Studies on the structure and function of hemoglobin using mutant hemoglobins with one amino acid substitution

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学位授与の題目	Studies on the structure and function of hemoglobin using mutant hemoglobins with one amino acid substitution（1アミノ酸置換変異体を用いたヘモグロビンの構造と機能に関する研究）
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学 位 論 文 要 旨

In order to study the structure and function of human adult hemoglobin (Hb A), the isolated chains of Hb A and their recombined tetramer, the natural mutant Hbs, Hb Hirose (β37Trp→Ser) and Hb Rouen (α140Tyr→His), and the artificial mutant Hb (β15Trp→Leu), rHb (Wβ15L), produced from *E. coli.* were measured for their UV circular dichroism (CD) spectra; the natural mutant Hb, Hb M Iwate (α87His→Tyr) was examined for its UV and visible resonance Raman (RR) spectra.

1. UV CD studies on Hb A

Circular dichroism is a sensitive physical technique for determining structures and monitoring structural alterations of biomolecules. The near-UV CD spectra of Hb A not only reflect the contribution of aromatic side chains, but also the heme configuration (1, 2). Hb A shows a pronounced change of the CD spectra in the 280~300 nm aromatic region. However, the attribution of the change of CD spectrum of Hb A in this region to quaternary structure remains still unsolved. In Hb A, there are 3 Tyrs and 1 Trp in α

chain, and 3 Tyrs and 2 Trps in β subunit. Among them, α42Tyr, α140Tyr, β37Trp and β145Tyr are located at the α1β2 subunit interface and remain invariant throughout the evolution of α and β chains (3). One of the characteristics of these residues is easily formation of hydrogen bond with the other residues in protein, which is the main chemical force to maintain the stability of Hb molecule. For example, β15Trp or α14Trp (A12) that is outside of subunit interface locates at A helix and forms a hydrogen bond with β72Ser or α67Thr in E helix, which remains unchanged in both oxyHb and deoxyHb. The indole ring of β37Trp forms a hydrogen bond with the carboxylate of α94Asp in deoxyHb A, which is broken in oxyHb A and considered to stabilize the T-structure (4, 5). The hydrogen bond between α42Tyr and β99Asp also changes upon the R→T quaternary structure alteration. α140Tyr and β145Tyr are located at both the subunit interface and the carboxy terminus of the α or β subunit, not only form the intrasubunit hydrogen bond but also have many contacts with another subunit. These contacts change upon the quaternary structure transition (5). The environmental change of aromatic residues upon the quaternary structure transition explains that these aromatic residues play very important roles in the allostery of Hb A. On the basis of static and time-resolved RR spectroscopy of Hb A and of Hb Kempsey (α99Asp→Asn) (6), being responsive to forces generated by ligation and deligation in the heme, E and F helices are moved to cause the A and H helices to move through the H-bonds, thereby repositioning the N- and C-termini. The H-bonds between the helices are formed by aromatic residues with another amino acid residues, and the penultimate of both the α and β subunits are

tyrosine residues. From the present results of CD measurements of Hbs, oxygen binding in the α chain brings about local environmental alteration of both Tyr and Trp residues due to tertiary structure change and in the β chain induces distinctively that of Trp. The environmental change of aromatic residues in the tertiary structure alteration is the first step and certain to make further that in quaternary structure transition and then causes the allostery. In the quaternary structure alteration of Hb A, α1β2 (β1α2) subunit interface is considered to play a pivotal role (3, 4). In the CD study

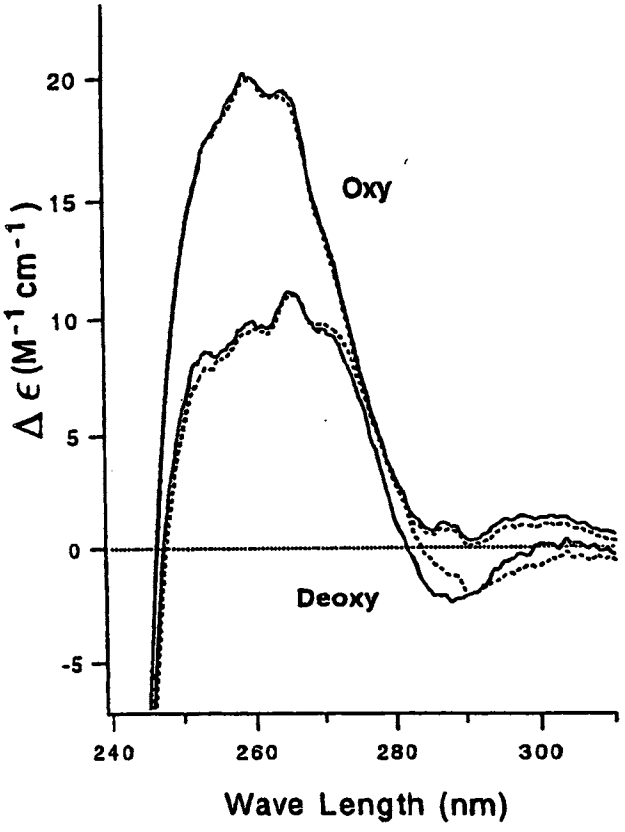

Figure 1. Comparison of CD spectra of the recombined Hb with the arithmetic mean of the spectra of α and β chains in the near-UV region. Recombined Hb was prepared by mixing α chain and β chain at equimolar concentration. The arithmetic mean was calculated from 1/2 (CD spectrum of α + CD spectrum of β). The dotted line refers to the arithmetic mean of α and β chains and the solid one to recombined Hb. The spectra shown here are the mean of two spectra (each spectrum was an average 20 scans). The Hb solution was 100 μM (in heme) in 0.1 M phosphate buffer, pH 7.0.

on change of the 287 nm CD band from a small positive signal to a negative trough that is the characteristic marker for the quaternary structure transition of Hb A from the R to T form, from the difference between CD spectrum of the arithmetic mean of deoxy isolated chains and that of deoxyHb tetramer (Figure 1), the contribution of tertiary structural change to the negative CD band at 287 nm in deoxyHb A was estimated to be 50%. This finding has revealed that the net contribution of quaternary structure transition to the negative band is 50%, and among which, the contributions of β37Trp and α140Tyr are assumed to be 56% from the CD studies of Hb Hirose (β37Trp→Ser) and Hb Rouen (α140Tyr→His) (Figure 2 and 3). This implies that at least one another aromatic residue at α1β2 subunit interface, α42Tyr or β145Tyr, is involved in the contribution to this negative 287 nm CD band. β15Trp located outside the subunit interface almost does not

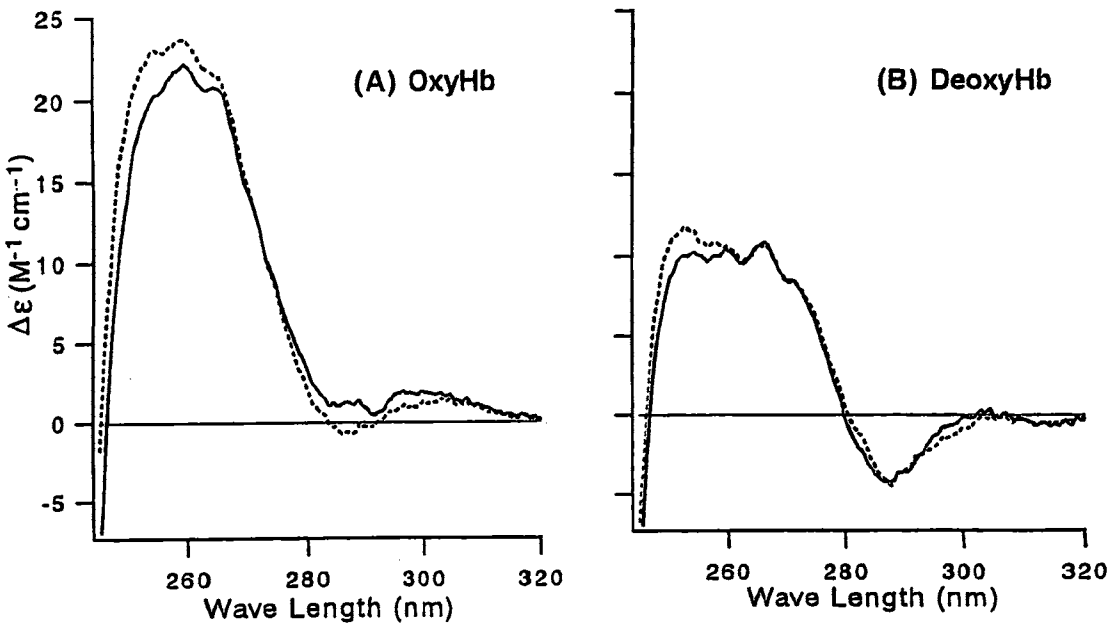

Figure 2. Spectra of Hb Hirose and Hb A in oxy (A) and deoxy form (B) at the near-UV region.

Dotted line: Hb Hirose. Solid line: Hb A. The spectra shown here are an average of three spectra. Hb solution was 100 μM (in heme) in 0.1 M phosphate buffer, pH 7, containing 1 mM IHP.

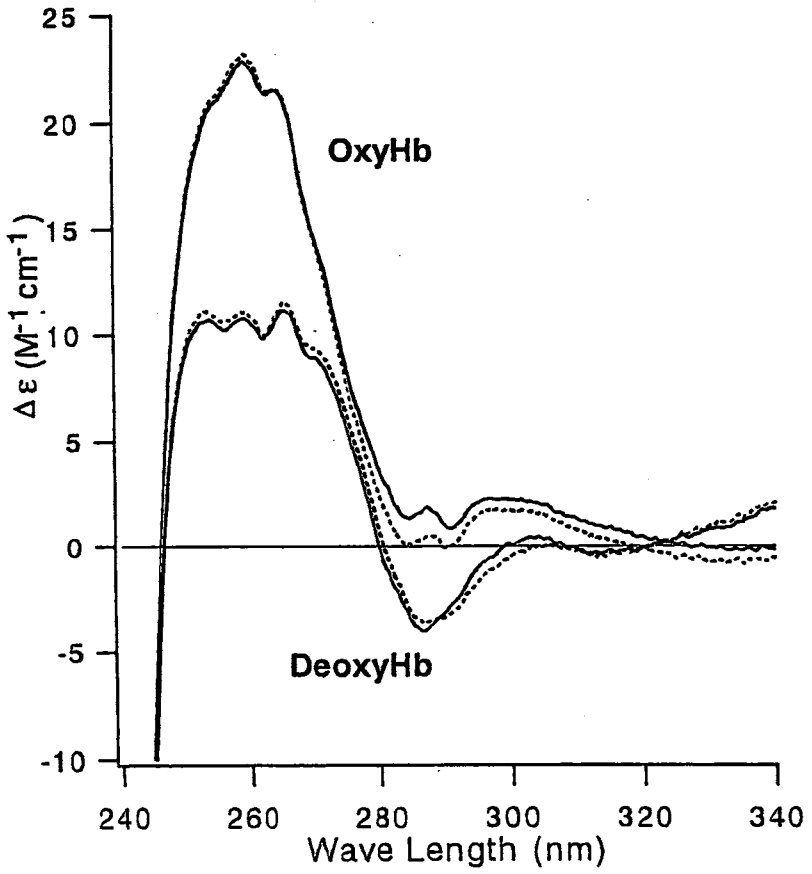

Figure 3. CD spectra of Hb Rouen and Hb A at the near-UV region. Dotted line: Hb Rouen. Solid line: Hb A. The spectra shown here are average of three spectra. Hb solution was 100 μM (in heme) in 0.1 M phosphate buffer, pH 7.

contribute to this CD change. As the composition of the subunit interface, aromatic residues play important role upon the quaternary structure transition. That is why those aromatic residues are invariant in the evolution. The relationship between allostery and aromatic residues in Hb A molecule can be summarized as following:

Ligation (deligation) → Change of heme state → Tertiary structure change (aromatic residues) → Quaternary structure change (α1β2 subunit interface)

↓

Allostery

The environmental changes of aromatic residues upon the quaternary structure transition of Hb A are also detected in the far-UV CD spectra as a negative band at 225 nm. The environmental changes of Trp residues at β37 and β15 are reflected at 230 nm CD band, and that of α140Tyr at 220 nm. The β15Trp might contribute to the changes of the CD band at 225 nm by 10%.

Owing to the replacement of A12Trp by nonpolar Leu in the rHb (Wβ15L), the hydrogen bond between A and E helices through β15Trp and β72Ser is lost. From the difference CD spectrum between deoxyHb A and the deoxy rHb (Wβ15L) at near 260 nm, it is shown that the state of the heme in rHb (Wβ15L) change slightly. This is possibly due to the slight movement of E helix owing to the loss of the hydrogen bond between A and E helices (6). These structural changes might not affect the subunit interface and Fe–His (F8) bond, so the cooperativity of rHb (Wβ15L) is preserved but the oxygen affinity increases.

2. RR studies of Hb M Iwate

Resonance Raman is a technique to observe the vibrational spectrum of a chromophore selectively by tuning the wavelength of Raman-exciting laser light to an absorption band of the molecule, hence is a sensitive physical technique for determining the heme structure and monitoring environmental change of aromatic residues in Hb A (8). The heme structure of Hb M Iwate in which the α87His (F8His) is replaced by Tyr was examined by UV and visible RR spectroscopies. Whether the heme iron binds to the proximal Tyr (F8Tyr) or the distal His (E7His) is the key for understanding the heme structure of Hb M Iwate. The comparison of UVRR or visible RR among Hb

A, Hb M Iwate and the model compounds, including the comparison with isolated chains of Hb, indicated that the heme Fe(III) of abnormal α chain in Hb M Iwate was 5-coordinated high spin and covalently bound to the F8-tyrosine that adopts a deprotonated form, which affected greatly the peripheral groups of porphyrin ring. This makes the normal β chain in Hb M Iwate have very low oxygen affinity and remain in the T-quaternary structure even in the fully oxidized form. When Hb M Iwate was fully reduced, the tyrosinate-Fe(III) bond in abnormal α chain was broken and the tyrosinate was changed to a tyrosine. The heme Fe(II)–distal His (E7His) bond was proved by the presence of $\nu_{Fe\text{-}His}$ RR band (Figure 4). The

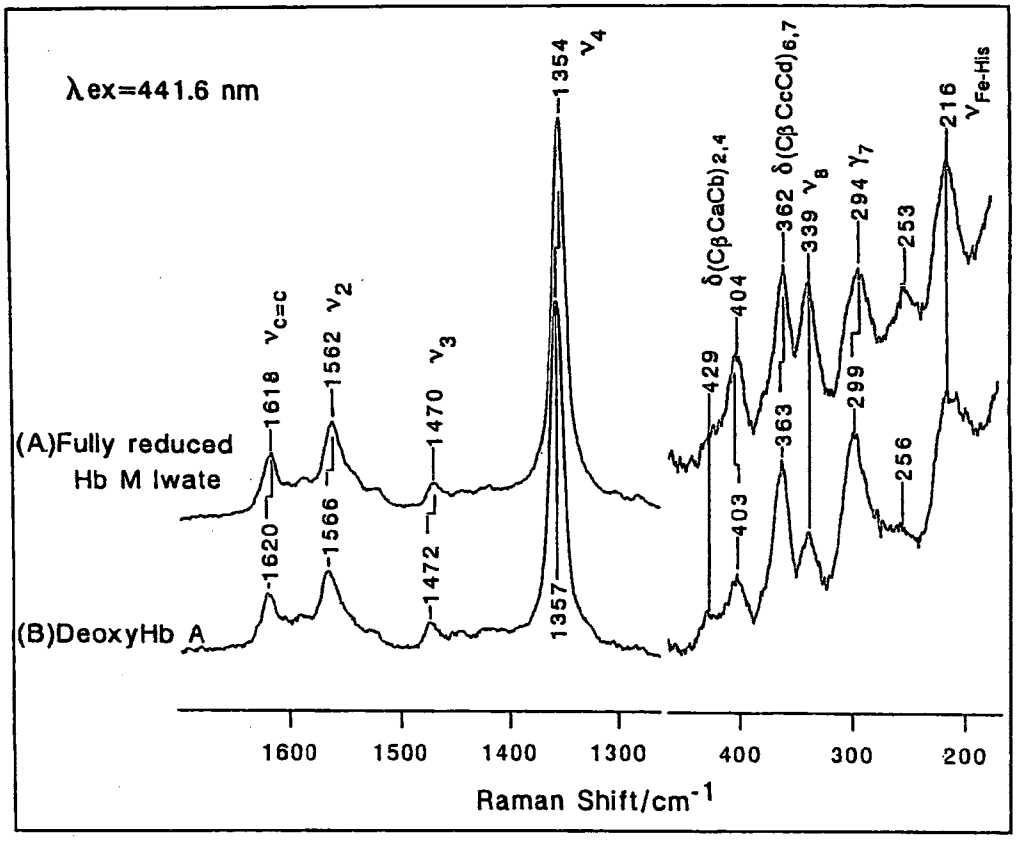

Figure 4. 441.6-nm excited resonance Raman spectra of the fully reduced Hb M Iwate (A) and deoxyHb A (B).

The concentration of Hbs was 100 μM (in heme) in 0.1 M phosphate buffer, pH 7.0, containing 2 mg of sodium dithionite/mL. Raman spectrum was measured under anaerobic conditions.

Fe(II)–E7His bonding caused some changes of the heme structure. The heme structures of abnormal α chain in Hb M Iwate in both met and reduced forms, especially in the relation between the Fe and the proximal Tyr or the distal His, were clarified through the present RR study.

3. Allosteric transition

On the basis of previous studies on hemoglobin and our results above, the oxygen affinity of Hb A is dependent on the heme condition, especially on the states of the heme iron, the proximal His (F8) and the distal His (E7). Any affecting to these states, directly or through the structure change in the globin (mutation), can change oxygen affinity. The ligation or deligation of the heme gives the strains on the Fe–His (F8) bond, which will be communicated to the subunit interface. These communications are a key of hemoglobin for the allostery. About the communication pathway from the F8His–Fe bond to the subunit interface, we suppose the following:

1. When oxygen binds to the heme of one subunit and the F8His moves, the tertiary structure change of the subunit must start. The changes of the residues in tertiary structure certainly induce the quaternary structural changes in the contact between the subunits. For example, as shown as in Figure 5, neutral α89His that is near to α87His (proximal) can be hydrogen bonded with ε-NH_3^+ of α139Lys. As the F8His moves, the possible movement of α139Lys in the communication may induce some movements of the next residue α140Tyr that is known to contact with β37Trp (3). The environmental changes of those aromatic residues located at the α1β2 subunit interface in tertiary and quaternary structure have been shown in the present UV CD studies.

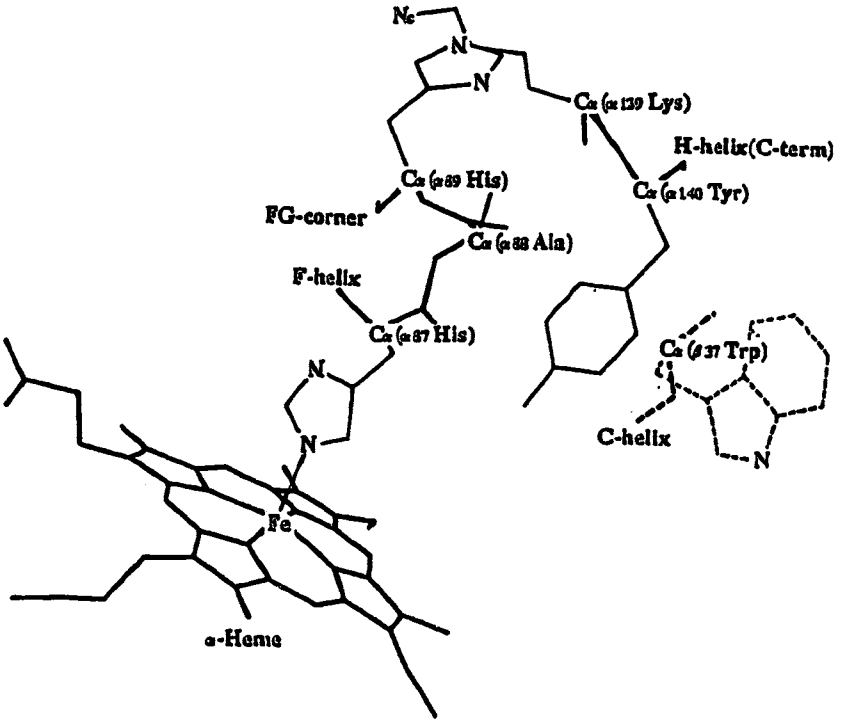

Figure 5. The structure of deoxyHb near α87–α89, α139–α140, and β37 residues [ref. (8)].

2. The strains generated by ligation on heme make the movements of E and F helices through E7His and F8His. The helices act as "lever" to cause A and H helices to move through the H–bonds between A and E, and between F and H helices. These movements of the "levers" reposition the chain termini and change the state of subunit interface. The movement of the helices in Hb A has been indicated in the study using static and time-resolved resonance Raman spectroscopy for hemoglobins (6). The increased oxygen affinity of the present rHb (Wβ15L) has shown that the H-bond between A12 (β15Trp) and E16 (β72Ser) is also involved to the communication pathway.

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学位論文審査結果の要旨

本研究は，ヒトヘモグロビン(Hb)の1アミノ酸置換変異体を用いて，Hbのアミノ酸置換による構造変化と機能との関連を追及したものである。変異体としては，個人に起こった天然型と大腸菌によるリコンビナントHb(rHb)の両方を用いた。天然型は患者血液から分離精製し，rHbについては大腸菌によるヒトHb生産システムを確立し，遺伝子組み換えにより変異体を作成した。蛋白質の構造変化は円二色性(CD)を用い，チロシン(Tyr)，トリプトファン(Trp)等の芳香族アミノ酸の変化として280～300nm域の近紫外CDで調べた。本研究においてdeoxy 型で特徴的な負のCD帯は，その1／2 は3次構造変化に由来し，残りの1／2が4次構造変化に因ることを初めて明らかにした。サブユニット界面に存在する芳香族アミノ酸の変異体、Hb Hirose(β37Trp →Ser)，Hb Rouen(α140Tyr→His)のCD実験から，4次構造変化の1／2はβ37Trpとα140Tyrによることを示した。しかし，サブユニット界面から離れ分子表面に位置するβ15Trpはその変化に全く寄与していないことを，rHb(β15Trp→Leu)を用いて示した。また，このHbの4次構造変化に寄与する芳香族アミノ酸の変化は，遠紫外域(200～250nm)でも認められることを，今回初めて明らかにした。ヘム鉄に直接結合している近位ヒスチジン(His)がTyrに置換した天然変異Hbがあり，そのα鎖変異体はHb M Iwate(α87His→Tyr)と呼ばれ，異常鎖ヘム鉄は酸化されている。このヘムの構造を紫外及び可視光励起共鳴ラマン分光法で調べ，置換Tyrはチロシネートの形でFe(Ⅲ)と結合していること，還元によりこのFe(Ⅲ)－チロシネート結合は消失し，Fe(II)は遠位Hisと結合することを初めて明らかにした。以上の結果は3つの論文にまとめられ全て国際誌に掲載された。したがって，本研究は博士(理学)に充分値すると判断し合格とした。