氏　　名	郭 浩 (GUO Hao)
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論 文 審 査 委 員	主　査　教授　加藤 晃一 准教授　古谷 祐詞 教授　岡本 裕巳 教授　秋山 修志 教授　神取 秀樹　名古屋工業大学

論文内容の要旨

Infrared (IR) spectroscopy is a powerful method for investigating the structures of proteins and other biomaterials. Major contributions to the molecular understanding of reaction mechanisms, particularly those that involve membrane proteins, have demonstrated the effectiveness of the method on the basis of difference spectra calculated from the spectra obtained before and after applying several stimuli, such as light, electric potential, and ions. In conventional IR spectroscopy, stacks of membrane protein layers are usually used to achieve an adequate signal from the sample. However, such stacks of layers do not represent an appropriate model of membrane proteins, which usually exist as a monolayer in the cellular membrane. Proteins in a monolayer and their functional changes are difficult to detect by a conventional Fourier-transform IR spectrometer because the amount of protein is on the order of a few picomole per square centimeter (10^{-12} mol/cm^2).

To overcome this difficulty, this thesis aims to apply surface-enhanced IR absorption spectroscopy (SEIRAS) to structural studies of light-receptive proteins, i.e., microbial rhodopsins, at single-monolayer level. The introduction chapter (Chapter 1) provides a summary of the current SEIRAS researches on biological systems. SEIRAS was firstly developed in the field of electrochemistry and has been recently used in biophysics. It is a phenomenon in which molecules adsorbed onto metal

island films or particles exhibit IR absorption 10–1000 times more intense than that expected from conventional measurements without using the metals. This technique has two advantages. First, the acute sensitivity of SEIRAS enables the detection of minute spectral changes of the adsorbed protein even at the monolayer level. Second, the enhancement is restricted to within a short distance from the surface, which results in the selective detection of signals only from the adsorbed monolayer. The attenuated total reflectance (ATR) geometry is preferred in my setup, with a gold-thin metal film overlayer atop the reflection surface of the ATR prism. The advantage of this optical geometry is that the sample surface is freely accessible during the IR measurements.

The preparation of a thin-metal film is the critical part of a successful SEIRA experiment. In Chapter 2, SEIRA-active thin-gold films were prepared by high-vacuum evaporation of gold onto a Si-ATR prism. The enhancement is strongly dependent on the size, shape, and particle density of the gold island film. Such surface properties can be partially tailored by adjusting the experimental conditions during film fabrication, specifically, the thickness and the rate of film deposition. It was confirmed by Raman spectroscopy and atomic force microscopy observations that a so-called island structure that consists of well -separated gold particles provides the best surface enhancement.

Chapter 2 also describes a methodology for the surface immobilization

and reconstitution of membrane proteins. Halorhodopsin from *Natronomonas pharaonis* (*p*HR), which has seven-transmembrane helices and binds an all-*trans* retinal, was chosen as a model protein for the initial studies. This method comprises various steps, including: (1) self-assembly of the active ester dithiobis(succinimidylpropionate) on the gold support, (2) formation of the nitrilotriacetic (NTA) layer by cross-linking of the active ester with aminonitrilotriacetate and (3) complexation with Ni^{2+}, (4) adsorption of the 6 × His-tagged *p*HR onto the Ni-NTA layer, and (5) embedding of *p*HR into lipid layers by incubated with lipid vesicles and microporous Bio-Beads. The observed vibrational bands related to the each surface reaction step can demonstrate the successful immobilization and reconstitution of the oriented monolayer of *p*HR onto the gold surface.

It was found that the shapes of the bands in the enhanced IR spectra can be dramatically changed with respect to their magnitude, symmetry, and even direction by the presence of the metal surface. Normal, bipolar, and inverted shapes of amide I and II bands of *p*HR became prominent with an increase in film thickness. Chapter 3 describes the nature of this impressive phenomenon. The amide I and II bands were determined as a function of the angle of incidence, the polarization of the incident beam, and the deposition rate of the gold films. The results illustrated that the optical properties of the gold film itself are essentially responsible for the

anomalies in the IR bands. Reflectivity measurements revealed that the band shapes are normal before the percolation threshold and that bipolar shapes occur when an anomalous absorption by the films is strong, whereas inverted shapes begin to appear in the spectra of the films in which surface scattering is predominant.

Chapter 4 describes the controllable oriented immobilization of *p*HR onto gold surfaces. Variation in the orientation was achieved by engineering the His-tag on the C- or N-terminus of *p*HR. After the C-terminal His-tagged *p*HR was bound, its cytoplasmic side faced the gold surface, whereas the N-terminal His-tagged *p*HR exhibited a contrary orientation, i.e., the extracellar side, where the β-sheet in an interhelical loop is located, faced on the gold surface. The attachment was then characterized by ATR-SEIRA spectroscopy. On the basis of the near-field effect of SEIRAS, key differences between these two opposite orientations were distinguished. Deconvolution and curve fitting of the SEIRA spectra suggested that more anti-parallel β-sheet structures were detected in the case of N-terminus binding. The difference in the absorption properties of these two orientations of the anti-parallel β-sheet structures was also explained by the theoretical background of the SEIRA spectra on the basis of the structural information of *p*HR obtained crystallographically. To corroborate the results, the binding experiments were repeated under the same conditions using β-sheet-deleted *p*HR

mutants, where the enhancement of the β-sheet bands was significantly decreased. Thus, the full control of the orientation of *p*HR was confirmed by SEIRAS. In addition, the surface coverage and molecular orientation of the immobilized *p*HR were also determined.

Chapter 5 contains details of investigations on the light-induced structural changes of sensory rhodopsin II from *Natronomonas pharaonis* (*p*SRII), which is a microbial rhodopsin and whose active state has a relatively longer lifetime than that of *p*HR. Functional studies of the C- or N-terminal His-tagged *p*SRII adsorbed onto gold surfaces were performed by comparison of the surface-enhanced IR difference absorption spectroscopy (SEIDAS) of the initial non-active state and the active state. Structural changes in the protein backbone and the retinal cofactor were resolved by SEIDAS at a single-monolayer level. SEIDA spectra obtained at different orientations and pH values were compared and discussed.

Finally, the general conclusions and outlook of the present thesis are provided in Chapter 6.

On the whole, the author has succeeded in constructing a SEIRA measurement system for investigating the functionality of a protein monolayer tethered to a gold substrate. The approach renders high-surface sensitivity by enhancing the signal of the adsorbed protein by a factor of 25. From the systematic preparation of the gold films, the

film thickness suitable for SEIRA measurements was examined. The orientation of the membrane protein was shown to be controlled by introduction of a His-tag at the N- or C-terminal position of a seven-transmembrane protein. Finally, the author observed the light-induced absorption changes of a *p*SRII monolayer with a satisfactory signal-to-noise ratio when the protein was adhered onto a modified gold surface. To probe membrane proteins, a concept was introduced for the oriented incorporation into solid supported lipid bilayers. A recombinant protein solubilized in detergent was immobilized on a chemically modified gold surface *via* the affinity of its His-tag toward a nickel-chelating NTA surface. The protein monolayer was reconstituted into the lipid environment by detergent removal. A change in the orientation of the protein with respect to the metal surface was achieved by inserting the His-tag on either side of the membrane protein surface. The presented methodology opens new avenues for the study of the molecular mechanisms of membrane proteins, such as ion channels, transporters, and sensors, at the atomic level.

博士論文の審査結果の要旨

膜タンパク質は、脂質二重膜に存在するタンパク質であり、膜を介した物質輸送や情報伝達など様々な役割を果たしている。郭氏は、膜タンパク質が機能する分子機構の解明を目的に、表面増強赤外分光法の条件検討を行い、膜タンパク質の一種である光受容タンパク質ロドプシン（光駆動イオンポンプであるハロロドプシンと光センサータンパク質であるセンサリーロドプシン）に適用した。表面増強赤外分光法（Surface-Enhanced Infrared Absorption Spectroscopy; SEIRAS）は、金や銀などの薄膜に吸着した分子の赤外吸収が10～1000倍程度に増強する現象を利用した赤外分光法である。また、ロドプシンは7本のαヘリックスが膜を貫通した膜タンパク質であり、光受容部位としてレチナールを結合している。本研究では、表面増強効果が薄膜表面に近いほど強く、表面近傍（数ナノメートル程度）に存在する分子を選択的に検出するという特性を生かし、ロドプシンのN末端およびC末端に固定用のタグを導入することで、細胞の内側と外側に向いた領域のタンパク質構造やその光誘起変化を選択的に検出することに挑戦している。

第一章には膜タンパク質の生理的な役割、赤外分光法、SEIRASの原理、SEIRASを膜タンパク質へ適用する意義、本研究の目的などが記述されている。第二章はSEIRASに適用する金薄膜の作製方法、特に真空蒸着による手法が記されており、原子間力顕微鏡や顕微ラマン計測による金薄膜の評価についても記述されている。また、金薄膜表面への膜タンパク質の固定方法、特にヒスチジンタグとNi-NTAを用いた手法について詳細に記述されている。

第三章はタンパク質骨格に由来するアミドIおよびアミドIIバンドの強度および形状に対する金薄膜の膜厚依存性の解析結果が記述されている。赤外光の入射角依存性、sおよびp偏光依存性などの結果から、バンド形状の変形や反転はs偏光によるものと結論づけている。また、膜タンパク質がリンカー分子を介して吸着されていることや、金薄膜との相互作用が弱い水の吸収バンドでも同様の現象が見られることから、金薄膜自体の光学特性によってもたらされる現象であると結論づけている。

第四章はハロロドプシンのC末端もしくはN末端にヒスチジンタグを導入し、SEIRASによってアミドIバンドを解析し、βシート構造がN末端試料で増大することが記されている。ハロロドプシンはβシート構造を細胞外側にのみ１つ持つため、細胞外側に存在するN末端側で金薄膜に吸着させた場合に良く増強することで定性的に説明されている。さらに、ハロロドプシンの結晶構造に基づいた表面吸着モデルから計算した増強度と近い値を示すことから定量的にも記されている。

第五章はセンサリーロドプシンのC末端もしくはN末端にヒスチジンタグを導入し、光刺激の有無によって生じる赤外吸収スペクトルの変化についてタグの位置の違いによる影響について議論されている。また、pHによる光反応への影響についても議論されている。

以上のように、本論文ではSEIRASの膜タンパク質への適用のために、出願者が主体的に、

金薄膜の作製条件、タンパク質の固定方法を検討し、二種類の膜タンパク質の主鎖の構造解析や光誘起構造変化の解析に成功している。膜タンパク質の配向を制御することでSEIRASの特徴を生かした実験結果も得られている。本論文は英語で記述されており、その一部は国際学術雑誌1報に報告されている。従って、本審査委員会は本論文が博士（理学）の授与に値すると全員一致で判断した。