Form 3

Abstract of Thesis

Name （Shanyan Chang）	
Title	Time-resolved Resonance Raman Studies on Quaternary and Tertiary Dynamics of Human Adult Recombinant Hemoglobin (時間分解共鳴ラマン分光法を用いたHuman Adult Recombinant Hemoglobinの四次および三次構造ダイナミクスに関する研究)

Abstract of Thesis

1. Introduction

Human adult hemoglobin (HbA) is the oxygen-transport protein in red blood cells. HbA is a tetramer, composed of two α- and two β-subunits. Within each subunit, there is a heme group. The iron in the center of the heme plane is the binding site for oxygen and other ligands. HbA has two distinct quaternary structural states, the low-affinity (T or tense) state and the high-affinity (R or relaxed) state. The quaternary transition between R and T states is the structural basis of the cooperative oxygen (O_2) binding, which plays important physiological roles.

Time-resolved resonance Raman (RR) spectroscopy is a very useful technique to investigate protein dynamics. Visible excitation near the Soret absorption band of heme produces the selectively and greatly enhanced Raman signals of the heme vibrational modes. In this study, I utilized heme-resonant Raman spectroscopic technique to monitor the dynamics of HbA.

2. Experimental section

In my study, I used recombinant Hb (rHb) instead of purifying protein sample from blood donors. During transformation, a certain type of plasmid was taken up by *Escherichia coli* (*E. coli*). Then, cultivate *E. coli* and induce the production of rHb. The rHb was purified from the lysate using affinity and ion exchange chromatography. The purity of the protein sample was verified by absorption spectra and electrophoreses.

Nanosecond time-resolved RR measurements were performed to study the dynamics of rHb after photodissociation of CO. Here, CO rather than O_2 was used as the ligand, because the allosteric transition after CO photolysis is very similar as compared with O_2 photolysis, and because the quantum yield of CO photolysis is higher. Two nanosecond pulse lasers were used, a 436-nm probe laser and a 532-nm pump laser. The powers of the probe and pump pulses were 0.5 and 185 μJ/pulse, respectively.

3. The first study - Effect of the N-terminal residues on the quaternary dynamics of HbA

(1) Background

In order to study the structure-function relationships of HbA, an efficient expression system is needed. In many respect, *E. coli* expression system is the best choice. However, the endogenous methionine aminopeptidase (MAP) of *E. coli* cannot cleave the initiator methionine (Met) from both α- and β-subunits of rHb. The extensions of the N-termini interfere with some functional and structural properties of the whole protein.

Two strategies have been used to circumvent this problem. One strategy is to add a Met-AP gene into the rHb expression plasmid. The initiator Met of rHb may be cleaved by a mass of coexpressed Met-AP. A much simpler strategy is to replace the N-terminal Val codons by the initiator Met codons to yield the V1M mutant. Although the kinetic and thermodynamic properties of the V1M mutant closely approximate those of HbA, the structural dynamics along the R→T transition pathway of the V1M mutant has not been examined yet.

(2) Results and discussion

In this study, I measured heme-resonant Raman spectra of the V1M mutant and the normal rHb expressed in *E. coli*, then compared their spectra with those of HbA purified from blood. It is indicated that the data of the normal rHb were quite similar to those of HbA. In contrast, the dynamic behavior of the α(V1M)/β(V1M)

double mutant was different from that of HbA. Apparently, the main dynamic difference was in the time region of tens of microseconds.

The present data showed that the V1→M mutation accelerates the second step in the R→T quaternary transition. A possible molecular mechanism was proposed. α_1V1 is sandwiched between the H helices of the α_1 and α_2 subunits. These H helices shift away from each other upon the R→T transition. Since two H-helices are much closer in R state, the side-chain of αV1 suffers more steric hindrance in R state. Once the N-terminal residue is mutated from Val to Met, the side chain becomes larger, indicating that steric hindrance increases. Since there is more steric hindrance in R state, R state is more destabilized than T state. Therefore, R-T transition is accelerated.

(4) Conclusion

The spectral changes of the V1M mutant were distinct from those of HbA in the tens of microseconds region, whereas the spectral changes of normal rHb and HbA were similar. The present study demonstrated that structural changes in the N-termini are involved in the second step of the R→T quaternary transition of HbA. These findings help further characterize the protein dynamics regulating the allosteric pathway of Hb.

4. The second study - Time-resolved resonance Raman studies on tertiary dynamics in R and T quaternary structures of human adult hemoglobin

(1) Background

Tertiary structural change means structural change of a single protein subunit. To realize the quaternary transition, certain tertiary structural changes (tertiary dynamics) are also indispensable.

In this study, I selectively studied the tertiary dynamics of rHb by fixing the quaternary state of rHb. Mutation of certain amino acid at the $\alpha_1\beta_2$ interface may fix the quaternary state of rHb. The R and T mutants used in this study are two examples. The R mutant is fixed in R quaternary state, while T mutant is fixed in T quaternary state. Consequently, the tertiary dynamics in R or T quaternary structure can be selectively investigated.

(2) Results and dicussion

The dynamics of R mutant is compared with wild-type rHb. The results indicate that the tertiary structural changes under R quaternary structure complete within 50 μs.

The dynamics of T mutant is also compared with wild-type rHb. The results predict that tertiary dynamics in T quaternary state complete at several hundreds of microseconds.

Obviously, the tertiary changes in T quaternary state is largely decelerated as compared with the tertiary changes in R quaternary state.

(3) Discussion

Figure 1 compares the structures of R and T states. The constraints of E and F helices by the switch and hinge contacts are probably the reason why tertiary dynamics in T quaternary state is decelerated.

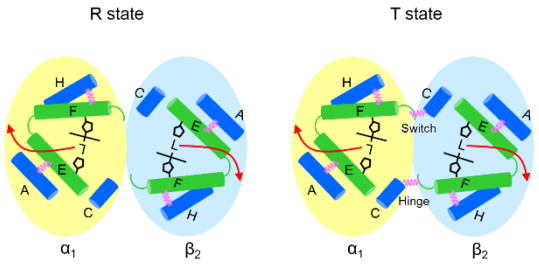

Figure 1. Comparison of R and T states.

(4) Conclusion

The results show that the tertiary dynamics in T quaternary state is much decelerated as compared with R quaternary state, which indicates that tertiary structural changes is not independent from quaternary structure. These findings help further characterize the coupling between tertiary and quaternary dynamics of Hb.

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論文審査の結果の要旨及び担当者

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論文審査の結果の要旨

タンパク質ダイナミクスの特徴は、幅広い時間スケール・空間スケールに変化の素過程が存在し、さらにそれらが互いに連動していることにある。このような性質はアロステリーとして知られ、タンパク質の機能発現の要である。しかし、連動性をもつことは分子として自明の性質ではない。分子内で、なぜ複数の素過程がうまく連動しているのかを明らかにすることは、分子と生命現象の理をつなぐ重要な課題である。

Shanyan Chang さんは、ヒトヘモグロビンの機能に重要な連動性を、時間分解紫外共鳴ラマン分光法を用いて明らかにした。本論文はその研究成果をまとめたものである。ヒトヘモグロビンは赤血球に含まれる酸素運搬体タンパク質で、αおよびβサブユニットが 2 つずつ会合した四量体構造をとっている。その酸素運搬に重要な性質は協同的酸素親和性であり、それは相互作用を介したサブユニット間の連動性によって生じる。

Chang さんは、N 末端残基の置換によって、四次構造の転移速度が 2.5 倍速くなることを見いだした。これは、N 末端残基が形成するサブユニット間相互作用が四次構造転移に影響を及ぼすことを示している。スペクトル変化に基づいた議論によって、Chang さんはその影響について明確な分子論的説明を与えた。サブユニット間相互作用における N 末端残基の重要性は、タンパク質構造化学研究のパイオニアである Max Perutz によっても指摘されていたが、それをリアルタイム観測したのはこの研究が初めてである。また、サブユニット界面に存在するアミノ酸残基を置換することによって四次構造転移を起こさないヘモグロビン変異体を作製し、この三次構造変化を詳しく調べた。その結果、三次構造変化のダイナミクスは四次構造に依存することを明らかにした。これはサブユニット内とサブユニット間の構造変化の連動性を示す重要な結果である。

本論文の研究成果は、タンパク質ダイナミクスの観測に基づき、連動的な構造変化の機構を明らかにしたものであり、タンパク質の物理化学研究として意義深い。よって、本論文は博士（理学）の学位論文として十分価値あるものと認める。