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学位（専攻分野）　博士(理学)

学位記番号　総研大甲第487号

学位授与の日付　平成１２年９月２９日

学位授与の要件　数物科学研究科　機能分子科学専攻

学位規則第４条第１項該当

学位論文題目　Resonance Raman Studies of Cytochrome *c* Oxidase and Heme-Binding Protein23

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論文内容の要旨

[Part I]

A new measurement system for Ultraviolet Resonance Raman (UVRR) spectra was designed so as to achieve 1) high throughput, 2) low possibility of sample damage, 3) high sensitivity, and 4) no interference by visible fluorescence. To satisfy these requirements, the author adopted the following ideas; 1) the use of a single spectrograph for a high throughput, 2) the use of quasi CW excitation light based on a mode-locked laser to reduce a peak power, 3) the use of a spinning cell with a stirring function of the sample to protect denaturation cased by illumination of the UV light, and 4) the use of a solar blind multichannel UV detector for high efficiency in accumulation of Raman signals and to reject visible light emitted from the sample, if any.

As an excitation light, the author adopted a frequency doubled mode-locked Ar^+ laser (Spectra Physics, 2045 laser and 342A mode-locker). The mode-locking was operated at ~82 MHz and the average power at 488.0 nm was typically 1 W with the pulse width between 200 to 500 ps. The peak power of each pulse was over 100 W. This visible light was frequency-doubled with an AR-coated BBO (β-BaB_2O_4) crystal. The average and the peak powers at 244.0 nm were ~1 mW and ~100 mW, respectively. Raman scattering was collected by UV microscope lens, which was expected to reduced the incorporation the light arising from scattering at unnecessary part of the sample such as the bottom of the cell. For dispersion scattered light, the author adopted a SPEX 1269 single monochromator (f=1260 mm, F=9) with a 3600 grooves/mm holographic grating blazed at 250 nm. As a solar-blind multichannel detector, the author adopted an intensified charge-coupled device (Princeton Instruments, 1024MG-E/1, 1024 x 256 pixels) with CsTe as the photocathode, which has sensitivity only between 200 and 300 nm.

Regarding the capability of spectrometer it is noted that the new system has made it possible to observe a RR spectrum of rhodamin 6G solutions with sufficiently high S/N ratios, although the measurements of RR spectra of its solutions in the visible region has been practically impossible due to strong fluorescence. This is owed to the use of the solar-blind detector and partly due to the use of the 250 nm-blazed grating. This system is also applied to cytochrome *c* with a molecular weight of 12 kDa and its spectrum, which was same as report, was observed without Raman saturation effect. By the use of a liquid filter to reject the stray light due to Rayleigh scattering, the application of the new system has further been extended to a much larger enzyme, cytochrome *c* oxidase (CcO) with an apparent molecular weight Mr = 420 kDa. Whereas the amount of sample is limited, continuous illumination of the probe light to the sample for long time may denature the protein. Even when a spinning cell is used, the molecules on the cell surface, which are mainly illuminated by laser light, are little exchanged with molecules inside. To over come this problem, the author has added a stirring function to a spinning cell during spinning of the cell. The effective exchange of the sample in the cell protected from losing the enzyme activity by the illumination of UV light and enabled high quality UVRR spectra of CcO.

[Part II]

Cytochrome *c* oxidase (CcO, EC 1.9.3.1) is a terminal enzyme of respiratory chain, having redox-active two iron centers (heme *a* and heme a_3) and two copper centers (Cu_A and Cu_B), and catalyzes the four electron reduction of dioxygen to water at the heme a_3-Cu_B binuclear site. The O_2 reduction is coupled with proton translocation across the mitochondrial inner membrane against the concentration gradient of protons to generate electrochemical potential for synthesis of ATP from ADP. The ratio of the number of protons to that of electrons transferred were determined to be 4 to 4 in one catalytic cycle, but this does not mean that every electron transfer is accompanied by a proton transfer. The reaction mechanism of this enzyme has been studied extensively using time-resolved absorption, EPR, and resonance Raman spectroscopy. One of important problems to be solved is the determination of proton carriers and the structural changes of protein moiety caused by the redox and coordination changes at the metal sites. UVRR spectroscopy is also expected to serve as a powerful tool for exploring protein structural changes, particularly on aromatic amino acid residues like Tyr-244 which is covalently bound to His-240 at the *ortho* position of phenoxy ring and expected to serve as an acid/base catalysis for dioxygen reduction.

The new UVRR measurement system described in Part I, brought about successful observation of high quality UVRR spectra of CcO excited at 244 nm. The RR bands of tryptophan (Trp) - and tyrosine (Tyr) - residues dominated the observed spectra with an extra band appeared at 1656 cm^{-1}. The extra band was assigned to the *cis* C=C stretching mode of linoleoyl side chains of phospholipid by using model compounds of some phospholipids. The UVRR spectra of CcO excited at 244-nm was almost the same as that of the amino acid mixed solution in which Tyr and Trp are mixed in the same ratio as that in CcO, except to the presence of the 1656 cm^{-1}. Therefore, titration experiments of the linoleyol phospholipid to the amino acid mixed solution were carried out and Raman intensity of Trp/Tyr band at 1618 cm^{-1} was used as an internal intensity standard. On the basis of the 1656 cm^{-1} band intensity vs number of *cis* C=C double bands curve, the intensity ratio observed for CcO means that there are 42 *cis* C=C bands per CcO molecule, indicating the presence of 21 linoleyol side chains. This corresponds to 5 cardiolipin molecules, if all come from cardiolipins. The redox difference spectrum ($a^{3+}a_3{}^{3+}$ - $a^{2+}a_3{}^{2+}$) exhibits a small positive peak at 1656 cm^{-1}, indicating that the *cis* C=C stretching band of the fully reduced form is weaker than that of the fully oxidized form, while the peak position remains unaltered. This change in the intensity of *cis* C=C stretching mode of linoleoyl type presumably reflects a change in a torsion angle around the CH_2-CH_2 single-bond adjacent to the CH=CH double bond, since the presence of weak coupling between the vibrations of the polymethylene chains at both ends and central CH = CH-CH_2CH = CH group has been noted. On the other hands, the ligation difference spectrum ($a^{2+}a_3{}^{2+}$ - $a^{2+}a_3{}^{2+}$ · CO) did not give any peaks for the 1656 cm^{-1} mode as well as for the Tyr and Trp modes. The intensity change of the 1656 cm^{-1} band would suggest that some conformational change of the polymethylene chains of the phospholipid takes place in coupling with the redox change of the metal centers. It is reported that

some cardiolipin molecules cannot be removed from the enzyme preparation without loss of the enzyme activity. There is a suggestion that some cardiolipin in CcO may be functioning in the internal electron transfer between heme *a* and heme a_3-Cu_B site. If the cardiolipins have linoleoyl side chains, the conformational change in the acyl chains of phospholipids discovered in the present study could have a crucial role in controlling the internal electron transfer.

The pH difference spectra, pH 9.1 minus pH 6.8, yielded a positive peak at 1600 cm^{-1} and a negative peak at 1620 cm^{-1}. This difference peak demonstrated the decrease of tyrosine (1620 cm^{-1}) and the corresponding increase of tyrosine (1600 cm^{-1}) at alkaline pH, although the 1600 cm^{-1} band is not so intense as that of ordinary tyrosinate. The same pH difference spectra for the fully reduced form and fully reduced- and CO-bound form of CcO generated no such a differential peek, indicating deprotonation of a Tyr residue near heme a_3. It is likely that only Tyr-244 which was covalently bound to His-240 at *ortho* position of phenoxy ring and located at heme a_3-Cu_B site, was deprotonated at pH 9.1, because the pKa value of mode compound of Tyr-244 was deduced to be lower than those of other Tyr residues by 1.3 in the next part.

[Part III]

Recent crystallographic analysis of cytochrome *c* oxidase has revealed that Tyr-244 is covalently bound to the ε nitrogen of His240, which is one of the ligands of Cu_B, at ortho position. The covalently linked imidazole group, which is expected to work as a π electron-donor to a phenolic π orbital, would reduce the acid dissociation constant of the phenolic OH group than that of normal tyrosine, of which pKa at 10.5. And Tyr-244, which is located at the end of a possible proton channel of the surface (K-channel), is expected to serve as an acid/base catalysis for dioxygen reduction. Since the author detected deprotonation of a tyrosine residue(s) in the previous part, which is most likely to be Tyr-244, the author tried to characterize the physical properties of Tyr-244 by using a model compound, which is 2-imidazole-1-yl-4-methylpenol. A member of Prof. Y. Naruta's group (Kyushu Univ.) kindly synthesized the model compound.

To evaluate the modulation induced by the cross-link, the author examined UVRR spectrum of 2-imidazole-1-yl-4-methylpenol, its phenol-^{18}O and imidazole-d_3 isotopomer, which can be a model compound of Tyr244 of CcO. This model compound has a lower pKa than that the covalent linkage; 9.2 for phenolic OH group than pKa of 10.5 in 4-methylpenol. For Radical form, the character of 1587 and 1530 cm^{-1} bands are similar to 4-substituted phenoxyl radicals. The C=O double-bound character, which frequency is known sensitive for density of π electron on a phenol ring, is highest among all 4- and 4,2-substituted radicals. The lower pKa and the highest frequency of C=O double-bound of modes compounds represents imidazole group should work as a π electron-donor to a phenolic π orbital. The bands of imidazolic vibrations are intensified by the mixing of phenolic π orbital with imidazolic π orbital and its intensities depended on dihedral angle of the phenolic and imidazolic rigs in the 240-nm excitation. For anion and neutral forms, the bands of phenolic vibrations, which are insensitive to

different mixing of phenolic π orbitals with imidazolic π orbitals, are observed in addition imidazolic vibrations. The band of 1610 cm^{-1} in the spectrum of anion form is sharp and 4 times stronger than that neutral form, but the frequency is almost same. In case of 4-methylpenol, the band of 1610 cm^{-1} in the spectrum of anion form is 20 times stronger than that neutral form and the frequency is 14 cm^{-1} lower. The relative intensity of the 1610 cm^{-1} band between anion and neutral form of the model compound is closed to the difference spectra between those of fully oxidized CcO at pH 9.1 and 6.1, except for behavior of frequency. The bands of deprotonated of tyrosine revealed, reviled by the difference spectra between those of fully oxidized CcO at pH 9.1 and 6.1 should arise form Tyr244, based on the character of anion and neutral form of the model compounds.

[Part IV]

Heme-Binding Protein 23 (HBP23) is a protein with a molecular mass of 23 kDa, which is purified from rat liver cytosol using heme-affinity chromatography, and binds heme (ferriprotoporphyrin IX) with a high affinity (K_d = 55 nM). Although its heme affinity is higher than that of abundant cytosolic heme-binding proteins like liver fatty acid-binding protein and the glutathione S-transferases, the function of heme is not known. According to X-ray crystallographic studies and the analysis of mutation for the heme-free form, it forms a homo dimer through a disulfide bond between Cys-52 of one monomer and Cys-172 of the other, and these residues are associated with peroxidase activity. However, the heme-bound form has not been crystallized so far and a heme-binding site is not deduced from the structure of heme-free form. HBP23 binds heme in a 1:1 molar ratio of heme/protein. In order to know structures of heme bound form, the author examined visible resonance Raman and absorption spectra.

The Soret band of Fe^{3+}-heme bound HBP23 was observed at 410 nm, and its resonance Raman spectrum excited at 413 nm provided the ν_3 (spin state maker) band at 1510 cm^{-1}. However many preparations yielded two bands in the ν_3 region (1501 - 1490 cm^{-1}), and the intensity of the low frequency components was varied with every preparation. The Soret band of Fe^{2+}-heme bound form was observed at 425 nm, and its Raman spectrum excited at 413 nm gave ν_3 and ν_4 (oxidation state maker) bands at 1468 and 1355 cm^{-1}, respectively. These frequencies suggest that the heme adopts the five coordinate high-spin state. Nevertheless, no band appeared between 200 and 230 cm^{-1} where Fe-His stretching band is expected. The Fe-CO stretching mode of Fe^{2+} · CO-heme bound form was observed at 492 cm^{-1} upon excitation at 423 nm. This frequency is similar to that of the Fe-CO stretching mode of mutant myoglobin in which distal His is replaced by hydrophobic residues but is distinctly lower than that of native COMb. It indicates that the environments in the distal side of heme-pocket of distal side are non-polar.

論文の審査結果の要旨

本論文は４章から成る英語で書かれた論文で、第１章は紫外共鳴ラマン散乱測定装置の製作と性能評価、第２章はそれをチトクロムｃ酸化酵素に適用した結果、第３章はチロシン－244 のモデル化合物に関する紫外共鳴ラマン分光の測定結果、第４章はラット肝臓のヘム結合蛋白質 HBP23 のヘム結合形の可視共鳴ラマンスペクトルの測定結果で、第１章と第２章の一部が既にアメリカ化学会の論文誌にフルペーパーとして印刷されている。

240nm 付近の紫外レーザー光を用いて分子量の大きいタンパク質のラマン散乱を測定するために克服しなければならない装置上の基本条件をまず説明し、現に研究室にある Ar レーザーを用いながらその基本条件を満足させるために工夫していった実際的な問題が第１章で詳しく説明されている。レーザーにモードロックをかけて倍波を出やすくし、繰り返しの高い紫外レーザー光源を現実的な手法で得た事は、４年前の研究スタート時点では新しい考えであり、評価されるべきであろう。現在ではイントラキャビティレーザーとして CW 発振の紫外レーザー光源が市販されるようになったが、相当高価である。またホログラフィックフィルターのつかえない波長領域であるので、液体のシャープカットフィルターの使用を考え、そのカット波長がレーザーの波長に合うように溶液条件を調節している点は、本研究のオリジナリティとしてカウントされる。集光レンズに紫外顕微鏡用対物レンズを用いた事、200～300nm しか感受性をもたないインテンシファイアーをつけた CCD 検出器を用いた事など、装置全体としてのユニークさ、新しさは随所に見られる。また試料セルに関して、申請者独特の発想による仕掛けがあり、成功につながっている。それは高速回転した時に内部で溶液が撹拌されるようにしている点である。このようにして組立てた装置の性能を、既知のタンパク質や、可視の蛍光が強くてラマンスペクトルが観測されたことのないローダミン 6G に適用すると共に、回転セルに撹拌子をいれた効果についても調べ、装置としては完成した事を示した。

申請者の主論文としてはこの装置をチトクロム酸化酵素に用いたが、これをヘムタンパクの一酸化窒素付加体やヘモグロビンの４次構造変化に使った結果も共著の副論文として国際誌に発表されている旨記載があった。分子量 20 万の膜タンパク質であるウシのチトクロム酸化酵素に適用した結果が第２章である。ポイントの１つは、pH9.1 で脱プロトン型チロシンのバンドが観測され、それをチロシン－244 に帰属した事であり、もう１つは高度に精製され可溶化したタンパクであるにもかかわらず、脂質のラマン線が観測され、それがリノール酸型の脂肪酸である事をモデルを用いて明らかにした。また酵素１分子当り脂肪酸が 21 本結合している事を定量した。この情報はチトクロム酸化酵素に関しては新しい情報であり、本装置を用いて初めて得られた事は評価に値する。第３章はチロシン 244 がヒスチヂンと共有結合した特殊なチロシンなので、そのモデル化合物として o－イミダゾール化クレゾールを合成し、紫外共鳴ラマンスペクトルでそのアニオン形とラジカル形を観測した研究である。イミダゾールの結合によりフェノールの pKa の下る事を示すと共に、フェノールの $\pi\pi^*$ 励起に共鳴してイミダゾールのラマン線も共鳴効果を受けること示した。第４章は独立な部分であるが、１～３章だけで学位論文として十分内容のあるものであり、合格と判断した。

口述試験は、申請者が研究成果を１時間あまりで説明し、その後２時間質疑応答する形

式で実施した。申請者は第１章～第３章部分を説明した。装置の各部分の設計指針や性能についての質問には的確に答えた。チトクロム酸化酵素に関して、ここに述べられた結果が新しい情報で重要なものであることに異論はないが、『プロトンポンプの機構解明』と申請者がうたっている事に関して、内容からプロトンポンプの機構解明という題名の適切さに質問が出され、申請者が論文を一部修正する事に同意した。しかし発表した１～３章の内容に関してはオリジナリティの高いもので、それに関する周辺知識も十分もっていると判断した。また論文は英語で書かれており、英語力についても十分な水準であると判断した。

公開審査に於いても質問に十分答えることができた。従って試験には合格ということで全委員の意見が一致した。