Abstract of Dissertation submitted by Rajib Acharjee

Biochemical Studies of Mitochondrial Malate:Quinone Oxidoreductase from *Toxoplasma gondii*

トキソプラズマ原虫由来ミトコンドリア型リンゴ酸：キノン酸化還元酵素の生化学的解析

Rajib Acharjee, Keith K. Talaam, Endah D. Hartuti, Yuichi Matsuo, Takaya Sakura, Bundutidi M. Gloria, Shinya Hidano, Yasutoshi Kido, Mihoko Mori, Kazuro Shiomi, Masakazu Sekijima, Tomoyoshi Nozaki, Kousuke Umeda, Yoshifumi Nishikawa, Shinjiro Hamano, Kiyoshi Kita and Daniel K. Inaoka.

International Journal of Molecular Sciences, 22(15), 7830, 22 July 2021
〔Total Page Number: 16〕

Program for Nurturing Global Leaders in Tropical and Emerging Communicable Disease, Infection Research, Graduate School of Biomedical Sciences, Nagasaki University

Supervisor：Professor Shinjiro Hamano, MD. PhD

Introduction

Toxoplasma gondii, is an intracellular coccidian parasite that causes cosmopolitan zoonotic infections in humans as well as virtually all warm-blooded animals. The capacity of *T. gondii* to adapt to different environmental conditions ranks it as one of the most successful parasites. Globally, one-third population is seropositive to *T. gondii* and 95% of them do not develop any symptoms. The deleterious impact of primary infection during pregnancy, as well as reactivation of the disease in immunocompromised patients are the main burdens of this disease. Limited treatment options, severe side effects and poor efficacy of the drugs, and emergence of drug-resistant parasites makes imperative the identification and development of new drug candidates with novel mechanism of action and fewer side effects. Like *Plasmodium falciparum*, several mitochondrial electron transport chain (ETC) enzymes of this parasite can be potential drug targets, since in several aspects they exhibit similar metabolic traits. However, amongst the five ETC dehydrogenases found in *P. falciparum* and *P. berghei*, malate:quinone oxidoreductase (MQO) is a flavin adenine dinucleotide (FAD)-dependent inner membrane bound enzyme essential for optimal growth and pathogenicity, whereas its orthologue is absent in human genome. Moreover, MQO is a tri-functional enzyme important for tricarboxylic acid (TCA) cycle, ETC and fumarate cycle. In TCA cycle, MQO oxidize malate to oxaloacetate, and reduces the respiratory ubiquinone to ubiquinol that contributes to formation of electrochemical gradient and ATP synthesis. Oxaloacetate is transported to cytosol, converted into aspartate and used to feed the fumarate cycle. To date, the mitochondrial MQO have never been purified and characterized. Because of high amino acid identity of apicomplexan MQO to *Campylobacter jejuni* and *Helicobacter pylori* MQO, it has been proposed that those parasites acquired MQO from ε-proteobacteria. In *T. gondii*, a putative MQO (TgMQO) is expressed in tachyzoite and bradyzoite stages and is also considered to be a potential drug target. In my PhD, I have developed a new expression system for TgMQO using FN102(DE3)TAO, a strain deficient in respiratory cytochromes and dependent on an alternative oxidase. This system allowed for the first time the expression and purification of a mitochondrial MQO family enzyme, which was used for steady-state kinetics, substrate specificity analyses, and inhibition kinetics.

Materials and Methods

The TgMQO gene (DQ457183), lacking the mitochondrial targeting signals (MTS; Δ1-37 residues) was codon optimized and expressed into two different bacterial expression system namely,

BL21Star™(DE3) and FN102(DE3)TAO, of which the later was the new bacterial expression system developed in my study. The collected bacterial membrane fractions containing MQO, was solubilized with detergent OG (n-Octyl-β-D-glucopyranoside) and purified by metal-affinity chromatography (cOmplete-His-Tag resin, Roche). The oligomeric state and purity of the purified TgMQO was determined using high resolution clear native electrophoresis (hrCNE) and SDS-PAGE, respectively. TgMQO assay parameters such as buffer, pH and temperature was optimized. The steady-state kinetics of TgMQO was performed with malate and different ubiquinones adapting the protocol reported for *P. falciparum* MQO (PfMQO). The reaction mechanism was also determined using fixed concentrations of malate at varying concentration of decylubiquinone (dUQ). Ferulenol, the only inhibitor reported for mitochondrial MQO, was used to get insight to inhibition mechanism of purified TgMQO.

Results

The bacterial membrane fraction overexpressing TgMQO from FN102(DE3)TAO showed almost three folds higher specific activity than the BL21Star™(DE3). For the first time we could successfully purified mitochondrial type of MQO from *T. gondii* with higher activity and purity over 90%. The oligomeric state showed that TgMQO remained either tetrameric or dimer of tetrameric state in solution, the later being the most stable and predominant. High optimum temperature for TgMQO than the PfMQO was observed and at 37° C, TgMQO could retain 70% of its maximum activity. The affinity (K_m) to malate was three times higher in TgMQO than PfMQO. Furthermore, TgMQO shower higher affinity to ubiquinones with longer than shorter side chain. The reaction mechanism of TgMQO was determined to be sequential. TgMQO was 14-fold less-sensitive to ferulenol than PfMQO, displaying an IC_{50} of 0.822 µM and 0.057 µM, respectively. Inhibition kinetics indicates the presence of a third binding site for ferulenol that is distinct from malate and ubiquinone.

Discussion

In conventional bacterial expression systems, ubiquinol produced by the enzymatic reaction of MQO is readily re-oxidized by three heme-dependent terminal oxidases, cytochrome *bo*$_3$, *bd*-I and *bd*-II. This makes difficult to accurately measure the rate of ubiquinone reduction of MQO. To avoid this problem, I have developed new expression system of MQO using FN102(DE3). This strain is heme-deficient and does not contain any terminal oxidase, and does not growth unless a non-heme diiron trypanosomal alternative oxidase (TAO) is expressed [FN102(DE3)TAO]. As expected, three-folds higher MQO specific activity was obtained in the membrane fraction of FN102(DE3)TAO than the BL21Star™(DE3), clearly demonstrates that quinone/quinol-dependent activity assayed in the conventional *E. coli* expression system was underestimated. My study presents for the first time, the biochemical characteristics of a purified mitochondrial type MQO. The tetramer and dimer of tetramers observed for TgMQO indicates different oligomeric state between bacterial MQOs (monomer). The optimum temperature for TgMQO was unusually high (50°C), but coincides with reported optimum temperatures of mammalian respiratory complex enzymes (complex II, II-III, IV, and V) and reported mitochondria temperature. Interestingly, after invasion of *T. gondii* into the host cytosol, the host mitochondria surround the parasite in a process known as mitochondria recruitment. Compared to PfMQO, TgMQO showed lower affinity to ubiquinones but higher affinity to malate. The sequential reaction mechanism of TgMQO and PfMQO, suggested that this kind of mechanism is conserved among mitochondrial-type MQOs. Unlike PfMQO, the inhibition of TgMQO by ferulenol was 14 times higher, and displayed mixed-type inhibition mechanism for both substrates. This result implies that ferulenol can bind to free TgMQO as well as to TgMQO-malate or TgMQO-ubiquinone complexes. Such feature can be advantageous for drug development because such kind of compound can inhibit the enzyme regardless of substrate concentrations in the parasite. As mentioned, MQO is conserved between apicomplexan parasites and not present in their mammalian hosts, hence my study will help to develop new drug candidates with novel mechanism of action with fewer side effects.

(Word count: 1004)