氏名
奥野 大地

学位（専攻分野）
博士（理学）

学位記番号
総研大甲第731号

学位授与の日付
平成15年9月30日

学位授与の要件
先導科学研究科 光科学専攻

学位規則第4条第1項該当

学位論文題目
FTIR Studies of Bovine Cytochrome c Oxidase; Proton Pumping Mechanism and Reduction by CO

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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 CO₂.

CcO is the terminal enzyme in the respiratory chain and it has four active metal centers: Cu₅₉, heme a, heme a₃ and Cu₆ in the order of electron flow. CcO receives electrons from cytochrome c and transports them via the active metal centers to O₂ which is bound to heme a₃. CcO catalyzes to reduce O₂ to H₂O and at the same time pumps a proton in coupling with the O₂ 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 O₂ catalytic reaction starts from O₂ binding to Fe₅₉ and finishes at leaving an OH group from Fe₅₉, ligation to Fe₅₉ 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 CO₂ 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 hemeproteins. 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 Fe₅₉-O and CO₂ 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 Cu₅₉ moiety mainly affected two carboxyl groups. Two bands were observed at 1749 and 1737 cm⁻¹ for H₂O solution only in the oxidized state of heme a and Cu₅₉. In D₂O solution, a single peak was present around 1742 cm⁻¹ only for the oxidized form of heme a and Cu₅₉. 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, Fe₅₉-O and Cu₆-O stretching were observed at 1963 (-) and 2063 (+) cm⁻¹, respectively, while the carboxylate band was observed at 1749 (+) and 1741 (-) cm⁻¹ as a differential pattern. These peaks were shifted to lower wavenumbers (1743/1735 cm⁻¹) in D₂O 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, Fe₅₆C-O and Cu₉C-O stretching
were observed at 1965 (-) and 2061 (+) cm⁻¹, respectively. Furthermore, an extra band of Cu₉CO
was found at 2040 cm⁻¹. This band was not due to α/β conformers as seen for bacterial CcO, but was
ascribed to the species in which electrons were flowed back from heme a₂⁻ to heme a²⁺. In the
carboxyl region, while a small positive peak at 1749 cm⁻¹ and a swelled negative peak at 1743 cm⁻¹
were seen for the H₂O solution, only a negative peak was observed at 1735 cm⁻¹ for D₂O 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 FRCC photolysis) and a proportion of by 25 %
back transfer of electrons from heme a₂ to the heme a and Cu₉ moiety upon CO dissociation from
heme a₂. 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 CO₂ are described in Chapter 3. Oxidized CcO
was converted into MV by incubation under CO atmosphere in the absence of O₂. Accordingly, CO
acted as a reductant for CcO and CO₂ 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 CO₂ was less in high pH than that in low pH. The generation of CO₂ was promoted by the
presence of O₂. Unexpectedly, however, the use of isotope C¹⁸O/¹⁸O₂ in this reaction resulted in only
the formation of C¹⁶O₂. Dissolution experiments of isotopically labeled CO₂ into water demonstrated
that the oxygen atom of CO₂ is rapidly exchanged with oxygen atom of H₂O. Therefore, it is
understandable why isotopically labeled CO₂ (C¹⁶O¹⁸O or C¹⁸O¹⁸O₂) could not be detected when
C¹⁸O/¹⁸O₂ was used.

CcO is a versatile enzyme. One feature is an ability of proton pump coupled to O₂ 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 O₂ reduction cycle. Therefore, MV is necessary to investigate a certain
intermediate of the catalytic cycle. Two calboxylic groups are involved in pumping a proton and
other calboxyl groups are affected by a ligation to heme a₂.
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多く分かった。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、発現したCO₂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を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として論文の総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せ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、試料室や検出器ブレアングを自作し、吸光度にして10⁻⁶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価値と感じた。したがって、論文構成に少し疑問を感じるもの、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らていない。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⁻¹のC=O伸縮振動に注目して調べた。その結果、Cu₄とheme-α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事を見つかった。また、heme-αのリガンドの脱着により、水素結合状態の変化する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、Fe₃やCu₈に結合したC=O伸縮振動をモニターシート、Cu₈に結合したC=O伸縮振動数がFe₃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がメカニズムをCOやH₂Oの同位体を用い、発生するCO₂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、結果は水の酸素とCO₂の酸素との交換があまりに速く、CO₂の酸素の起源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。