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In biological systems, many proteins possess prosthetic groups which are indispensable for their functions. Myoglobin (Mb) and hemoglobin (Hb) are oxygen-storage and -carrier proteins, respectively, whereas terminal oxidases are oxygen reducing proteins. All of these proteins contain a heme as a prosthetic group where oxygen molecules bind, even though they play different roles in biological systems. There are some similarities and differences in the physicochemical properties of these heme proteins which are very important for understanding the functions of these proteins.

Spectroscopic techniques are very useful to obtain kinetic as well as steady state information of biomolecules, including heme proteins. Especially, resonance Raman (RR) spectroscopy provides information on bond characters of the heme group if assignments have been established. In this thesis, RR technique is adopted to examine these oxygen binding heme proteins, namely, Mb, Hb, and terminal oxidases. One of the most useful information for ligand-bound heme proteins obtained by RR spectroscopy is the ligand bond characters. The force constant of the ligand bending mode reflects the energy required to bend the Fe-ligand unit in the protein. Therefore, the assignment of the ligand bending mode is very important to know the ligand character in ligand-bound heme proteins.

This thesis consists of two parts, Parts I and II. Parts I treats reassignment of the Fe-ligand stretching and bending vibrational modes of various ligand-bound heme proteins. In chapter I-1, the assignments of the ligand related vibrational modes, especially those obtained by RR spectroscopy, are reviewed.

Chapter I-2 describes the first detection of δ_{FeOO} at 425 and 435 cm^{-1} for HbO_2 and $CcO{\cdot}O_2$ respectively. The δ_{FeOO} frequencies for HbO_2 and $CcO{\cdot}O_2$ were very similar, suggesting that HbO_2 and $CcO{\cdot}O_2$ have similar Fe-O-O geometries for their FeO_2 units even though they differ in functions. The ν_{Fe-O_2} bandwidth of $CcO{\cdot}O_2$ was narrower than those of HbO_2 and MbO_2. This indicates that the Fe-O-O geometry is more fixed in $CcO{\cdot}O_2$ which could have relation with its oxygen reactivity, although these three O_2-bound heme proteins seem to have similar Fe-O-O geometries. O_2-and NO-bound heme proteins have very similar ligand-binding geometries, and thus ν_{Fe-O_2} and δ_{FeOO} frequencies of O_2-bound heme proteins have frequencies similar to ν_{Fe-NO} and δ_{FeNO} frequencies of NO-bound heme proteins.

Chapters I-3, I-4, and I-5 discuss the reassignment of the δ_{FeCO} RR band. The CO-isotope-sensitive band around 575 cm^{-1} has been assigned heretofore to δ_{FeCO} for CO-bound heme proteins, but the frequency is higher

than the ν_{Fe-CO} frequency. Chapter I-3 describes the detection of a new CO-isotope-sensitive band around 365 cm^{-1} for various CO-bound heme proteins. This CO-isotope-sensitive band at 365 cm^{-1} was undetectable for MbCO, while it was detected for all other CO-bound heme proteins examined, including HbCO, its isolated chains, CcO·CO, and P-450·CO. In Chapter I-4, the ^{54}Fe and ^{15}N isotope shifts of this new CO-isotope-sensitive band, the ν_{Fe-CO} band, and the 575 cm^{-1} band for CO-bound cytochrome *bo* from *Escherichia coli* (*E. coli*) are discussed. The ^{54}Fe-isotope shifts of the 575 cm^{-1} and ν_{Fe-CO} bands were 1.5 and 3.5 cm^{-1}, respectively. These isotope shifts were unable to be reproduced by normal coordinate calculation of the isolated FeCO unit if the 575 cm^{-1} band was assigned to δ_{FeCO}, but were well reproduced when the new CO-isotope-sensitive band around 365 cm^{-1} was assigned to δ_{FeCO}. The force constants for ν_{Fe-O_2} and ν_{Fe-CO} were very similar, while that of δ_{FeOO} was larger than that of δ_{FeCO}. The non-equilibrium geometry of the Fe-C-O unit in CO-bound heme proteins would have a reduced bond-strength and a flatter potential curve for the bending mode. This would lower the δ_{FeCO} force constant than that in the equilibrium geometry. The detection of nonfundamental $Fe-O_2$ and Fe-CO vibrations are discussed in chapter I-5. The overtone mode of the 365 cm^{-1} band and a combination mode of this band with ν_{Fe-CO} were detected for HbCO and MbCO, but the overtone mode of the 575 cm^{-1} band was undetectable. These results support the assignment of the new CO-isotope sensitive band around 365 cm^{-1} to δ_{FeCO}. The δ_{FeOO} band was also undetectable for MbO_2, although they were detected for HbO_2 and $CcO·O_2$. There must be some structural origins that make the ligand bending mode undetectable in the heme pocket of Mb, although they are not known at the present stage.

Chapter I-6 discusses the ν_{Fe-CN^-} and δ_{FeCN^-} frequencies of several CN^--bound heme proteins systematically. The CN^--isotope-sensitive band around 452 cm^{-1} is assigned to ν_{Fe-CN^-}, and the difference peaks present in a range from 340 to 440 cm^{-1} of the CN^--isotope difference spectra are attributed to δ_{FeCN^-} coupled with porphyrin modes for the CN^--bound heme proteins examined. As the ligand-binding geometries of CN^- and CO-bound heme proteins are very similar and their electronic characters are also alike, their ligand vibrational frequencies should have similarities. As δ_{FeCN^-} appeared around 340-440 cm^{-1}, it is more reasonable to assign the CO-isotope-sensitive band around 365 cm^{-1} to δ_{FeOO} rather than to assign the 575 cm^{-1} band to it.

Chapter I-7 discusses the observation of the ν_{Fe-OH^-} bands of the low-spin species for hydroxy-Mb and -Hb at 549 and 552 cm^{-1}, respectively. The ν_{Fe-OH^-} frequencies of the low-spin species are reported to be higher by 60 cm^{-1} than those of the high-spin species. This character was similar to that obtained for a hydroxy model compound. $Fe(TMPPyP)(OH)_{2(aq)}^{3+}$ (Fe(TMPPyP);

[tetrakis-5,10,15,20-(2-N-methyl-pyridyl)porpyriato]iron(III), to have the ν_{Fe-OH^-} frequency of the low-spin species higher by 50 cm^{-1} than that of the high-spin species.

Part II treats some similarities and differences in the physicochemical properties of terminal oxidases. One of the major differences of terminal oxidases from Hb and Mb is that they have a heme-copper binuclear center at the oxygen binding site. Another special character is that the oxidases involve the intramolecular heme to heme electron transfer during the oxygen reduction. Chapter II-1 gives a review of the terminal oxidases.

Chapters II-2, II-3, and II-4 each treat RR spectra of a different kind of terminal oxidases. Chapter II-2 discusses the observation of ν_{CO} for CO-bound bovine *aa*$_3$-type cytochrome *c* oxidase (CcO·CO) by RR spectroscopy, and the measurement of the CO-recombination of CO-photodissociated CcO·CO by time-resolved RR spectroscopy. The bandwidths of the ν_{Fe-CO} and ν_{CO} RR bands of CcO·CO were narrower than those of CO-bound myoglobin (MbCO). This character was the same as the ν_{Fe-CO} and ν_{CO} bands of CO-bound *E. coli* cytochrome *bo*-type ubiquinol oxidase, having very narrow bandwidths. This suggests that CO takes a more fixed CO conformation in the heme pocket for CO-bound terminal oxidases than for MbCO. The CO-recombination rate was well fitted with a single exponential curve, and the lifetime of the photodissociated species was 30 ms. This lifetime was very long compared with those of MbCO and HbCO which were in the order of ms. No new ν_{Fe-CO} RR band was observed during the CO-recombination. This suggests that CO relaxes to its equilibrium form as soon as CO binds to the heme, although the CO binding rate is slower than those of MbCO and HbCO by an order.

Chapter II-3 describes the observation of the reaction intermediates in dioxygen reduction by the *E. coli* cytochrome *bo*-type ubiquinol oxidase detected by time-resolved RR spectroscopy using the artificial cardiovascular system, and compares the results with those obtained with bovine *aa*$_3$-type cytochrome *c* oxidase. At 0~20 μs following photolysis of the enzyme-CO adduct in the presence of O_2, the Fe-O_2 stretching Raman band was observed at 568 cm^{-1} which was shifted to 535 cm^{-1} with $^{18}O_2$. These frequencies were remarkably close to those of other oxyhemoproteins, including O_2-bound hemoglobin and *aa*$_3$-type cytochrome *c* oxidase. In the later time range (20~40 μs), other O_2-isotope-sensitive Raman bands were observed at 788 and 361 cm^{-1}. The 781 cm^{-1} band was assigned to the Fe^{IV}=O stretching mode, since it exhibited a downshift by 37 cm^{-1} upon $^{18}O_2$ substitution, but its appearance was much earlier than the corresponding intermediate of bovine cytochrome *c* oxidase (>100 μs). The 361 cm^{-1} band showed the $^{16}O/^{18}O$ isotopic frequency shift of 14 cm^{-1} similar to the case of bovine *aa*$_3$-type cytochrome *c* oxidase reaction. The detection of the

intermediates for *E. coli* cytochrome *bo*-type ubiquinol oxidase has significance since it enables us to apply the time-resolved investigation of the reaction to enzymes obtained by site-directed mutagenesis. This will be a future subject.

Chapter Ⅱ-4 describes the RR spectra of the ^{54}Fe- and ^{56}Fe-labeled *E. coli* cytochrome *bd*-type ubiquinol oxidase at the reduced and oxidized states. For the reduced enzyme, the 227 and 250 cm^{-1} bands detected in the 441.6 nm excitation and the 397 cm^{-1} bands detected in the 427.0 nm excitation were Fe-isotope-sensitive. For the 406.7 nm excitation of the oxidized enzyme, bands at 391 and 349 cm^{-1} were Fe-isotope-sensitive. The band at 227 and 349 cm^{-1} are assignable to the Fe^{2+}-His and Fe^{3+}-S^{-}(Cys) stretching vibrations, respectively. Accordingly, these results suggest that a histidine is the axial ligand of heme *d* similar to that of heme a_3 of CcO, and a cystein is the axial ligand of one of the heme *b*, and the heme iron of each cytochrome adopts a five coordinated structure.

審査結果の要旨

本論文はヘムタンパク質の共鳴ラマン分光に関する研究結果を記述したもので、Part Ⅰ と Part Ⅱの２部に分かれている。Part Ⅰではヘムタンパク質の鉄イオンに２原子分子（ＸＹ）が結合したときのＦｅＸＹ部分の振動の帰属を問題にしており、ヘムタンパク質としてはヘモグロビン（Hb）、ミオグロビン（Mb）、チトクロム酸化酵素（CoO）、チトクロムP-450（P-450）、チトクロムｂｏ（Cyt bo）、ＸＹ分子としてはO_2、ＣＯ、CN^-、OH^-をとり上げている。第１章はこの分野のこれまでの研究の背景の記述で文献をよく読み、本研究の位置づけや意義を十分理解していると判断できた。第２章はＦｅ－Ｏ－Ｏ変角振動によるラマンバンドをはじめて観測したという研究で、$^{16}O_2$、$^{18}O_2$、$^{16}O^{18}O$による同位体シフトの観測により帰属を明確にしている。第３章では Ｆｅ－Ｃ－Ｏ変角振動のラマンバンドの新しい帰属を提案している。すなわち本研究により観測された新しい実験データに基づいてこれまで国際的に信じられている帰属をくつがえす新しい考えを提案し、基準振動計算により観測した^{13}C及び^{18}O同位体シフトを合理的に説明した。またこの帰属を用いると結合音や倍音の説明が合理的にできるが、これまでの帰属ではそれが困難なことを第５章で説明している。これらの結果はすでに３報の論文として国際誌に印刷されている。第６章ではＦｅ－Ｃ－Ｎの伸縮及び変角振動、第７章Ｆｅ－ＯＨの伸縮振動をラマン分光で観測し、帰属していて、いづれも同位体シフトを観測して帰属を明確なものとした。これらの研究はヘムタンパク質の分光学的意義の高い基礎研究として評価された。Part Ⅱは末端酸化酵素の構造と機能の相関をラマン分光で調べる研究で、第１章では末端酸化酵素の説明とその研究の背景が説明されている。第２章ではウシのチトクロム酸化酵素のＣＯ光解離系を共鳴ラマン分光で調べ、ヘムポケットの構造的特色に言及している。第３章は大腸菌のチトクロムｂｏの反応中間体を検出したというもの、第４章は大腸菌のチトクロムｂｄの共鳴ラマンスペクトルを調べたものである。各章が一つの論文に対応するので、印刷された論文がすでに６報、投稿準備中のものが３報あり、学位論文として量は十分すぎるほどある。その内、第一著者のものが４報あり、いづれも一流の国際誌に印刷されているので内容も国際レベルのものと判断される。論文は分かりやすい英語で書かれており、各パートの第１章に書かれた背景を読むと、本研究の周辺に関する知識も十分勉強していると判断された。従って本論文は理学博士の学位論文として十分であると判断された。

また、口述試験における研究発表は博士論文の一部についてのみ行われたが、たいへんよく準備されており、その内容は充分よく理解できるものであった。また、学問的内容も高度であった。質問に対する答えも、ほぼ的確であり、満足できるものであった。博士論文はたいへん素直な英文で書かれており、英語の能力は高いものと判定された。さらに、発表・討論の過程において、物理化学の基礎的学力もたいへん高いものと判定できた。

公開発表会の発表はよく準備されており、内容も充実したものであった。質問に対する返答もきわめて的確であった。

これらを総合して、試験は合格であると判定された。