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REVIEW

A role of heme side-chains of human hemoglobin in its function revealed by circular dichroism and resonance Raman spectroscopy

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Abstract

Structural changes of heme side-chains of human adult hemoglobin (Hb A) upon ligand (O_2 or CO) dissociation have been studied by circular dichroism (CD) and resonance Raman (RR) spectroscopies. We point out the occurrence of appreciable deformation of heme side-chains like vinyl and propionate groups prior to the out-of-plane displacement of heme iron. Referring to the recent fine resolved crystal structure of Hb A, the deformations of heme side-chains take place only in the β subunits. However, these changes are not observed in the isolated β chain (β_4 homotetramer) and, therefore, are associated with the α–β inter-subunit interactions. For the communications between α and β subunits in Hb A regarding signals of ligand dissociation, possible routes are proposed on the basis of the time-resolved absorption, CD, MCD (magnetic CD), and RR spectroscopies. Our finding of the movements of heme side-chains would serve as one of the clues to solve the cooperative O_2 binding mechanism of Hb A.

Keywords Human hemoglobin · Subunits interactions · Circular dichroism · Resonance Raman · Deformations of heme side-chains · Cooperative oxygen binding

Introduction

Hemoglobin (Hb) is a well-known O_2-transporting allosteric protein. Human adult Hb (Hb A) is a heterotetramer consisting of two α (141 residues) and two β (146 residues) subunits. The active center which binds O_2 is a heme (protoporphyrin IX Fe-complex, protoheme) (Fig. 1a). Hb binds O_2 in the lung and releases it in tissues. When an O_2 molecule is bound to deoxyHb in the lung, its O_2 affinity increases and, when a second one binds, its O_2 affinity increases further. In this way, O_2 affinity increases with increasing number of bound O_2 molecules. This phenomenon is called cooperativity and it is a characteristic of Hb. Upon release of O_2, the opposite features appear. Structural elucidation of a mechanism of cooperativity is the subject of many studies of Hb.

There are several allosteric effectors of Hb, such as CO_2, proton, Cl^- (chloride ion), and organic phosphate, similar to other allosteric proteins. However, the most effective regulator for the O_2 affinity of Hb is the concentration of O_2 itself (partial pressure of O_2) (Edelstein 1975; Imai 1982; Bunn and Forget 1986; Dickerson and Geis 1983). When oxyHb, $[Hb(O_2)_4]$, releases one molecule of O_2, the O_2 affinity of the remaining O_2-bound subunits becomes lower and, thus, the release of one molecule of O_2 accelerates the release of the remaining O_2. This change of O_2 affinity has been interpreted in terms of the quaternary structure transition from relaxed (R) to tense (T), which was first proposed by Monod, Wyman, and Changeux as a two-state model (MWC model) (Monod et al. 1965). Perutz (1970) revealed structures of Hb A with X-ray crystallography and pointed out that oxyHb and deoxyHb correspond to the R and T structures, respectively.

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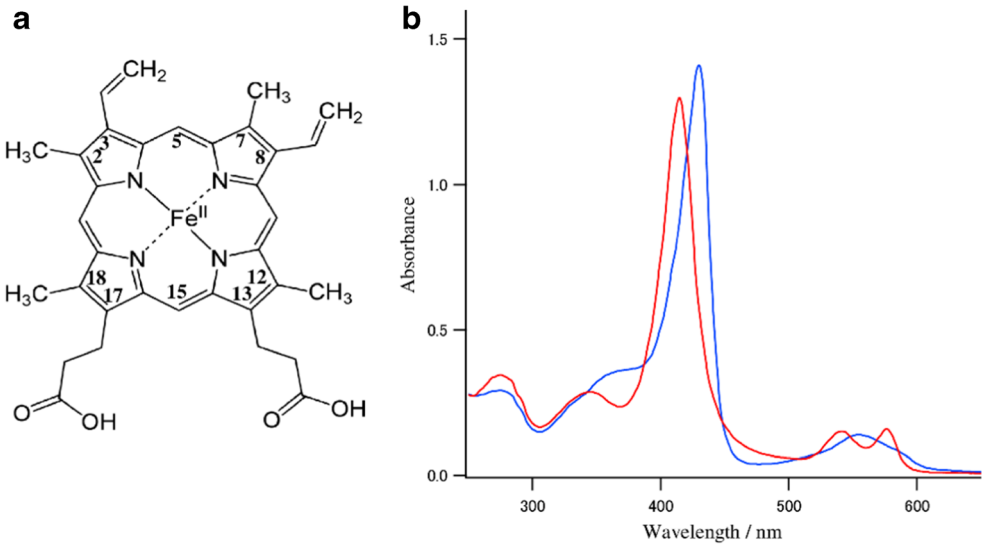

Fig. 1 **a** The structure of protoheme. **b** Absorption spectra of oxy (red) and deoxy forms (blue) of Hb A in 0.05 M phosphate buffer, pH 7.0

Hb A can also be considered to consist of two αβ dimers. There are two kinds of subunit interfaces, $\alpha_1\beta_1$ (= $\alpha_2\beta_2$) and $\alpha_1\beta_2$ (= $\alpha_2\beta_1$) (Dickerson and Geis 1983). The $\alpha_1\beta_1$ subunit interface is strong and hardly changes, but the $\alpha_1\beta_2$ interface is relatively weak and changes upon O_2 binding, resulting in the quaternary structure changes (Perutz 1970). Baldwin and Chothia (1979) have suggested that the rearrangements of the $\alpha_1\beta_2$ subunit interface are essential to the change of O_2 affinity of Hb, and, thus, to the cooperativity. In the $\alpha_1\beta_2$ interface, there are two important contacts in the deoxy state; one is an H-bond between Aspα94 and Trpβ37 and the other is an H-bond between Tyrα42 and Aspβ99. The former is called the "flexible joint" or "hinge region" and the latter is called the "switch region" (Dickerson and Geis 1983). These two H-bonds are important for stabilizing the T quaternary structure, but disappear by rotation of one αβ dimer by 15° with regard to the other αβ dimer upon O_2 binding, and, thus, by the quaternary structure change from T to R.

Arisaka et al. (2011) examined the tetramer–dimer equilibrium of Hb by analytical ultracentrifugation. The sedimentation coefficient of Hb A and Mb show 4.27S and 2.22S, respectively (Arisaka et al. 2011; Schuck and Demeler 1999). Dimer Hb known as Hb Hirose (Trpβ37 → Ser) gives 3.05S. Using mutant Hbs at the $\alpha_1\beta_2$ interface, they obtained the following s-values and also showed practical effects of an allosteric effector, IHP (inositol hexaphosphate) on the s-value; Hb A 4.27S (+IHP, 4.27S), Hb Hirose 3.05S (+IHP, 4.19S), rHb (Trpβ37 → His) 3.34S (+IHP, 3.82S), and rHb (Tyrα42 → Ser) 3.82S (+IHP, 4.12S) (Arisaka et al. 2011). All these mutants tend to dissociate into two dimers in different ways and the addition of IHP to the dissociated Hbs shifts the equilibrium toward tetramer, and, concomitantly, some mutant Hbs restore cooperativity. These results indicate that the tetramer–dimer equilibrium could be influenced by the substitution of only one amino acid residue. The equilibrium depends on where the substitution occurs and which kind of amino acid is introduced.

Since 1970, the cooperative O_2 binding of Hb has been explained in terms of the transition between two quaternary structures (T and R) (Perutz 1970), based on quaternary structure. Recently, Henry et al. (2002) proposed the "tertiary two-state model", in which tertiary structure is more important rather than quaternary structure, and Yonetani et al. (2002) and Yonetani and Laberge (2008) interpreted the cooperativity by the fluctuation of F- and E-helices as the "global allostery model", in which quaternary structure is not so important. However, we do not discuss them in this review. Readers who are interested in them are suggested to refer to other reviews (Eaton et al. 1999; Yonetani and Kanaori 2013; Yuan et al. 2015).

The O_2-bound heme iron is covalently bound to the proximal (F8) His at the opposite site and stays in the heme plane, but moves out-of-plane (~ 0.5 Å) toward His(F8) upon O_2 dissociation (Dickerson and Geis 1983). The movement of iron is conveyed to the other portion of protein via HisF8 and this induces the quaternary structure change. This movement was thought to be skeletal distortion of heme itself (Perutz 1970) and little attention had been paid to the contribution of the individual peripheral group (side-chains) of the heme molecule. We have studied them with circular dichroism (CD) and resonance Raman (RR) spectroscopy and found appreciable changes of heme side-chains occurring prior to the movement of iron upon O_2 dissociation (Nagai and Nagai 2011; Nagatomo et al. 2011). Indeed, in the recent 1.25 Å resolution X-ray crystallography of Hb A (Park et al. 2006), we noticed that the changes of heme side-chains occur in the β subunits of Hb A. On the other hand, in an O_2 sensor heme protein, HemAT-Bs, an H-bonding of the heme side-chain with a near-by residue seems to be important for selective O_2 binding and signal transduction (Yoshimura et al.

2006). These facts may suggest that the participation of heme side-chains in determining the function is common in heme proteins. Therefore, we focused on structural changes of heme side-chains upon ligand dissociation in this review.

Circular dichroism of hemoglobin

Hb A displays absorption spectra in the UV and visible regions (Fig. 1b). The characteristic visible absorption of Hb, called the Soret band, appears at 400–450 nm and derives from an allowed $\pi\pi^*$ transition of porphyrin. Soret bands of the isolated α and β chains are alike in both the oxy and deoxy forms, but are different from that of tetrameric Hb. When these isolated chains are mixed, the Soret absorption band profile becomes similar to that of native $\alpha_2\beta_2$ tetramer Hb (Hb A), but the intensity is significantly larger than their sum, especially in the deoxy form (Brunori et al. 1968). This spectral change by reconstitution is derived from some changes in the electronic properties of heme caused by protein conformational change due to interactions between α and β chains. This is the so-called "heme–heme interaction".

Heme alone cannot exhibit a CD spectrum because the π-orbital of heme has higher symmetry. However, a characteristic CD spectrum appears when the heme is incorporated into the globin. Interaction between the heme and amino acid residues in the globin could induce asymmetry into the π-orbital of heme. Figure 2 shows the CD spectra of the isolated α and isolated β chains of Hb A in the Soret region (Nagai et al. 1969). Although electronic absorption spectra of both isolated chains are alike, different features of CD spectra between the isolated α and isolated β chains probably reflect the different environment around heme in the protein interior. Myoglobin (Mb) exhibits similar CD spectra to those of the isolated α chains (Nagai et al. 1969). In the X-ray crystal structure of Hb A, the orientations of the heme side-chains (vinyl and propionates) are different between the α and β subunits (Park et al. 2006). The different features of CD between the α and β chains partially arise from these differences of orientation of the heme side-chains.

Figure 3 shows the CD spectra of Hb reconstituted from the isolated α and isolated β chains. The CD spectra of reconstituted oxyHb and deoxyHb are almost the same as those of native Hb A, but are not the same as the arithmetic mean of those of isolated α and isolated β chains (Nagai et al. 1969). Especially, the reconstituted deoxyHb exhibits strong intensity in the Soret region compared with the arithmetic mean. Similar results were also reported by Geraci and Li (1969). The results indicate that the π electronic symmetry of heme changes greatly by tetramerization of α and β chains. In the X-ray crystal structure of Hb A, the heme propionates of the β subunit change the orientation upon ligand dissociation (Park et al. 2006). The intensity enhancement of the Soret CD band in the deoxyHb A might be due to the orientation changes of heme propionates of the β subunit.

For the origin of induced heme optical activity in Hb and Mb, Hsu and Woody (1971) proposed a coupled oscillator interaction between heme transition and allowed $\pi\pi^*$ transition of near-by aromatic residues, and this idea has been widely accepted. However, this model is contradicted by new observations that Hb with the reversed heme exhibits a negative CD in the Soret region (Nagai et al. 2008). Even if the heme is reversed in the globin pocket, the positions of aromatic residues remain unchanged. Therefore, we proposed a different interpretation. The optical activity of heme is influenced by the orientation of the heme side-chains. Rotation of the heme by 180° about the 5,15-meso axis interchanges the methyl groups at positions 2 and 7 with the vinyl groups at positions 8 and 3, respectively. The 13- and 17-propionates are the same as the 17,13-propionates. However, the orientation of the two propionates are opposite between the normal and reversed hemes, and, therefore, the optical activity will be changed.

In order to examine the effect of heme side-chains on the Soret CD band, we reconstituted Mb with apoMb and

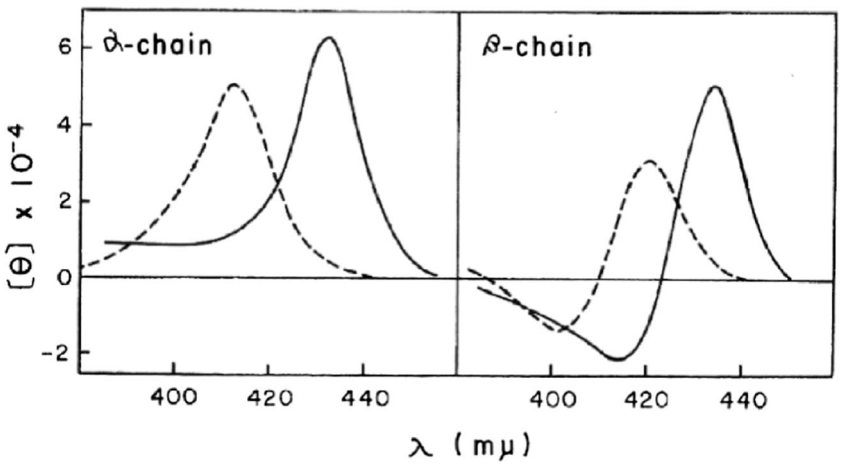

Fig. 2 Circular dichroism spectra in the Soret region of the isolated α and β chains at pH 7.0. *Dashed line*, oxy form; *solid line*, deoxy form (Nagai et al. 1969)

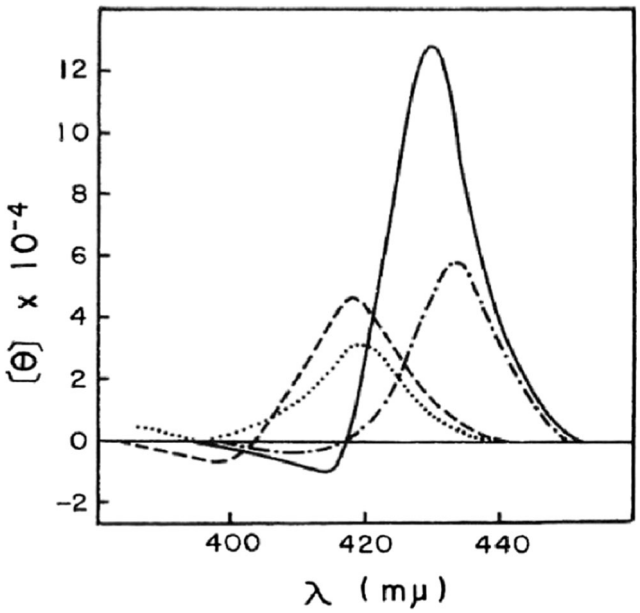

Fig. 3 Circular dichroism spectra of the reconstituted Hb. *Dashed line*, reconstituted oxyHb; *solid line*, reconstituted deoxyHb; *dotted line*, arithmetic mean of the oxy α and β chains; *dot-dashed line*, arithmetic mean of the deoxy α and β chains (Nagai et al. 1969)

unnatural hemes having the substituted side-chains for 3,8-vinyl and 13,17-propionates (Nagai et al. 2015). The upper part of Fig. 4 shows the structures of the artificial hemes used in this experiment: heme **3**, 3,8-methyl, 13,17-propionates; heme **5**, 3,8-methyl, 13,17-acetates; heme **6**, 3,8-methyl, 13,17-carboxylates. The lower part of Fig. 4 shows the CD spectra of the reconstituted Mbs, that is, Mb3 (heme **3**), Mb5 (heme **5**), and Mb6 (heme **6**), respectively.

The CD spectrum of Mb3 is similar to that of native Mb but the intensity of the Soret band was 70% of that of the native Mb, indicating that 3,8-vinyl side-chains contribute partly (~ 30%) to the chirality of the heme, as suggested by Woody and Pescitelli (2014). The change of the 13,17-propionate to acetate (Mb5) shows little effect, but that to carboxylate (Mb6) considerably diminished the heme chirality. This indicates that the length of 13,17 side-chains is important for the induced CD in the Soret region.

According to X-ray crystallographic analysis of Hb A, two sets of orientations are present for propionates at the 13- and 17-positions; in one, the two side-chains are directed up (proximal side) and down (distal side) against the heme plane (anti-parallel) and in the other, they adopt the same direction (parallel). We performed theoretical analysis on the effect of the propionate orientations on the Soret CD using time-dependent density functional theory (TDDFT) (Nagai et al. 2015). It became clear that up and down orientations of a propionate gave a positive and negative CD, respectively, and their magnitudes are different between the 13- and 17-positions. The great

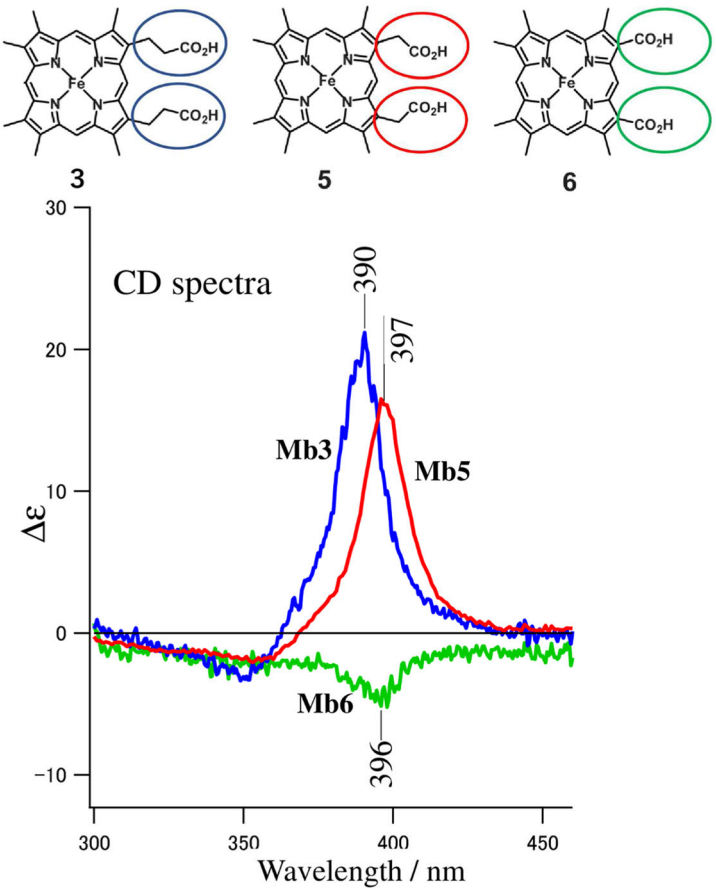

Fig. 4 The Soret circular dichroism spectra of Mbs reconstituted with 3,8-methyl heme modified at 13,17-propionate. Mbs are in the aquomet form at pH 7.0 (Nagai et al. 2015)

enhancement of the Soret CD band of the deoxyHb A might be caused by an orientational change of the 13,17-propionates upon O_2 dissociation, and this will be quantitatively discussed later.

Although Hb and Mb generally exhibit a positive CD band in the Soret region, monomeric Hb from *Chironomus thummi thummi* (CTTHb) gave a negative Soret CD band (Formanek and Engel 1968). To explain this, Woody and Pescitelli (2014) modified their original idea so that the orientation of the vinyl side-chains of heme is more influential on the induced Soret CD than the interactions between the $\pi\pi^*$ transitions of porphyrin and near-by aromatic residues. In the crystal structure of CTTHb (Steigemann and Weber 1979), two vinyl side-chains of heme stand 180° from the heme plane and the 13-propionate extends toward the proximal site, while the 17-propionate extends toward the distal site. This structure is different from those of Mbs (Nagai et al. 2015). These orientations of heme side-chains of CTTHb look similar to that of the Hb A with the reversed heme, suggesting that the orientation of heme side-chains influences the Soret CD.

The most recent refinements of the crystallographic structures of oxy, deoxy, and COHb A with 1.25 Å resolution have revealed the movements of heme side-chains upon O_2 dissociation in either α or β subunits, which are depicted in Figs. 5 and 6 (Park et al. 2006). In these figures, protein parts are shown by the ribbon model, and the heme moiety is shown by a molecular model. In the heme of the α subunit in the oxyHb A, 3,8-vinyl and 13,17-propionate side-chains seem to be unchanged upon O_2 dissociation (Fig. 5). In contrast, these side chains of the β subunit in oxyHb A change greatly upon O_2 dissociation; the 3-vinyl side chain changes direction from the proximal side (up) to the distal side (down) against the heme plane, and the 13,17-propionate groups change from the same direction (parallel orientation) to the up and down orientation against the heme (anti-parallel orientation) (Fig. 6). These alterations observed for the β subunit of oxyHb A are the same in COHb A (Park et al. 2006). The isolated β chain forms a homotetramer, β_4. The crystal structures of the deoxy and CO forms of the isolated β chains were refined by Borgstahl et al. (1994a, b), who noted that the propionate side-chains of all four β chains take an anti-parallel orientation in both the deoxy and CO forms.

Propionates of Mb form the H-bond networks in the upper and lower parts of the heme plane, and these H-bond networks do not change with or without ligand (Hayashi et al. 2002). Therefore, characteristic changes from parallel to anti-parallel orientation of the propionate upon ligand dissociation occur only in the β subunit of the heterotetramer, $\alpha_2\beta_2$ (Hb A).

Gas-sensor proteins such as HemAT-Bs (O_2) (Yoshimura et al. 2006), CooA (CO) (Aono et al. 1998), *Ec* Dos (O_2) (Sasakura et al. 2002), and FixL (O_2) (Rodgers and Lukat-Rodgers 2005), and P450cam (Hayashi et al. 2009) all have a protoheme (*b*-type) the same as Hb A. In HemAT-Bs, an H-bond between 13-propionate and His86 is known to be important for O_2 sensing and signal transduction, because the mutation of His86 to Ala loses the signal transduction upon O_2 binding (Yoshimura et al. 2007). CooA (transcription factor), *Ec* Dos (phosphodiesterase), and FixL (histidine kinase) are activated by the binding of a specific gas molecule, but there is no report on the contributions of their heme propionates to functions. For the activity of P450cam, 17-propionate is considered to make a salt-bridge with Arg299, regulating the uptake of water molecules to the active site (Hayashi et al. 2009), but the 13-propionate is believed to be essential, because deletion of the 13-propionate changes P450cam to inactive P420 (Hayashi et al. 2009).

Carbon monoxide (CO) is dissociated from HbCO by light irradiation. The change in the absorption spectrum of Hb after photodissociation of CO was pursued using time-resolved absorption spectroscopy (Hofrichter et al. 1983, 1985; Murray et al. 1988; Gelin et al. 1983; Goldbeck et al. 1996), which showed that fast absorption change (0.1 μs) due to conformational change of heme (distortion) was followed by a slow (20–30 μs) quaternary structure change of the protein. Björling et al. (1996) examined the nanosecond time-resolved near-UV CD spectral changes (TRCD) of Hb after CO photodissociation and observed the early time changes of

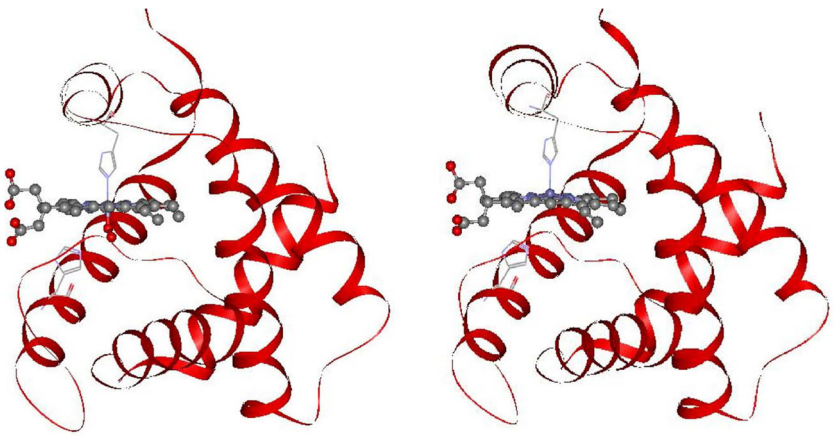

Fig. 5 Comparison of crystal structures of the α subunits in the oxyHb A (left, PDB: 2DN1) and deoxyHb A forms (right, PDB: 2DN2) (Park et al. 2006). Anti-parallel orientation of the propionate side chains against the heme plane do not change upon O_2 dissociation

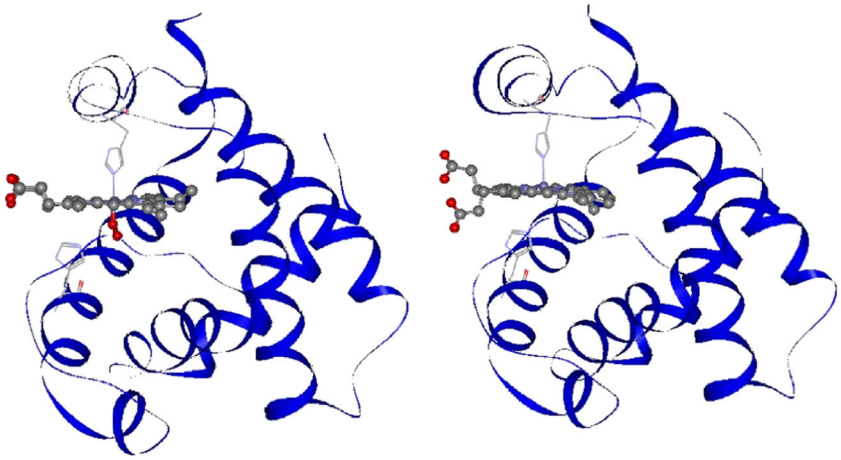

Fig. 6 Comparison of the crystal structures of the β subunits in the oxyHb A (left, PDB: 2DN1) and deoxyHb A forms (right, PDB: 2DN2) (Park et al. 2006). The two propionate side-chains of the heme change from parallel (left) to anti-parallel orientation (right) against the heme plane upon O_2 dissociation

both the heme and the aromatic region of the protein. Perutz et al. (1974) found a negative CD band around 285 nm in the deoxyHb, and called this band "the T-state marker", because its appearance is correlated to an increase of cooperativity. It is deduced that the 285-nm negative CD band derives from the H-bond formation of Trpβ37-Aspα94 and/or Tyrα42-Aspβ99 at the $\alpha_1\beta_2$ subunit interface (Perutz et al. 1974).

We identified the contribution of aromatic residues at the $\alpha_1\beta_2$ subunit interface contributing to the negative CD band to be Tyrα42 (4%), Trpβ37 (18%), Tyrβ145 (28%), and Tyrα140 (32%), using Hbs, which involves mutation at one of these residues (Aki-Jin et al. 2007; Li et al. 2000a). The negative CD band is not due to the Tyrα42 and Trpβ37, but arises from changes of the penultimate Tyr of both the α and β subunits (Tyrα140 and Tyrβ145). Moreover, the negative CD band also contained an appreciable contribution from the protein tertiary structure changes upon ligand dissociation (Li et al. 2000b). Accordingly, the CD change of the aromatic region observed by Björling et al. (1996) probably reflects the tertiary structure changes after ligand photodissociation. Goldbeck et al. (2002) examined the aromatic region (285–295 nm) of HbCO after CO photodissociation by the time-resolved magnetic CD (TRMCD). They found red-shift of the Trp band at early time (2 μs) and suggested that this red-shift is due to the H-bond formation between Trpβ37 and Aspα94, and that the H-bond formation is the first step of the quaternary structure transition.

As clarified by time-resolved absorption, CD, and MCD studies of Hb mentioned above, fast conformational change of the heme (ns) occurs immediately after CO photodissociation, and the protein tertiary and quaternary structure changes (μs) follow it.

Raman scattering of hemoglobin

Raman scattering is a vibrational spectroscopy like an infrared absorption spectroscopy. Resonance Raman (RR) is a technique to enhance the Raman scattering from a chromophore of a molecule by approaching the Raman excitation wavelength to the absorption maximum of the target molecule. Accordingly, the fine structure of heme could be examined using the Soret band excitation. If there is a sample of αoxy-βdeoxy Hb, we can obtain the RR spectrum for the α subunit with the 413-nm excitation (absorption peak of oxyHb) and obtain the RR spectrum of the β subunit with the 441.6-nm excitation (near the absorption peak of deoxy Hb).

As cited earlier, Hb with the reversed heme showed a negative Soret CD band (Nagai et al. 2008). In the reversed heme, the methyl groups at positions 2 and 7 interchange for the vinyl groups at positions 3 and 8, but the propionate groups at positions 13 and 17 remain unchanged. Since the spectrum of Mb3 in Fig. 4 suggested that the contribution from vinyl group to Soret CD is approximately 30% (Nagai et al. 2015), it is unlikely to ascribe the Soret negative CD to the exchange of vinyl groups with methyl groups (Nagai et al. 2015). However, it is possible that the orientations of propionates at positions 13 and 17 are opposite to those of native Hb. To examine this possibility, the RR spectrum of Hb with the reversed heme was compared with that of Hb with normal heme, as shown in Fig. 7 (Nagai et al. 2016).

Many peaks are observed in Fig. 7 and each peak has been assigned to a specific vibration of a heme experimentally and theoretically (Hu et al. 1996; Kitagawa and Teraoka 1979; Li et al. 1990). Assignments of RR bands in Fig. 7 are the ones determined by Hu et al. (1996), who assigned the RR bands on the basis of reconstitution of Mb with isotopically labeled heme. The strong ν_4 band at 1357 cm^{-1} is known as an oxidation state marker and varies with the oxidation of the heme and ligand binding. The ν_2 band at 1567 cm^{-1} and the ν_3 band at 1472 cm^{-1} are called the spin-state and coordination-state markers, respectively. In the higher frequency region (Fig. 7 left panel), RR bands of Hb with the reversed heme (upper spectrum) are the same as those of Hb A with normal heme (lower spectrum), except for the vinyl C = C band around

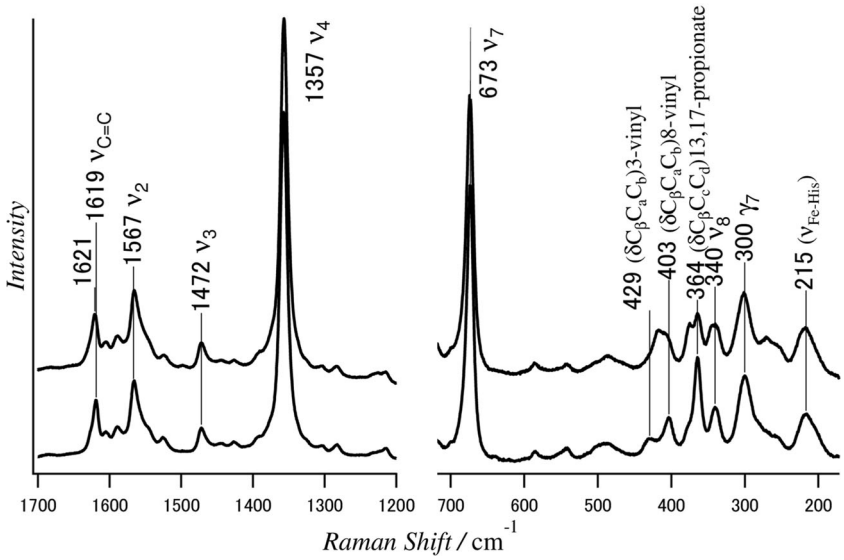

Fig. 7 The 441.6-nm excited resonance Raman spectra of native Hb A (lower spectrum) and rHb A with the reversed heme (upper spectrum) in the deoxy form (Nagai et al. 2016)

1620 cm^{-1}. It means that the in-plane skeletal vibrations of the heme are hardly affected by the orientation of heme.

In the low frequency region (Fig. 7 right panel), RR bands at 300 and 340 cm^{-1} were assigned to the out-of-plane (γ_7) and in-plane modes (ν_8) of the heme, respectively. The Fe-His mode at 215 cm^{-1} and the porphyrin ring modes are the same between Hb As with normal and reversed hemes. For side-chain modes, however, the RR band at 364 cm^{-1} was assigned to the propionate skeletal bending $\delta(C_\beta C_c C_d)$, and the RR bands at 403 and 429 cm^{-1} were assigned to the 8-vinyl and 3-vinyl bending $\delta(C_\beta C_a C_b)$ modes, respectively. The latter bands merge into a single band at around 418 cm^{-1} in the spectrum of Hb A with the reversed heme. Moreover, a bending mode $\delta(C_\beta C_c C_d)$ of the 13,17-propionate groups of Hb A with normal heme at 364 cm^{-1} splits into two bands at 364 cm^{-1} and 374 cm^{-1} in the spectrum of Hb A with the reversed heme. The shifts of vinyl $C_a = C_b$ stretching mode at 1619 cm^{-1} of Hb A with normal heme by 2 cm^{-1} to higher frequency in Hb A with the reversed heme is probably caused by the change of the orientation of vinyl side-chains. As no change was observed for the γ_7 and ν_8 bands between Hb A with normal heme and that with the reversed heme, planarity of the heme must be maintained between them. The characteristic negative CD band in the Soret region of Hb A with the reversed heme is probably related to the changes of orientation of the heme side-chains.

In Fig. 8, RR spectra of the isolated α and isolated β chains in the low frequency region are compared with that of native

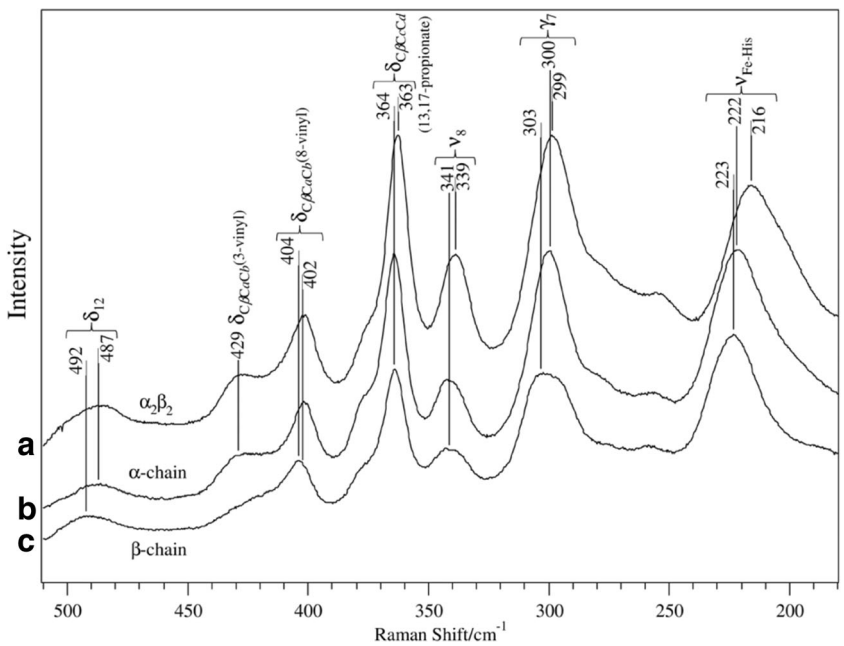

Fig. 8 Low frequency resonance Raman spectra of: **a** Hb A, **b** the isolated α chains, and **c** the isolated β chains in the deoxy form (Nagatomo et al. 2011). Excitation wavelength: 441.6 nm

Hb A ($\alpha_2\beta_2$) in the deoxy form (Nagatomo et al. 2011). The $\nu_{Fe\text{-}His}$ band of deoxyHb A (T) is observed at 215–216 cm^{-1}, but the corresponding bands of isolated chains appear at 222–223 cm^{-1}, while the frequencies of all other bands are similar between native Hb A and the isolated chains. The frequency of the $\nu_{Fe\text{-}His}$ band depends on the O_2 affinity: Hb Chesapeake (Argα92 → Leu) with high O_2 affinity and low cooperativity (R) gives the $\nu_{Fe\text{-}His}$ band at 222 cm^{-1}, although Hb Chesapeake in the presence of IHP with decreased O_2 affinity and a significant cooperativity (T) gave the $\nu_{Fe\text{-}His}$ at 215 cm^{-1} (Imai 1974; Matsukawa et al. 1985).

It is a question of whether the $\nu_{Fe\text{-}His}$ band of the α subunit is the same as that of the β subunit in the deoxyHb A or not. Nagai and Kitagawa (1980) have determined the $\nu_{Fe\text{-}His}$ of each subunit in the T-state Hb using valency hybrid Hbs (ferric heme in either α or β subunit) and metal hybrid Hbs (Ni-heme in either α or β subunit) (Nagai and Kitagawa 1980; Shibayama et al. 1986). They showed that, in the T-state Hb, the $\nu_{Fe\text{-}His}$ band appeared at 201–203 cm^{-1} for the α subunit and at 217–220 cm^{-1} for the β subunit, respectively. This result indicates that, in the slow relaxation process following CO photolysis, the $\nu_{Fe\text{-}His}$ of α subunit shifts to much lower frequencies (222 → 203 cm^{-1}) than that of β subunit (223 → 217 cm^{-1}), and, thus, their relaxation energies would be different.

The relation between Raman intensity and the structure of vinyl side-chain were examined through the selective deuteration of vinyl side-chain by Uchida et al. (1988) and Rwere et al. (2014). They interpreted that the Raman intensity of vinyl side-chain depends on their co-planarity with pyrrole ring, that is, the bands are weaker when vinyl side-chains are out-of-plane. As shown in Fig. 8, the 8- and 3-vinyl $\delta C_\beta C_a C_b$ bands are observed at 402 and 429 cm^{-1}, respectively, for Hb A and the α chain. However, the 429 cm^{-1} band is much weaker for the β chain, suggesting that the 3- and 8-vinyl side-chain of both Hb A and its α chain are in-plane, while 3-vinyl of the β chain is out-of-plane.

Figure 9 shows the RR spectra of equilibrated HbCO (green), deoxyHb (blue), and the time-resolved RR spectra (red) following CO photodissociation (Mizutani and Nagai 2012). The red spectra represent the difference between spectra observed at a specific delay time (Δt) against the spectrum of HbCO. In the spectrum of HbCO (Fig. 9 bottom, green), the RR band at 503 cm^{-1} is assigned to Fe-CO stretching ($\nu_{Fe\text{-}CO}$) (Tsubaki et al. 1982). It is noted that the spectrum at Δt = 1000 ps is still different from the spectrum of deoxyHb, particularly around the $\nu_{Fe\text{-}His}$ band. This means that the structural relaxation of Hb A is extremely slow. With regard to the side-chain modes, 3-vinyl $\delta(C_\beta C_a C_b)$ is present at 429 cm^{-1} for HbCO, while 8-vinyl $\delta(C_\beta C_a C_b)$ is observed at 404 cm^{-1} for deoxyHb, suggesting that the co-planarity of vinyl groups with pyrrole ring changes upon CO dissociation. The propionate bending mode, $\delta(C_\beta C_c C_d)$, on the other hand, appears at

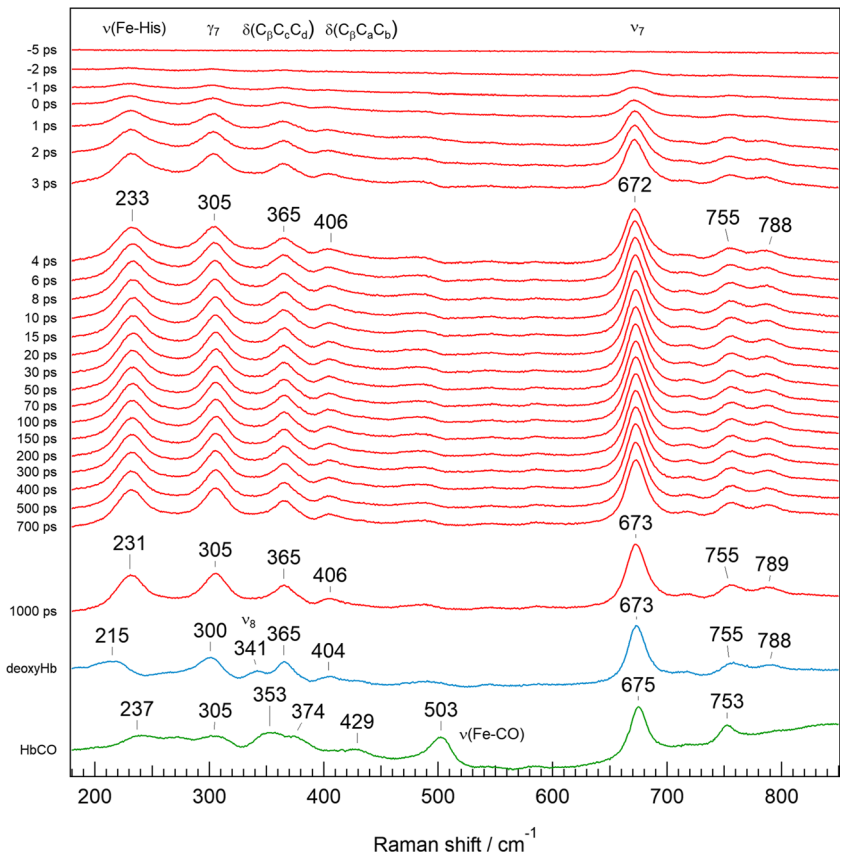

Fig. 9 Pico-second time-resolved resonance Raman spectra of photodissociated HbCO in the 180–850 cm^{-1} region. Spectra of the equilibrated states of deoxyHb (blue) and HbCO (green) are depicted at the bottom for comparison (Mizutani and Nagai 2012). The time-resolved spectra (red) are differences of the spectrum observed for specific Δt against the spectrum of HbCO (green)

365 cm^{-1} for deoxyHb but splits into two bands at 374 and 353 cm^{-1} in HbCO, suggesting that the orientation of the propionates changes upon CO dissociation. These changes of RR bands for the heme side-chains upon ligand dissociation are similar to those of Hb A with the reversed heme, as shown in Fig. 7.

The propionate bending mode $\delta(C_\beta C_c C_d)$ is known to shift to a higher frequency when the terminal carboxylate of propionate forms an H-bond (Nakashima et al. 1998). As shown in Fig. 6, the 13,17-propionates of β subunit in oxy-Hb A take a parallel orientation and their carboxylates can make an H-bond via a water molecule (Park et al. 2006). Upon O_2 dissociation, orientation of the propionates changes from parallel to anti-parallel, and the H-bond disappears. Accordingly, the propionate bending mode, $\delta(C_\beta C_c C_d)$, is shifted to a lower frequency upon ligand dissociation. On the other hand, the 13-propionate of the α subunit of Hb A makes an H-bond with Hisα45, but the H-bond does not change in any of the oxy, CO, and deoxy forms (Park et al. 2006).

In the pico-second time-resolved RR spectra shown in Fig. 9, the $\nu_{Fe\text{-}His}$ band appears at 233 cm^{-1} at $\Delta t = 1$ ps and is down-shifted only 2 cm^{-1} in 1000 ps (Mizutani and Nagai 2012). The 2 cm^{-1} down-shift of the $\nu_{Fe\text{-}His}$ band at 1000 ps was also observed in Mb and the isolated α and β chains, but was not seen in the heme model compound (CO-hemin 2-methyl imidazole) (Mizutani and Kitagawa 2001). Therefore, this 2 cm^{-1} down-shift is due to protein relaxation. The other RR bands, at 305 cm^{-1} (out-of-plane mode, γ_7) and 406 cm^{-1} (vinyl bending mode, $\delta C_\beta C_a C_b$), do not change until 1000 ps, but these bands are not the same as the RR bands at 300 cm^{-1} (γ_7) and 403 cm^{-1} ($\delta C_\beta C_a C_b$) of the deoxyHb. Moreover, the 341 cm^{-1} band (in-plane mode, ν_8) observed in the deoxyHb is not seen even at $\Delta t = 1000$ ps. The quaternary structure transition to the T structure takes place at $\Delta t = 20$ μs (Yamada et al. 2013).

Recently, Jones et al. (2014) have shown an interesting result using the proto-meso heme hybrid Hb. An RR spectrum of the subunit having proto-heme could be obtained upon excitation at 441.6-nm, because the Soret absorption peak of the meso-heme is shifted to a shorter wavelength than that of the proto-heme by 10 nm. From the RR spectra after photodissociation and theoretical calculations with quantum mechanics/molecular mechanics (QM/MM), using the PELE program, it became clear that the shift of the $\nu_{Fe\text{-}His}$ band to the frequency of the T structure is much faster (3 μs) in the β subunit than that in the α subunit (20 μs).

The ultra-violet RR (UVRR) spectroscopy allows us to monitor the conformational changes of proteins (Miura et al. 1988; Chi and Asher 1998). Upon excitation at 235 nm, the vibrations of tyrosine (Tyr) and tryptophan (Trp) are selectively detected. Accordingly, we examined the UVRR spectral changes of these residues due to the quaternary structure transition upon ligand dissociation, and observed high-frequency shifts for Tyr residues and intensity enhancement for Trp residues (Nagai et al. 1995, 1996). We clarified that the frequency shifts of Tyr are due to Tyrα42 and Tyrα140 and that the intensity enhancement of Trp RR bands is due to Trpβ37 (Nagai et al. 1995, 1996, 2012). All these aromatic residues are located at the $\alpha_1\beta_2$ subunit interface and form an intra- or inter-subunit H-bond in deoxyHb. Thus the frequency shifts and intensity enhancements of RR bands upon ligand dissociation seem to be caused by H-bond formations or change of hydrophobic circumstances around these aromatic residues in proteins. Specific H-bond formations of Tyrα42 and Trpβ37 in the deoxyHb were clarified by the ^{1}H NMR spectra. The characteristic ^{1}H NMR signals of these residues are used as "T-state marker bands" (Ho 1992).

Rodgers and Spiro (1994) have examined the conformational changes of protein after photodissociation by time-resolved UVRR and revealed that tertiary structure changes occur at 0.3 μs and quaternary structure changes occur at 20 μs. The tertiary H-bond changes arise from Trpα14-Thrα67 and Trpβ15-Serβ72 (Balakrishnan et al. 2004b), although these authors improved their data (Balakrishnan et al. 2004a), which indicated that the T-state quaternary contacts are formed in two steps, with time constants at 2.9 μs and 21 μs, instead of a single 20-μs process. The first step involves the hinge region by the formation of an H-bond between Trpβ37 and Aspα94 and the second step involves the switch region by an H-bond formation between Tyrα42 and Aspβ99 at the $\alpha_1\beta_2$ subunit interface. Kavanaugh et al. (2005) have investigated how the Tyrα42 contributes to the cooperativity using a mutant with Tyrα42 replaced by alanine, rHb (Tyrα42 → Ala). This mutant Hb showed increased O_2 affinity but exhibited a significant cooperativity (Hill's n = 1.9). Intensity enhancement of Trp RR bands upon O_2 dissociation was almost the same as those in native Hb. From these results, they concluded that Tyrα42 significantly stabilizes the quaternary T structure but is not a major player in yielding quaternary constraints in the deoxyHb.

Conformational changes of the heme upon ligand dissociation and its relation to cooperativity

Cooperativity in O_2 binding of Hb has been attributed to the T–R quaternary structure transition. It was known from the crystal structure of the porphyrin model compounds that a five-coordinated high-spin Fe(II) sits roughly 0.45 Å out-of-heme plane, whereas a six-coordinated low-spin Fe(II) sits on the heme plane (Perutz 1979). Fermi et al. (1984) pointed out that, in the deoxyHb with T structure, the iron atom stays out-of-plane of the porphyrin ring toward the proximal HisF8 by ~ 0.4 Å. RR measurements indicated that the Fe-N_ε(HisF8) stretching mode ($\nu_{Fe\text{-}His}$) of deoxyHb appeared at 215–

218 cm^{-1} for the T state and at 220–221 cm^{-1} for the R state (Nagai et al. 1980; Nagai and Kitagawa 1980). This suggested that the Fe-N_ε(HisF8) bond was weakened in the T state due to a strain exerted by globin. The binding of O_2 to Hb would force the iron atom to move into the heme plane, pulling the HisF8 and, thus, the F-helix toward the heme against the strain. This motion could trigger an eventual T-to-R (deoxy-to-oxy) structure transition. The Perutz group (Perutz et al. 1974; Fermi et al. 1984) proposed this trigger mechanism, in which the displacement of iron is the first event for the quaternary structure change.

However, our finding suggests that the changes of heme side-chains upon O_2 dissociation precede the quaternary structure transition. The shift of the $\nu_{Fe\text{-}His}$ band to 215 cm^{-1} after CO photodissociation (233 cm^{-1}) takes 20 μs (Yamada et al. 2013), but the changes of heme side-chains start immediately after CO photodissociation (1 ps), as shown in Fig. 9. A possible transient structure of heme in the β subunit of Hb A upon O_2 dissociation is illustrated in Fig. 10.

As shown in Fig. 10, O_2 dissociation from the β subunit in Hb A changes the orientation of the propionate side-chains from parallel to anti-parallel, and also changes the orientation of the 3-vinyl side-chain from the proximal side to the distal side, in addition to the shift of heme iron from in-plane to out-of-heme plane. However, just after O_2 dissociation, heme planarity is supposed to be unstable because the RR band at 305 cm^{-1}, an out-of-plane mode (γ_7), is different from that of the deoxyHb (at 300 cm^{-1}), and the RR band at 340 cm^{-1}, in-plane stretching mode of the iron and pyrrole nitrogens (ν_8), does not yet appear in the *transient state*, though it appears in the deoxyHb (Mizutani and Nagai 2012).

The great intensity enhancement of CD and the changes of low frequency RR spectra suggest that structural changes of heme moiety take place immediately after O_2 dissociation and that they might derive from the alteration of the orientation of heme-side chains. According to X-ray crystallographic analysis of Hb A, the alterations of the vinyl and propionate side-chains of the heme upon ligand dissociation occur only in the β subunit of the $\alpha_2\beta_2$ tetramer but do not occur in the isolated β chains (β_4 homotetramer). The presumed changes of the β subunit would be transmitted to the α subunit via the $\alpha_1\beta_2$ subunit interface and then the α subunit would start to change. Thus, the constraints for the T structure of Hb would be completed.

We have shown the CD spectra of isolated α and β subunits, which are different from each other, and their arithmetic mean is not the same as that of $\alpha_2\beta_2$ tetramer, especially the two-fold intensity increase in the deoxy form (Nagai et al. 1969). We demonstrated with Fig. 4 that ca. 70% of the induced Soret CD of Hb arises from the 13,17-propionates (Nagai et al. 2015). As shown in Figs. 5 and 6, the propionates of α subunit always take anti-parallel orientation, but those of β subunit take parallel and anti-parallel orientations in the oxy and deoxy forms, respectively (Park et al. 2006). On the other hand, TDDFT calculations (Nagai et al. 2015) is compatible with the assumption that the contributions from 17- and 13-propionates to Soret CD are +ε and −3ε when both are oriented along the proximal side of heme.

Since the orientation of 17-propionate is along the proximal side in the deoxy and oxy forms, its contribution should be +ε. On the other hand, since the orientation of 13-propionate is along the proximal or distal sides, its contribution should be −3ε or +3ε, respectively. Then, the parallel and ant-parallel orientations of 13- and 17-propionates are expected to yield −2ε (= (+ε) + (−3ε)) and +4ε (= (+ε) + (+3ε)), respectively. This is the case of the β subunit. Since the propionates in both the deoxy and oxy forms of the α subunit adopt the anti-parallel orientation, their contributions are +4ε. This is illustrated in Fig. 11, where the CD band is assumed to be Gaussian, with the band center at the wavelength of Fig. 2. When the peak wavelengths of α and β subunits are close, the apparent CD intensities of $\alpha_2\beta_2$ tetramer would be +8ε and +2ε for the deoxy and oxy forms, respectively. This explains the strong CD intensity for deoxyHb. If we assume 1.8×10^{-4} for ε, we obtain ellipticity (θ) values of 14.4×10^{-4} and 3.6×10^{-4} for deoxy and oxyHb A, respectively. These values are very close to the observed values of Fig. 3.

Time-resolved RR spectra of HbCO indicate that the orientation change of heme side-chains occurs at early times (ps) after CO photodissociation, but that it takes a long time (μs) to stabilize the heme structure (Yamada et al. 2013). Stabilization of the heme structure and the shift of $\nu_{Fe\text{-}His}$ to low frequency (215 cm^{-1}) take place in almost the same time regime (20 μs) (Yamada et al. 2013; Jones et al. 2014). Presumably, the changes of heme side-chains initiate the tertiary structure changes of protein, which is followed by the quaternary structure transition.

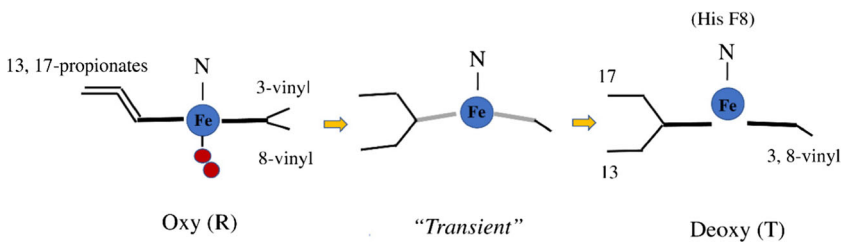

Fig. 10 A possible transient structure of heme in the β subunits of Hb A upon O_2 dissociation

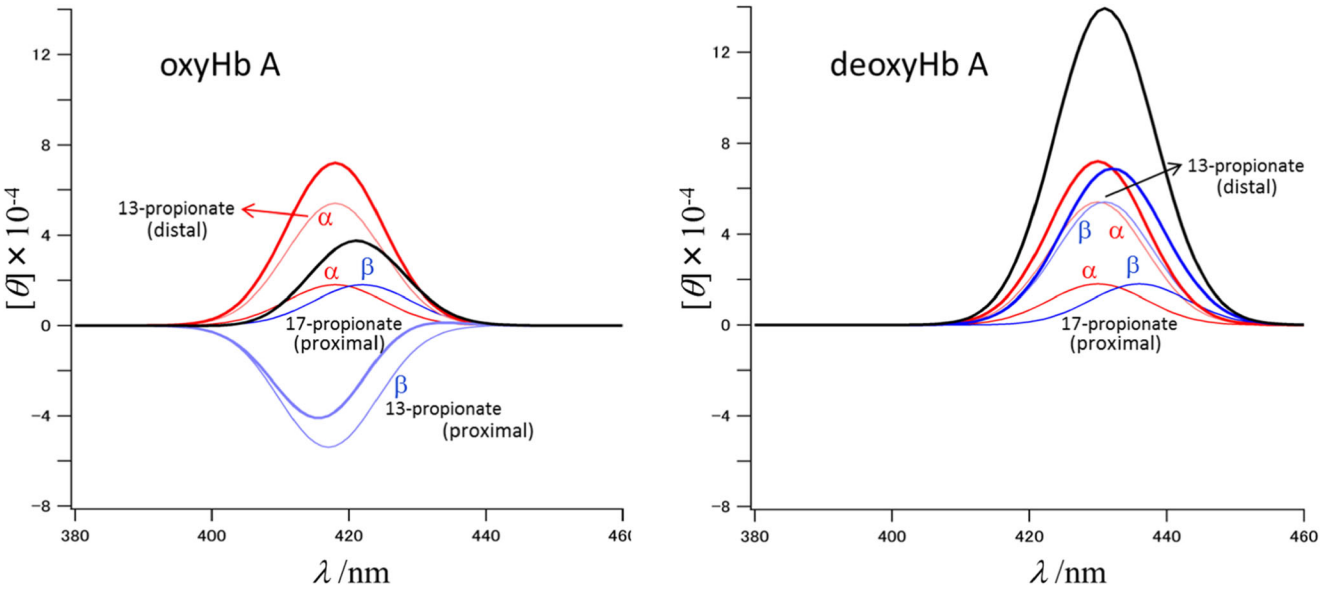

Fig. 11 Calculated circular dichroism spectra of oxyHb A (*left panel*) and deoxyHb A (*right panel*). For each band of propionate, Gauss functions are assumed. The thin lines are the spectra of 17-propionate and 13-propionate of the heme. The bold lines are the spectra of each subunit in $\alpha_2\beta_2$ tetramer. Red, α subunit; blue, β subunit; black, the sum of α and β subunits. Parameters used for the Gauss function, $\theta = A\exp(-(\lambda - \lambda_0)/$ *width*), are as follows: *width* = 10 nm; A = 1.8 for α subunit, 5.4 for β subunit (distal), and −5.4 for β subunit (proximal); λ_0 = 418 nm for α subunit (oxy), 430 nm for α subunit (deoxy), 417 nm for β subunit of 13-propionate (oxy), 422 nm for β subunit of 17-propionate (oxy), 431 nm for β subunit of 13-propionate (deoxy), and 436 nm for α subunit of 17-propionate (deoxy)

A possible route of signal transmission between α and β subunits upon O_2 dissociation is illustrated in Fig. 12. Changes of heme side-chains of the β subunit occur at 1 ps following CO photodissociation. The change of the $\nu_{Fe\text{-}His}$ band in the β subunit (2.9 μs) is much faster than that in the α subunit (20 μs) (Balakrishnan et al. 2004a; Jones et al. 2014). The H-bond formation at the hinge region (Trpβ37-Aspα94) (3 μs) is also faster than that of the switch region (Tyrα42-Aspβ99) (21 μs) (Jones et al. 2014). Accordingly, the first event in the sequence is orientation changes of the heme side-chains in the β subunit (1 ps) and the subsequent changes involve stabilization of the unstable heme (transient form) by tertiary structure changes, and then the shift of the $\nu_{Fe\text{-}His}$ band to lower frequency and an H-bond formation at the hinge region (3 μs) at the $\alpha_1\beta_2$ subunit interface take place. After these structure changes of the β subunit, the signal would be transmitted to the α subunit through the $\alpha_1\beta_2$ subunit interface. The changes of the α subunit begin with a shift of the $\nu_{Fe\text{-}His}$ band from 222 cm^{-1} (R) to 203 cm^{-1} (T). When the ligand dissociation occurs at the α subunit, the $\nu_{Fe\text{-}His}$ band must shift to a lower frequency and then forms an H-bond in the switch region, and the signal would be transmitted to the β subunit via the $\alpha_1\beta_2$ subunit interface.

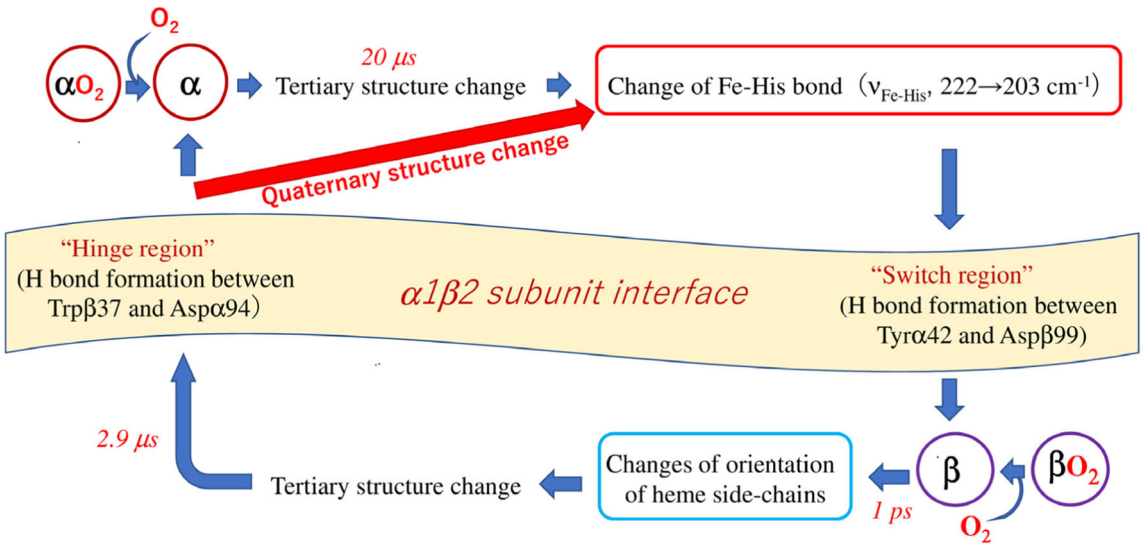

Fig. 12 A possible route of signal transmission between the α and β subunits of Hb A upon O_2 dissociation

Concluding remarks

Heme alone cannot exhibit a circular dichroism (CD) spectrum, but gives a distinct CD when it is incorporated into the globin. We have found experimentally that the deformation of propionate side-chains makes a considerable change in the Soret CD spectrum of hemoglobin (Hb), and it is supported by theoretical calculations. CD spectra of the isolated α and β chains are different from each other, and the CD spectrum of recombined $\alpha_2\beta_2$ tetramer (Hb A) is not the same as the arithmetic mean of α- and β-isolated chains, especially about the two-fold increased intensity in deoxyHb A. We could demonstrate theoretically that the enhanced positive CD in the deoxyHb A is due to the changed orientation of propionate in the β subunit. Changes of propionate side-chain were also detected by resonance Raman (RR) spectra. Time-resolved RR spectra obtained after the photodissociation of HbCO revealed that the deformation of propionates side-chains starts immediately after the ligand dissociation. Therefore, deformation of propionates would be associated with the cooperative O_2 binding of Hb as a trigger for initiating protein conformational transition prior to the movement of heme iron.

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Compliance with ethical standards

Conflict of interest Masako Nagai declares that she has no conflict of interest. Naoki Mizusawa declares that he has no conflict of interest. Teizo Kitagawa declares that he has no conflict of interest. Shigenori Nagatomo declares that he has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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