Bronder, J.R. Kincaid, J. Biol. Chem. 269 (1994) 31047–31050.
[30] D.L. Rousseau, J.M. Friedman, in: T.G. Spiro (Ed.), Biological Applications of Raman Spectroscopy, vol. 3, Wiley, New York, 1988 (Chapter 4).
[31] G. LaMar, J.D. Satterlee, J.S. DeRopp, in: K.M. Kadish, K.M. Smith, R. Guilad (Eds.), The Porphyrin Handbook, vol. 5, Academic Press, New York, 2000 (Chapter 37).
[32] S. Jeyarajah, J.R. Kincaid, Biochemistry 29 (1990) 5087–5094.
[33] K.M. Smith, D.W. Parish, W.S. Inouye, J. Org. Chem. 51 (1986) 666–671.
[34] T.R. Janson, J.J. Katz, in: D. Dolphin (Ed.), The Porphyrins. Physical Chemistry, vol. IV, part B, Academic Press, New York, 1979.
[35] G.M. Godziela, S.K. Kramer, H.M. Goff, Inorg. Chem. 25 (1986) 4286–4288.
[36] J.W. Buchler, in: D. Dolphin (Ed.), The Porphyrins. Structure and Synthesis, vol. I, part A, Academic Press, New York, 1978.
[37] Y.K. Yip, M. Waks, S. Beychok, J. Biol. Chem. 247 (1972) 7237–7244.
[38] B.D. Howes, J.N. Rodrigez-Lopez, A.T. Smith, G. Smulevich, Biochemistry 36 (1997) 1532–1543.
[39] P. Argade, M. Sassaroli, D.L. Rousseeau, T. Inubushi, M. Ikeda-Saito, A. Lapiolot, J. Am. Chem. Soc. 106 (1984) 6593–6596.
[40] A.W. Wells, J.T. Sage, D. Morikis, P.M. Champion, M.L. Chiu, S.G. Sligar, J. Am. Chem. Soc. 113 (1991) 9655–9660.
[41] M. Tsubaki, K. Nagai, T. Kitagawa, Biochemistry 19 (1980) 379–385.
[42] K. Inaba, K. Ishimori, J. Morishima, J. Mol. Biol. 283 (1998) 311–317.
[43] K. Gersonde, L. Noll, H.T. Gaud, S.J. Gill, Eur. J. Biochem. 62 (1976) 577–582.
[44] G.K. Ackers, V.J. LiCata, P.M. Dalessio, Proteins: Struct. Funct. Genet. 17 (1993) 279–296.
[45] E.S. Peterson, J.M. Friedman, E.Y.T. Chien, S.G. Sligar, Biochemistry 37 (1998) 12301–12319.
[46] C. Zamparelli, D. Verzili, A. Boffi, E. Chiancone, S. Takahasi, D.L. Rousseau, R. Regan, Q.H. Gibson, Arch. Biochem. Biophys. 339 (1997) 275–282.
[47] V. Jayaraman, T.G. Spiro, Biochemistry 38 (1996) 1243–1251.
[48] A.J. Sitter, J.R. Shifflett, J. Terner, J. Biol. Chem. 263 (1988) 13032–13038.
[49] W.E. Royer Jr., A. Pardanani, Q.H. Gibson, E.S. Peterson, J.M. Friedman, Proc. Natl. Acad. Sci. USA 93 (1996) 14526–14531.
[50] M. Mukai, S. Nagano, M. Tanaka, K. Ishimori, I. Morishima, T. Ogura, Y. Watanabe, T. Kitagawa, J. Am. Chem. Soc. 119 (1997) 1758–1766.
[51] A. Feis, M.P. Marzocchi, M. Paoli, G. Smulevich, Biochemistry 33 (1994) 4577–4582.
[52] K. Uchida, Y. Susai, E. Hirotani, T. Kimura, T. Yoneya, H. Takeuchi, I. harada, J. Biochem. 103 (1988) 979–985.
[53] M. Unno, J.F. Chrystian, J.S. Olson, J.T. Sage, P.M. Champion, J. Am. Chem. Soc. 120 (1998) 2670–2671.
[54] S. Hirote, T. Ogura, E.H. Appelman, K. Shinzawa-Itoh, S. Yoshikawa, T. Kitagawa, J. Am. Chem. Soc. 116 (1994) 10564–10570.
[55] E.A. Bonomo, J.B. Swaney, Biochemistry 29 (1990) 5094–5103.
[56] M. Nagai, M. Aki, R. Li, Y. Jin, H. Sakai, S. Nagatomo, T. Kitagawa, Biochemistry 39 (2000) 13093–13105.
[57] B. Shaanan, J. Mol. Biol. 171 (1983) 31–59.
[58] J.M. Baldwin, J. Mol. Biol. 136 (1980) 103–128.
[59] S. Hirota, T. Ogura, K. Shinzawa-Itoh, S. Yoshikawa, M. Nagai, T. Kitagawa, J. Phys. Chem. 98 (1994) 6652–6660.
[60] K. Imai, Allosteric Effects in Hemoglobins, Cambridge University Press, Cambridge, UK, 1982.
[61] K. Inaba, K. Ishimori, K. Imai, J. Morishima, J. Biol. Chem. 275 (2000) 12438–12445.
[62] T. Li, G.N. Phillips Jr., J.S. Olson, M. Mukai, T. Kitagawa, J. Am. Chem. Soc. 118 (1996) 7845–7846.
[63] M.L. Quillan, R.M. Arduin, J.S. Olson, J. Mol. Biol. 234 (1993) 140–155.
[64] H. Brunner, Naturwiss 61 (1974) 129–141.