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学 位 論 文 題 目 FTIR Studies of Bovine Cytochrome *c* Oxidase;  
Proton Pumping Mechanism and Reduction by CO

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## 論文内容の要旨

This thesis describes the infrared (IR) study of cytochrome *c* oxidase (CcO) regarding its proton pumping function and reduction by CO. Chapter 1 gives a background of CcO, and the aims of this study. Chapter 2 describes the main results of this thesis on the proton pumping mechanism of CcO. Chapter 3 describes the elucidation of a reduction mechanism of CcO with CO by examining isotope distribution of product CO<sub>2</sub>.

CcO is the terminal enzyme in the respiratory chain and it has four active metal centers: Cu<sub>A</sub>, heme *a*, heme *a*<sub>3</sub> and Cu<sub>B</sub> in the order of electron flow. CcO receives electrons from cytochrome *c* and transports them via the active metal centers to O<sub>2</sub> which is bound to heme *a*<sub>3</sub>. CcO catalyzes to reduce O<sub>2</sub> to H<sub>2</sub>O and at the same time pumps a proton in coupling with the O<sub>2</sub> reduction. A carboxylic side chain is a most likely candidate for a proton carrier in the proton translocation activity. Accordingly, this study focuses on IR spectra of COOH groups. Certainly, the results from X-ray analysis or mutation studies show that Glu and Asp play an important role in proton pumping. As the O<sub>2</sub> catalytic reaction starts from O<sub>2</sub> binding to Fe<sub>a<sub>3</sub></sub> and finishes at leaving an OH group from Fe<sub>a<sub>3</sub></sub>, ligation to Fe<sub>a<sub>3</sub></sub> seems to be an important factor associated with proton pump. CcO can be partially reduced to mixed valence state (MV) by CO and CO adducts of MV state (MV-CO) and CO<sub>2</sub> are generated during reduction of oxidized CcO with CO. This reaction is a unique reaction for binuclear site of CcO but does not occur to general mononuclear heme proteins. MV is a kind of intermediate in the catalytic cycle.

IR spectroscopy is a useful method to identify molecular species and their structures. Especially, since the C=O stretching mode of protonated carboxylate is not overlapped with other vibrational modes, IR spectroscopy is suitable for the measurement of the structural change of carboxylate. The CO stretching bands of Fe<sub>a<sub>3</sub></sub>C-O and CO<sub>2</sub> in solution also are easily observed without overlapping with other bands. Therefore, this technique is suitable to investigate CcO about the mechanism of proton pumping and reduction by CO. These are described in Chapter 1 as the general introduction of the thesis.

The structural changes of carboxylate upon redox and ligation changes are described in Chapter 2. The difference spectra between fully reduced state (FR) and oxidized one and between FR-CO and MV-CO were measured. The redox of heme *a* and Cu<sub>A</sub> moiety mainly affected two carboxyl groups. Two bands were observed at 1749 and 1737 cm<sup>-1</sup> for H<sub>2</sub>O solution only in the oxidized state of heme *a* and Cu<sub>A</sub>. In D<sub>2</sub>O solution, a single peak was present around 1742 cm<sup>-1</sup> only for the oxidized form of heme *a* and Cu<sub>A</sub>. From the results of X-ray analysis and mutants of bacterial CcOs, these bands were assigned to Glu242 and Asp51, respectively. The difference spectra between CO photodissociated and CO bound forms of FR-CO and MV-CO were measured in a photo-steady state by CW laser illumination at 590 nm. In the light-minus-dark difference spectra of FR-CO, Fe<sub>a<sub>3</sub></sub>C-O and Cu<sub>B</sub>C-O stretching were observed at 1963 (-) and 2063 (+) cm<sup>-1</sup>, respectively, while the carboxylate band was observed at 1749 (+) and 1741 (-) cm<sup>-1</sup> as a differential pattern. These peaks were shifted to lower wavenumbers (1743/1735 cm<sup>-1</sup>) in D<sub>2</sub>O solution but the intensity of positive peak was weakened. This asymmetrical pattern was not attributed to slow

deuteration, but most likely to be ascribed to the presence of more than two carboxylates with different susceptibility to deuteration. On the other hand, for MVCO,  $\text{Fe}_{a_3}\text{C-O}$  and  $\text{Cu}_B\text{C-O}$  stretching were observed at 1965 (-) and 2061 (+)  $\text{cm}^{-1}$ , respectively. Furthermore, an extra band of  $\text{Cu}_B\text{CO}$  was found at 2040  $\text{cm}^{-1}$ . This band was not due to  $\alpha/\beta$  conformers as seen for bacterial CcO, but was ascribed to the species in which electrons were flowed back from heme  $a_3^{2+}$  to heme  $a^{3+}$ . In the carboxyl region, while a small positive peak at 1749  $\text{cm}^{-1}$  and a swelled negative peak at 1743  $\text{cm}^{-1}$  were seen for the  $\text{H}_2\text{O}$  solution, only a negative peak was observed at 1735  $\text{cm}^{-1}$  for  $\text{D}_2\text{O}$  solution. The spectral change of carboxyl bands for MV was satisfactorily interpreted in terms of the sum of the spectral changes by just ligation change (for FRCO photolysis) and a proportion of by 25 % back transfer of electrons from heme  $a_3$  to the heme  $a$  and  $\text{Cu}_A$  moiety upon CO dissociation from heme  $a_3$ . The carboxyl groups that exhibited a frequency change and deprotonation upon redox change were assigned to Glu242 and Asp51, respectively. The carboxyl side chains which exhibited a frequency shift upon ligation change were distinct from Glu242 and Asp51.

The reduction of CcO by CO and generation of  $\text{CO}_2$  are described in Chapter 3. Oxidized CcO was converted into MV by incubation under CO atmosphere in the absence of  $\text{O}_2$ . Accordingly, CO acted as a reductant for CcO and  $\text{CO}_2$  was generated. Although it is known that the conversion of oxidized CcO into MV state is faster in high pH than in low pH, it was found that the amount of generated  $\text{CO}_2$  was less in high pH than that in low pH. The generation of  $\text{CO}_2$  was promoted by the presence of  $\text{O}_2$ . Unexpectedly, however, the use of isotope  $\text{C}^{18}\text{O}/^{18}\text{O}_2$  in this reaction resulted in only the formation of  $\text{C}^{16}\text{O}_2$ . Dissolution experiments of isotopically labeled  $\text{CO}_2$  into water demonstrated that the oxygen atom of  $\text{CO}_2$  is rapidly exchanged with oxygen atom of  $\text{H}_2\text{O}$ . Therefore, it is understandable why isotopically labeled  $\text{CO}_2$  ( $\text{C}^{16}\text{O}^{18}\text{O}$  or  $\text{C}^{18}\text{O}_2$ ) could not be detected when  $\text{C}^{18}\text{O}/^{18}\text{O}_2$  was used.

CcO is a versatile enzyme. One feature is an ability of proton pump coupled to  $\text{O}_2$  reduction, and the other is an enzymatic activity to catalyze the CO oxidation during reduction of oxidized CcO by CO (conversion to MV). MV is a two-electron reduced state and can be regarded as a kind of intermediates of the  $\text{O}_2$  reduction cycle. Therefore, MV is necessary to investigate a certain intermediate of the catalytic cycle. Two carboxylic groups are involved in pumping a proton and other carboxyl groups are affected by a ligation to heme  $a_3$ .

## 論文の審査結果の要旨

本論文は英文で書かれた4章から成る論文で、それ以外に自作の赤外分光装置の性能評価について述べた Appendix から構成されている。第1章は、研究の背景と呼吸酵素の説明や用いる方法論などの説明が詳しく記述されており、申請者が関連分野の基礎知識、および、文献をよくフォローしている事がよく分かった。第2章が主なテーマである、シトクロム酸化酵素のプロトンポンプ機構に関する記述で、この内容はアメリカ化学会のトップジャーナルである J. Am. Chem. Soc. に full paper として first author の論文として掲載されている。第3章は、第2章で使用した混合原子価シトクロム酸化酵素の生成メカニズムを解明せんとする実験であるが、発生した CO<sub>2</sub> の酸素と水の酸素の交換反応が予想外に早い事がわかり、所期の目的は果たせなかった。しかし、赤外分光で見つけたこの速い酸素交換反応は審査員を含む一般の人にとっては意外な実験結果である。第4章では General Conclusion として論文の結論が簡潔に記述されている。

蛋白質水溶液の赤外吸収の実験は、水の強い赤外吸収のため測定が難しい。申請者は強い吸収にのった弱い吸収変化を検出できるような感度の高い赤外吸収検出系を自作した。つまりフーリエ変換の干渉計部分は市販品を購入したが、試料室や検出器プレアンプ等を自作し、吸光度にして 10<sup>-6</sup> までの差を検出できるようにした。その性能等の評価が Appendix というかたちで論文に記載されている。立派な内容なので、独立した章として記述するに値すると感じた。したがって、論文構成に少し疑問を感じるものの、論文全体の研究内容としては十分な内容がある。

呼吸酵素が電子移動にカップルしてプロトンを能動輸送する事は知られているが、電子移動とプロトン輸送がどのようにカップルするかわかっていない。本研究では酵素の三つの酸化状態に対してカルボン酸側鎖のプロトン化を 1730~1750 cm<sup>-1</sup> の C=O 伸縮振動に注目して調べた。その結果、Cu<sub>A</sub> と heme-a の酸化状態変化により、Asp51 のプロトン化/脱プロトン化と Glu242 の COOH の水素結合状態が変わることを見つけた。また、heme-a<sub>3</sub> のリガンドの脱着により、水素結合状態が変わる COOH が上記2残基以外に存在する事を指摘した。これと同時に、Fe<sub>a3</sub> や Cu<sub>B</sub> に結合した C≡O 伸縮振動をモニターして、Cu<sub>B</sub> に結合した C-O 伸縮振動数が Fe<sub>a3</sub> の酸化状態で変わる事を初めて見つけ、これが CO 光解離による電子の逆流現象によると説明した。

第3章は、酸化形呼吸酵素に CO を入れて放置すると半還元状態ができる事のメカニズムを CO や H<sub>2</sub>O の同位体を用い、発生する CO<sub>2</sub> にどのように同位体が含まれるかを調べようとしたものであるが、結局は水の酸素と CO<sub>2</sub> の酸素との交換があまりに速く、CO<sub>2</sub> の酸素のオリジンを同定できなかった。しかし、実験データは意外性を持つ新しい結果である。このように、本研究は感度の高い赤外吸収検出系を自作して、困難な実験をやり遂げ、新しいいくつかの発見をしているので、学位論文としては標準以上の内容をもつものであるという結論に、全審査員の意見が一致した。それ故、合格と判断した。