博士論文調査報告書

氏名	Chen Li（チェン　リ）
学位の種類	博士（理学）
学位記番号	博理第１１０号
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学位授与年月日	平成３０年９月２７日
学位授与の要件	学位規則第４条１項該当
論文題目	Study of proton pumping mechanisms of bovine cytochrome *c* oxidase during oxygen catalytic cycle by time resolved IR spectroscopy 「時間分解赤外分光法による牛チトクローム酸化酵素の酸素触媒回路中のプロトンポンプ機構の研究」
論文審査委員	（主査）教授　舘野　賢 （副査）教授　阪口　雅郎 （副査）教授　城　宜嗣 （副査）教授　永野　真吾 （鳥取大学大学院工学研究科） （副査）Constantinos Varotsis (Professor,CyprusUniversityofTechnology, Cyprus)

Varotsis 委員の審査結果については別紙（英文）として添付する。

１．論文内容の要旨

チトクローム酸化酵素(CcO)は、分子状酸素を水分子に還元する反応を触媒する呼吸鎖電子伝達系の末端酵素であり、この触媒サイクルは膜間のプロトンポンプと共役している。このプロトンポンプ共役機構の解明のためには、反応中水溶液下でのプロトン移動を実時間で測定できる時間分解赤外分光法による観測が不可欠である。しかし、水の強い吸収のある中、反応中間体の極めて弱い信号を短時間で測定しなければならないため、これまでそうした研究は行われてこなかった。Li 氏は研究室で開発された水溶液中でも測定可能な赤外分光装置を用い、CcO 溶液で酸素還元反応をおこしてその中間体を時間分解できるようなフローセルシステムを接続し、その中間体の測定に初めて成功した。

これまで X 線構造により、ウシ CcO については哺乳動物 CcO においてのみ保存さ

れている H-pathway と呼ばれるプロトン経路が存在し、H-pathway の末端に位置するアスパラギン酸残基 D51 が redox 変化によりプロトンを放出するモデルが提唱されている。CcO の酸素還元反応はプロトンポンプと共役しているため、プロトン化されたカルボキシル基の反応中の動的挙動が明らかにされる最も重要なポイントとなる。こうした蛋白質内の構造変化は赤外分光法によって検出可能であるので、Li 氏は開発した高感度時間分解赤外分光法(TRIR)を用いて、CcO 触媒サイクルの酸化相における蛋白質構造変化を測定した。その結果反応中にプロトン化および脱プロトン化を示す D51 の活性が明らかになった。D51 のプロトン化は、**P** 状態と **F** 状態との間で見られ、また **O** 状態でも、D51 を介したプロトンポンプを示した。これは、反応中に D51 を介したプロトンポンプを直接的に検出した最初の例である。他には、E242、heme$_{a3}$ プロピオン酸基および Mg^{2+}/H_2O クラスターの動力学を観測した。 E242 および heme$_{a3}$ の挙動は、水生成のためのプロトンポンプ経路(D-pathway)の活性化を示した。Mg^{2+}/H_2 クラスターでは、プロトンポンプ用の H^+の放出に伴う極性低下が観測された。

このように Li 氏は TRIR システムの開発に成功し、酸素還元触媒サイクル中の CcO の蛋白質ダイナミクスを観察した。詳細な解析により、これまで不明であったプロトンポンプ共役機構の蛋白質部分の挙動が明らかになった。さらにこのシステムは、生理学的条件下での反応中の一般的な蛋白質構造変化を測定できるメリットがあり、今後の発展が期待できる。

２． 論文審査結果

チトクローム酸化酵素は呼吸鎖電子伝達系に限らず、生命活動のエネルギー代謝において最も重要な酵素の１つであり、その機能解明のための研究が世界的にも強力に推し進められてきている。特に、酸素還元反応のための触媒回路とプロトンポンプとの共役機構の解明が最重要課題となっている。しかし、その解明のためには反応の起こる水溶液中での時間分解した赤外分光測定が必須となるが、これまで実験上の困難さのため行われてこなかった。本研究は、研究室で開発された赤外分光装置に反応中の中間体を観測できるフローシステムを組み合わせて、酸素還元反応のプロトンポンプを直接観測した世界でも初めての研究であり、先鋭的な成果といえるものである。

本研究の成果は、チトクローム酸化酵素のプロトンポンプ共役機構に関与する蛋白質ダイナミクスを直接観測したものであり、蛋白質の動的過程を生理的条件下で測定することが可能であることを示した点にある。これは、チトクローム酸化酵素のプロトンポンプ共役機構の研究において全く新しい局面を開く成果であり、今後の研究の最も重要な基盤となるものである。このように本研究はプロトンポンプ共役機構の全貌を明らかにする上で、実験的に最も重要な部分を明らかにした研究であり、重要な意義を有する成果であると評価できる

よって、本論文は博士（理学）の学位論文として価値のあるものと認める。

また、平成３０年８月１日、論文内容およびこれに関連する事項について試問を行った結果、合格と判定した。

Evaluation Report for Doctoral Thesis

Title : Study of proton pumping mechanisms of bovine cytochrome *c* oxidase during oxygen catalytic cycle by time resolved IR spectroscopy

Applicant : Chen Li

1 . Abstract of the thesis

Cytochrome *c* oxidase (CcO) is the terminal enzyme in the cellular respiratory chain, which reduces molecular oxygen to water molecule. This catalyze cycle is coupled with proton pumping across the membrane. To elucidate this proton pump coupling mechanism, observation by time resolved infrared spectroscopy, which can measure proton transfer under aqueous solution in real time during the reaction, is indispensable. However, in the presence of strong absorption of water, such studies have not been carried out since very weak signals of reaction intermediates must be measured in a short time. Ms. Li used the infrared spectrometer measurable even in an aqueous solution developed in the laboratory, connected a flow cell system capable of oxygen reduction reaction with CcO solution in time-resolve manner, and has measured the intermediate states of CcO successfully for the first time.

From the X-ray structure analysis, a proton path called H-pathway which is conserved only in mammalian CcO has been proposed for proton pumping. In bovine CcO, an aspartic acid residue D51 located at the terminal of H-pathway is changed by redox of the enzyme and proposed to be the released site of the proton. Since the oxygen reduction reaction of CcO is coupled with the proton pumping, it is essential to clarify the dynamic behavior of the protonated carboxyl group during the reaction. Since the structural changes in these proteins can be detected by infrared spectroscopy, Ms. Li utilized the newly developed highly sensitive time resolved infrared spectroscopy (TRIR) to measure protein structural changes in the oxidative phase of the CcO catalytic cycle. As a result, the D51 was revealed to show protonation and deprotonation during the catalytic cycle. Protonation of D 51 was seen between the **P** and **F** states and also showed a proton pumping through D 51 even in the **O** state. This is the first example to directly detect the proton pumping through D51 during the reaction. In addition, the dynamics of E242, heme a_3 propionate group and Mg^{2+}/H_2O cluster were observed. The behavior of E 242 and heme a_3 showed activation of the proton pumping pathway (D-pathway) for water production. In the Mg^{2+}/H_2O cluster, a decrease in polarity due to the release of H^+ for the proton pump was observed.

In this manner, Ms. Li succeeded in developing the TRIR system and observed the protein dynamics of CcO during the oxygen reduction catalytic cycle. Detailed analysis revealed the behavior of the protein moiety of proton pumping coupling mechanism, which was previously unknown. Furthermore, this system has the merit of being able to measure changes in general protein structure during reaction under physiological conditions, and future development can be expected.

２．Evaluation of the thesis and the final examination

Since CcO is an essential enzyme for the respiratory chain, and is one of the most important enzymes in the energy metabolism, the researches to elucidate its function have been vigorously promoted. Especially, the clarification of the coupling mechanism between the catalytic cycle for the oxygen reduction reaction and the proton pumping is the most important problem. However, in order to elucidate it, infrared spectroscopic measurement with time resolve manner in the aqueous solution is essential, but it has not been performed because of experimental difficulties. This research is the first research in the world where the proton pumping of the oxygen reduction reaction was directly observed by combining the infrared spectroscopy device developed in the laboratory with a flow system capable of observing intermediates during reaction.

Accordingly, the results of this research gave a direct observation of the protein dynamics involved in the coupling mechanism of CcO proton pumping, and showed that it is possible to measure the dynamic process of protein under physiological conditions. This result opens a completely new phase in the study of the proton pumping coupling mechanism of CcO, which is the most important basis for future research. Thus, this study is a research that clarified the most important part experimentally in clarifying the whole picture of the proton pumping coupling mechanism, and it can be evaluated that it is an outcome with excellent significance

Thus, the reading committee members listed below hereby state our full approval of the thesis completed by Ms. Li in fulfillment of the requirements for the degree of Doctor os Science in the Graduate School of Life Science.

The committee also certifies that the applicant passed the final oral examination on her thesis and related issues held on Xxx yy in 2018.

The chief examiner : Masaru Tateno _____

The second readers : Masao Sakaguchi _____

: Yoshitsugu Shiro _____

: Shingo Nagano _____
（鳥取大学大学院工学研究科、教授）

: Constantinos Varotsis _____
(Professor, Cyprus University of Technology, Cyprus)

Τεχνολογικό
Πανεπιστήμιο
Κύπρου

RE: Review of PhD thesis of Ms. Li Chen

Dear colleagues

With this letter I wish to provide a short review concerning the Doctorate Dissertation of Ms. Li Chen. This thesis, titled "Study of proton pumping mechanism of bovine cytochrome c oxidase during oxygen catalytic cycle by time-resolved IR spectroscopy", presents the results of novel experimental research work, performed by the author, Ms. Li Chen, and is submitted as part of the requirements for fulfilling the PhD degree at the U. of Hyogo.

In the framework of his thesis research, supervised by the late Prof. T. Ogura, the candidate has employed time-resolved IR Spectroscopy to investigate, from a mechanistic standpoint, a number of distinct processes taking place during the redox action of the bovine cytochrome c oxidase in an effort to understand how the protein functions and performs key biochemical tasks related to respiration.

The thesis comprises seven (7) main chapters, preceded by a short Introduction and followed by an Appendix featuring specific experimental details. Relevant bibliography is provided at the end of the thesis. The total length of the thesis is 118 pages from cover to end. The whole document is written in the English language.

Introduction

The Introductory section provides briefly the motivation for the present study and the author explains in short that has focused on the use of time-resolved IR spectroscopy to follow kinetically the structural changes of the protein.

Chapter II: Construction of time-resolved IR system

In this Chapter the author presents in detail, the construction of the time-resolved system used for the study of CcO

Chapter III: Flow cell with oxygen lung system

The 3nd chapter of the thesis provides detail description of the flow cell in conjunction with the oxygen lung system that was successfully used in the past in the laboratory of Prof. Ogura

Chapter IV: System estimation

Estimation of the photolysis experiments and reliability of the system was described in this chapter

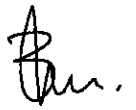
Chapter V: In this chapter the measurements of the time resolved IR measurements of CcO were reported.

Chapter VI: The coupling mechanism of CcO was analyzed and reported.

Overall, the research work presented in this thesis is quite systematic and the investigation quite detailed and thorough. The content and quality of this thesis demonstrates that the author has a very good knowledge of the subject and an excellent understanding of the critical issues and challenges involved in the study of complex biochemical systems.

Considering all the above, I am pleased to declare that the PhD thesis prepared by Ms. Li Chen fulfils, to my opinion, all the requirements of quality and innovative research to be accepted as a PhD dissertation at the University of Hyogo and I feel it can be presented in the context of the foreseen oral defense.

Sincerely



Constantinos Varotsis

Professor