รายงานวิจัยฉบับสมบูรณ์

โครงการวิจัยเอนไซม์ของวิถีการสังเคราะห์เบสไพริมิดีนเป็นตำแหน่งเป้าหมายของการพัฒนายาในเชื้อมาลาเรียของคน

Pyrimidine Biosynthetic Enzymes as Drug Target in Human Malaria Parasite

โดย

ศาสตราจารย์ ดร. จิระพันธ์ กรึงไกร

ภาควิชาชีวเคมี คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

กรกฎาคม 2551

สัญญาเลขที่ BRG4880006

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และคณะ

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

กิตติกรรมประกาศ

โครงการวิจัยเรื่อง "เอนไซม์ของวิถีการสังเคราะห์เบสไพริมิดีนเป็นตำแหน่งเป้าหมายของการพัฒนายาในเชื้อมาลาเรียของคน (Pyrimidine Biosynthetic Enzymes as Drug Target in Human Malaria Parasite)" ได้รับทุนสนับสนุนจากสำนักงานกองทุนสนับสนุนการวิจัย ตามสัญญาเลขที่ BRG4880006 ระยะเวลาดำเนินการ 3 ปี ตั้งแต่ 28 กรกฎาคม 2548 ถึง 27 กรกฎาคม 2551 ผู้รับทุนขอกราบขอบพระคุณสำนักงานฯ ที่ให้การสนับสนุนอย่างเต็มที่ทำให้โครงการวิจัยสำเร็จลุล่วงไปด้วย และขอขอบคุณบุคคลต่อไปนี้ที่มีส่วนสำคัญให้โครงการสำเร็จ ได้แก่ ผู้ร่วมวิจัยชาวไทย 2 ท่านคือ อ.ดร.สุดารัตน์ กรึงไกร (คณะวิทยาศาสตร์ มหาวิทยาลัยรังสิต) ผศ.ดร.พิสิฏฐ์ ประพันธ์วัฒนะ (คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย) นักศึกษา คือ น.ส.สวิราศจี พงศบุตร นายปานันท์ กาญจนภูมิ ผู้ร่วมวิจัยชาวต่างชาติ 2 ท่าน คือ Prof. Toshihiro Horii และ Tsuyoshi Inoue (Osaka University, Japan) เลขานุการ คือ น.ส.กาญจนา เคาวสุต รวมทั้ง ภาควิชาชีวเคมี คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ในฐานะสถาบันต้นสังกัดที่ให้ความสนับสนุนและอำนวยความสะดวกในโครงการวิจัยอย่างดียิ่ง

สารบัญ

บทคัดย่อ

รหัสโครงการ: BRG4880006

ชื่อโครงการ: เอนไซม์ของวิถีการสังเคราะห์เบสไพริมิดีนเป็นตำแหน่งเป้าหมายของการพัฒนายาในเชื้อมาลาเรียของคน

ชื่อนักวิจัย: ศาสตราจารย์ ดร. จิระพันธ์ กรึงไกร

ภาควิชาชีวเคมี คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

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ระยะเวลาโครงการ: 28 กรกฎาคม 2548 – 27 กรกฎาคม 2551

โครงการวิจัย:

โครงการวิจัยนี้ต้องการสร้างองค์ความรู้เกี่ยวกับวิถีการสังเคราะห์เบสไพริมิดีนในเชื้อมาลาเรียของคนให้มีความเข้าใจอย่างถ่องแท้ และใช้เป็นตำแหน่งเป้าหมายของการพัฒนายารักษาต่อไป จากผลงานวิจัยที่ผ่านมาแสดงว่าการสังเคราะห์เบสไพริมิดีนซึ่งเป็นสารตั้งต้นในกระบวนการสังเคราะห์สารพันธุกรรมมีความแตกต่างระหว่างเชื้อมาลาเรียและของคน ดังนั้นถ้ายับยั้งการทำงานของวิถีการสังเคราะห์เบสไพริมิดีนในเชื้อมาลาเรียได้ จะต้านการเจริญเติบโตของเชื้อมาลาเรีย สามารถนำสารยับยั้งดังกล่าวมาสู่การพัฒนายารักษามาลาเรียชนิดใหม่ได้อย่างมีประสิทธิภาพ

โครงการวิจัยนี้จึงมีวัตถุประสงค์ดังนี้ (1)ได้สร้างความรู้เกี่ยวกับชีวเคมีและอณูชีววิทยา ของคุณลักษณะวิถีการสังเคราะห์เบสไพริมิดีนในเชื้อมาลาเรียของคน ที่เกี่ยวกับไดไฮโดรออโรเทท (DHO) เอนไซม์ตัวที่สามของวิถี และ (2) มีความเข้าใจอย่างถ่องแท้ถึงโครงสร้างเอนไซม์ 2 ชนิดคือ ออโรเทท ฟอสโฟไรโบสิลทรานซ์เฟอเรส (OPRT) และ ออโรทิไดเลท ดีคาร์บอกซีลเลส (OMPDC) เอนไซม์ตัวที่ห้าและหกของวิถีการสังเคราะห์เบสไพริมิดีนในเชื้อมาลาเรีย โดยมีเป้าหมายในการได้มาซึ่งโครงสร้างสามมิติของเอนไซม์ที่ศึกษาอยู่เป็นตำแหน่งเป้าหมายใหม่ในการออกแบบและพัฒนายารักษามาลาเรีย

ผลการวิจัยที่ได้จากโครงการในระยะเวลา 3 ปีที่ผ่านนี้ โดยครอบคลุมวัตถุประสงค์ข้าง ต้น สรุปได้ดังนี้

1. ได้ทำการแยกเอนไซม์ DHO ซึ่งเร่งปฏิกิริยาการเปลี่ยน carbamoyl aspartate ไปเป็น dihydrorotate ได้อย่างบริสุทธิ์จากเชื้อมาลาเรียของคนที่เพาะเลี้ยงได้มากพอในห้อง ทดลอง และได้ทำการโคลนยีน DHO ซึ่งพบว่าอยู่บนโครโมโซมคู่ที่ 14 แล้วนำไปแสดงออกของยีนนี้ในแบคทีเรียอีโคไล ทำการแยกเอนไซม์รีคอมบิแนนท์ให้บริสุทธิ์และมีการทำงานได้ประสิทธิภาพสูง เอนไซม์ที่เตรียมได้ทั้งจากเชื้อมาลาเรียและรีคอมบิแนนท์ข้างต้นแสดงคุณสมบัติการเร่งปฏิกิริยาแบบโมเลกุลเชิงเดี่ยวได้เหมือนกับคุณสมบัติของเอนไซม์แบบที่ 2 ที่มีผู้ศึกษามาก่อน และยังแสดงคุณสมบัติทั้งในแง่จลนศาสตร์และผลการใช้สาร

ยับยั้งเอนไซม์ เช่น orotate และอนุพันธ์ของมันเหมือนกับคุณสมบัติของเอนไซม์แบบที่ 1 ที่พบได้ในเอนไซม์ของสิ่งมีชีวิตชั้นสูงรวมทั้งของคนด้วย ผลการวิจัยนี้ได้รับการตีพิมพ์และเก็บข้อมูลยีนไว้ในฐาน ข้อมูลสากล

2. ได้ทำการโคลนยีนส์ OPRT และ OMPDC จากเชื้อมาลาเรียของคน ซึ่ง OPRT เร่งปฏิกิริยาการเปลี่ยน orotate และ 5-phosphoribosyl-1-pyrophosphate ไปเป็น orotidine 5'-monophosphate (OMP) และ OMPDC เร่งปฏิกิริยาการเปลี่ยน OMP ไปเป็น uridine 5'-monophosphate (UMP) แล้วนำไปแสดงออกของยีนส์ทั้งสองในแบคทีเรียอีโคไล ทำการแยกเอนไซม์รีคอมบิแนนท์ทั้งสองให้บริสุทธิ์ เอนไซม์ที่ได้ทำงานมีประสิทธิภาพสูง ได้ศึกษาเปรียบเทียบกับเอนไซม์ของคนที่มีการศึกษาไว้ก่อนหน้านี้แล้วพบ ว่ามีคุณสมบัติแตกต่างกันระหว่างเอนไซม์นี้ในเชื้อมาลาเรียและในคนอย่างสิ้นเชิง จากนั้นนำเอนไซม์รีคอมบิแนนท์ *Pf*OMPDC ที่บริสุทธิ์สูงและมีปริมาณมากพอ ไปทำให้เกิดผลึกโดยอาศัยสาร polyethylene glycol เป็นตัวตกผลึก นำผลึกที่ได้ไปทำให้เกิดการหักเหของ X-ray โดยใช้เครื่อง synchrotron ที่กำลังขยาย 2.7 อังสตรอม สมมาตรของผลึกเป็น trigonal และ R3 space group ผลการวิจัยนี้มีการร่วมมือกับ Professor T. Horii และคณะ แห่ง Osaka University และได้รับการตีพิมพ์

3. ได้ทำการวิเคราะห์โครงสร้างสามมิติของเอนไซม์ *Pf*OMPDC ในรูปแบบของเอนไซม์อิสระ ของเอนไซม์ที่มี substrate OMP จับอยู่ และของเอนไซม์ที่มี product UMP จับอยู่แทน OMP โดยการนำผลึกของเอนไซม์ทั้ง 3 รูปแบบข้างต้นที่ได้ ไปใช้เครื่อง synchrotron ที่ Spring-8 Japan ที่กำลังขยายขนาด 2.7, 2.65 และ 2.6 อังสตรอมตามลำดับ โครงสร้างสามมิติของเอนไซม์ที่ได้ ทำให้ทราบกลไกการเร่งปฏิกิริยาเริ่มตั้งแต่การที่ substrate OMP จับกับ active site มีการจัดเรียงใหม่ของ hydrogen network มีการเปลี่ยนแปลงของโครงสร้างสามมิติของเอนไซม์ และการกำจัดหมู่คาร์บอกซีลออกจาก OMP ให้ได้ UMP ในที่สุด โครงสร้างสามมิติของเอนไซม์ที่ได้นำไปเป็นตำแหน่งเป้าหมายใหม่ในการใช้ออกแบบและการพัฒนายารักษามาลาเรีย ซึ่งกลุ่มของเราเป็นกลุ่มแรกที่ได้โครงสร้างสามมิติของเอนไซม์ OMPDC จากเชื้อมาลาเรียของคนชนิดฟัลซิพารัม ผลการวิจัยนี้มีการร่วมมือกับ Professor T. Horii และคณะ แห่ง Osaka University และได้รับการตีพิมพ์และเก็บข้อมูลโครงสร้างสามมิติของเอนไซม์ทั้ง 3 รูปแบบไว้ในฐานข้อมูลสากล

4. ได้ทำการโคลนยีนส์ OPRT และ OMPDC ที่ถูกตัดด้านปลายอะมิโนให้สั้นลงจากเชื้อมาลาเรียของคน ซึ่งทั้ง OPRT และ OMPDC ของเชื้อมีขนาดใหญ่กว่าเอนไซม์เดียวกันที่อยู่ในสิ่งมีชีวิตชั้นสูงรวมทั้งของคนโดยพบว่าที่ด้านปลายอะมิโนของเอนไซม์จากเชื้อมีจำนวนกรดอะมิโนเพิ่มขึ้น แล้วนำไปแสดงออกของยีนส์ที่สั้นลงทั้งสองในแบคทีเรียอีโคไล ซึ่งยีน truncated mutant OPRT เท่านั้นที่มีการแสดงออกและในปริมาณที่ต่ำมาก แต่ก็ทำ การแยกเอนไซม์รีคอมบิแนนท์ mutant OPRT ออกมาได้และมีความเสถียรและมี

ประสิทธิภาพที่ไม่ดีเมื่อเทียบกับเอนไซม์รีคอมบิแนนท์ที่เป็น wild type OPRT ที่ได้จากข้อ 2 งานวิจัยส่วนนี้จะมีการดำเนินต่อไปโดยเป็นวิทยานิพนธ์ของนิสิตระดับปริญญาเอก 2 คน

ผลงานวิจัยของโครงการนี้ในช่วงเวลา 3 ปีที่ได้รับทุนสนับสนุน มีการผลิตปริญญาโท-เอก มีการเสนอผลงาน มีการร่วมมือกับต่างประเทศ มีการเก็บข้อมูลยีนและข้อมูลโครงสร้างสามมิติของเอนไซม์ไว้ในฐานข้อมูลสากล มีผลงานวิจัยจากโครงการโดยตรงได้รับการตี พิมพ์จำนวน 4 เรื่อง (1-4) และจากโครงการทางอ้อมอีก 4 เรื่อง (5-8) ดังนี้

1) Crystallization and preliminary crystallographic analysis of orotidine 5'-monophosphate decarboxylase from the human parasite *Plasmodium falciparum*. *Acta Crystallographica* **F62**, 542-545 (2006)

2) Structural basis for the decarboxylation of orotidine 5'-monophosphate (OMP) by *Plasmodium falciparum* OMP decarboxylase. *Journal of Biochemistry* **143**, 69-78 (2008)

3) Dihydroorotase of human malarial parasite *Plasmodium falciparum* differs from host enzyme. *Biochemical and Biophysical Research Communication* **366**, 821-826 (2008)

4) Malaria parasite: genomics, biochemistry and drug target for antimalarial development. *Chulalongkorn Medical Journal* **50**, 127-142 (2006)

5) Malarial parasite carbonic anhydrase and its inhibitors. *Current Topics in Medicinal Chemistry* **7**, 909-917 (2007)

6) The alpha-carbonic anhydrase from the malarial parasite and its inhibition. *Current Pharmaceutical Design* **14**, 631-640 (2008)

7) Putative metabolic roles of the mitochondria in asexual blood stages and gametocytes of the malaria parasite. *Asian Pacific Journal of Tropical Medicine* **1**, 31-49 (2008)

8) Biochemistry research in Thailand: present status and foresight studies. *ScienceAsia* **34**, 1-6 (2008)

Abstract

Project Code: BRG4880006
Project Title: Pyrimidine Biosynthetic Enzymes as Drug Target in Human Malaria Parasite
Investigator: Prof. Dr.Jerapan Krungkrai, Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Rama 4 Rd., Pathumwan, Bangkok, Thailand.
E-mail Address: fmedjkk@md.ac.th.,jerapan.k@gmail.com.
Project Period: 28 July 2005–27 July 2008

Project Description:

This project is performed to better understanding of *P. falciparum* metabolic pathway with the goal of illuminating new chemotherapeutic targets for drug development. Since *P. falciparum* is totally dependent on *de novo* synthesis of pyrimidine nucleotides from small precursors, and differs from its human host, having both the *de novo* and salvage pathways. Thus, interference of this unique pathway in *P. falciparum* will result in an effective mean to control the disease.

The specific objectives of the project (TRF supported) are: (1) to conduct molecular and biochemical characterization of the third enzyme of the pathway, namely, dihydroorotase (DHO), catalyzing formation of dihydroorotate from carbamoyl aspartate, and (2) to characterize structural, kinetic, and functional properties of the recombinant orotate phosphoribosyltransferase (OPRT, reaction: [orotate + 5-phosphoribosyl-1-pyrophosphate (PRPP) → orotidine 5'-monophosphate (OMP) + PP_i]), and orotidine 5'-monophosphate decarboxylase (OMPDC, reaction: [OMP → uridine 5'-monophospate (UMP)]), the fifth and sixth enzymes of the pyrimidine pathway in human malarial parasite, *P. falciparum*. One of our ultimate goal for the ongoing project in my laboratory is structure-based design of antimalarial drug development based on the known 3-dimensional structure of the enzymes.

In the 3-year project, we have the following results to fulfill our objectives:

1. The enzyme DHO has been purified from *P. falciparum* obtained from *in vitro* cultivation, and the gene encoding *P. falciparum* DHO (*Pf*DHO) has been identified and located on chromosome 14, cloned, sequenced and functionally expressed as soluble protein in *Escherichia coli*. Both native and recombinant *Pf*DHO, exhibiting a monofunctional enzyme similarly to type II DHO, are shared kinetic properties and inhibitory effects by orotate and its 5-substituted

derivatives similarly to type I DHO which is found in higher organisms like human enzyme. The results were published in *Biochem. Biophys. Res. Commun*. vol. 366, pp. 821-826 (2008). The nucleotide sequence of the gene *Pf*DHO was deposited in GenBank/EMBL/DDBJ databases with accession number AB373011.

2. Genes encoding *P. falciparum* OPRT (*Pf*OPRT) and *P. falciparum* OMPDC (*Pf*OMPDC) were functionally expressed in *E. coli*.. The recombinant *Pf*OMPDC has been purified homogeneity in a large amount and then crystallized by the seeding method in the hanging drop using polyethylene glycol as the precipitant. The complete set of X-ray diffraction data from the crystal was collected to 2.7 Å resolution at 100 K using synchrotron radiation. The crystal exhibits trigonal symmetry (space group *R*3). This work was cooperated with Prof T. Horii at Osaka University, Japan. The results were published in *Acta Crystallogr*. vol. F62, pp. 542-545 (2006).

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Executive Summary

Project Title: Pyrimidine Biosynthetic Enzymes as Drug Target in Human Malaria Parasite **(**Project Code: BRG4880006)
Investigator: Prof. Dr.Jerapan Krungkrai, Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Rama 4 Rd., Pathumwan, Bangkok, Thailand.
E-mail: fmedjkk@md.ac.th.,jerapan.k@gmail.com.
Period: 28 July 2005–27 July 2008

Project Description:

Malaria remains one of the world's major public health problems. *Plasmodium falciparum*, the etiologic agent of the most lethal and severe form, is responsible for 1.5-2.7 million deaths annually. Chemotherapy of malaria is available but it is complicated both drug toxicity and widespread drug resistance. The need for more efficacious and less toxic agents, particularly rational drugs that exploits pathways and targets unique to the parasite, is therefore acute. This project is performed to better understanding of *P. falciparum* metabolic pathway with the goal of illuminating new chemotherapeutic targets for drug development. Since *P. falciparum* is totally dependent on *de novo* synthesis of pyrimidine nucleotides from small precursors, and differs from its human host, having both the *de novo* and salvage pathways. Thus, interference of this unique pathway in *P. falciparum* will result in an effective mean to control the disease.

The specific objectives of the project (TRF supported) are: (1) to conduct molecular and biochemical characterization of the third enzyme of the pathway, namely, dihydroorotase (DHO), catalyzing formation of dihydroorotate from carbamoyl aspartate, and (2) to characterize structural, kinetic, and functional properties of the recombinant orotate phosphoribosyltransferase (OPRT, reaction: [orotate + 5-phosphoribosyl-1-pyrophosphate (PRPP) → orotidine 5'-monophosphate (OMP) + PP_i]), and orotidine 5'-monophosphate decarboxylase (OMPDC, reaction: [OMP → uridine 5'-monophospate (UMP)]), the fifth and sixth enzymes of the pyrimidine pathway in human malarial parasite, *P. falciparum*. One of our ultimate goal for the ongoing project in my laboratory is structure-based design of antimalarial drug development based on the known 3-dimensional structure of the enzymes.

In the 3-year project, we have the following results to fulfill our objectives:

1. The enzyme DHO has been purified from *P. falciparum* obtained from *in vitro* cultivation, and the gene encoding *P. falciparum* DHO (*Pf*DHO) has been identified and located on chromosome 14, cloned, sequenced and functionally expressed as soluble protein in *Escherichia coli*. Both native and recombinant *Pf*DHO, exhibiting a monofunctional enzyme similarly to type II DHO, are shared kinetic properties and inhibitory effects by orotate and its 5-substituted derivatives similarly to type I DHO which is found in higher organisms like human enzyme. The results were published in *Biochem. Biophys. Res. Commun*. vol. 366, pp. 821-826 (2008). The nucleotide sequence of the gene *Pf*DHO was deposited in GenBank/EMBL/DDBJ databases with accession number AB373011.

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Project Description

1. **Rationale and Objectives**

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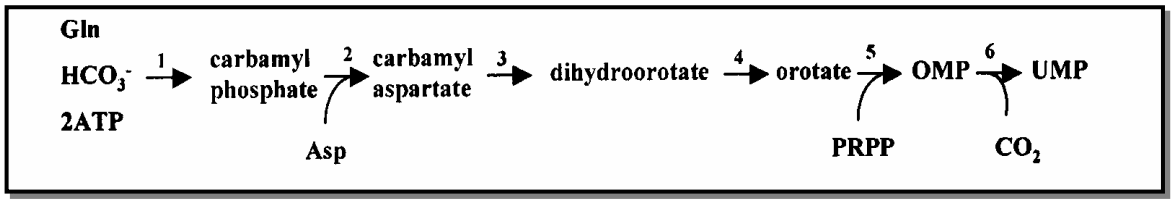
The *de novo* pyrimidine biosynthetic pathway of *P. falciparum* is an important target for drug development because the parasite, unlike its human host, is unable to salvage pyrimidine bases and nucleosides. The malarial parasite relies on the *de novo* pathway for its pyrimidine precursors. Thus, inhibitors of the pathway could be effective antimalarial agents, as the infected patient could synthesize their pyrimidine nucleotides mostly from salvage pathways *via* uridine and cytidine nucleosides. The activities of all six of the enzymes necessary to synthesize uridine 5'-monophosphate (UMP) have been identified in *P. falciparum* (see diagram).



The biochemical studies on the enzymes of the pathway in *P. falciparum* and *P. berghei* have revealed some interesting features. The first three enzymes, carbamyl phosphate synthase II (CPS II, pyr1), aspartate transcarbamylase (ATC, pyr2), and dihydroorotase (DHO, pyr3) appear to exist as discrete proteins similar to the activities in prokaryotic system. This is unlike the activities in the mammalian systems where the CPS II, ATC and DHO activities are found in a single multifunctional protein. The ATC and DHO are poorly characterized enzymes. Orotate analogues, potent inhibitors of the malarial DHO, 5-fluoroorotate and 6-thiodihydroorotate have strong antimalarial activities against both *P. falciparum in vitro* and also *P. berghei* in mouse model. However, molecular and biochemical properties for DHO in *P. falciparum* are presently not known.

Numerous studies have focused on the dihydroorotate dehydrogenase (DHOD, pyr4), the fourth enzyme in the pathway, particularly as a target of the antimalarial agents, hydroxynaphthoquinone. The DHOD in *P. falciparum* and *P. berghei* has been proved to be mitochondrial protein. The enzymatic reaction of the malarial DHOD requires coenzyme CoQ for catalysis. The DHOD gene homologue of *P. falciparum* has been cloned, and expressed in our lab (supported from TDR/WHO), and also by other groups.

The orotate analogues, particularly 5-fluoroorotate, have strong antimalarial activities. 5-Fluoroorotate and other orotate analogues do inhibit both the malarial DHO and DHOD, but they also inhibit thymidylate synthase, most likely following conversion to their respective nucleotide analogues by orotate phosphoribosyltransferase (OPRT, pyr5) and orotidine 5'-monophosphate (OMP) decarboxylase (OMPDC, pyr6), the fifth and sixth enzymes. An effective antimalarial agent pyrazofurin inhibits malarial OPRT

while its 5'-monophosphate derivative inhibits malarial OMPDC.

Supported from the TRF (BRG4580020), the two enzymes OPRT and OMPDC have been purified to homogeneity from *P. falciparum*. Characterizations of both enzymes have been studied, i.e., physicochemical and kinetic properties. The *P. falciparum* OPRT and OMPDC exist as a multienzyme complex having two subunits each of OPRT and OMPDC. We have cloned both pyr5 and pyr6 genes encoding *P. falciparum* OPRT and OMPDC, respectively. We have expressed both *P. falciparum* OPRT and OMPDC genes in *E. coli*. Using the recombinant proteins, the tight-associated enzymes were confirmed as the multienzyme complex, which are similar to those of the native *P. falciparum* enzymes. However, detailed kinetic, structural and functional characteristics of the multienzyme complex formation are poorly understood. A systemic study is necessary to understand the unique properties of the malarial OPRT and OMPDC enzyme complex.

In this funding project (BRG4880006), we have two main objectives as follows:

1. To conduct molecular and biochemical characterization of dihydroorotase (DHO)
2. To characterize structural, kinetic, and functional properties of the recombinant orotate phosphoribosyltransferase (OPRT) and OMP decarboxylase (OMPDC)

One of our ultimate goal for the ongoing projects in my laboratory is structure-based design and optimization of antimalarial drug development based on the known 3-dimensional structure of the pyrimidine enzymes.

2. Methods, Results and Discussion

During the 3-year period of the proposal we have carried out the experiments simultaneously to serve our two objectives. The experimental results are described in ordering the objectives of the project.

2.1 Molecular and biochemical characterization of *P. falciparum* DHO

Molecular and biochemical properties of *P. falciparum* DHO are poor understood. The malarial DHO seems to have similar properties to the bacterial enzyme. Thus, we intend characterize this monofunctional enzyme in *P. falciparum* using both molecular and biochemical approaches to identify the unique features.

2.1.1 Purification of *P. falciparum* DHO

The *P. falciparum* culture is maintained continuously and is scale up for mass cultivation using a modified procedure of Trager and Jensen**.** The native enzyme DHO has been purified from mass culture. Purification was performed using FPLC techniques. The enzyme activity was assayed by using radioactive substrate and TLC technique.

2.1.2 Cloning and expression of *pyr3* gene encoding *Pf*DHO

The gene *pyr3* encoding *Pf*DHO was identified and localized on chromosome 14. The amino acid sequence is 30% identity with the known crystal structure *E. coli*. The ORF of *pyr3* was cloned by PCR technique. The sequence of the *pyr3* gene was verified. Expression of the *pyr3* gene was performed in *E. coli* as soluble active protein. Purification of the recombinant enzyme was performed using nickel-affinity columns.

2.1.3 Physical and kinetic characterization of native DHO

We measured the activity of DHO from synchronized *in vitro* cultures of *P. falciparum*. The total activity in the trophozoite (87.4 ± 10.2 nmol min^{-1} per 10^8 parasites) and schizont (108 ± 13 nmol min^{-1} per 10^8 parasites) stages were increased ~23-fold and ~28-fold, respectively, as compared to the activity in the ring stage

(3.8±0.5 nmol min^{-1} per 10^8 parasites). This finding confirms stage-dependent activity of the pyrimidine enzyme in *P. falciparum*. From the crude extract preparation of the parasite to FPLC on Mono Q, Superose, and finally Mono P columns, the DHO was purified to near homogeneity at ca. 356-fold and 26% yield. The purified enzyme appeared as a dominant band at 42 kDa on SDS-PAGE. Gel filtration chromatography on the Superose column of the purified DHO obtainable from the Mono P column showed the enzyme activity eluted as a single symmetric peak at 40±4 kDa, indicating that the native enzyme exists in the monomeric form.

Comparison the *P. falciparum* and *P. berghei* amino acid sequences with the crystal structure in *E. coli* to determine structural features revealed two salt bridges and three hydrophobic interactions on dimeric interface, responsible for the subunit-subunit interaction in the bacteria. These residues are not conserved in the *P. falciparum* and *P. berghei* sequences, partly supporting our observation that the native malarial enzyme is a monomeric protein, as we have premised based on *P. berghei*. DHO from the bacterial *A. aeolicus* and the apicomplexan *T. gondii* that appear to function in monomeric form, likewise, lack this conserved residues for the subunit-subunit interaction.

2.1.4 Physical and kinetic characterization of recombinant DHO (*Pf*DHO)

Gel filtration chromatography of the recombinant enzyme shows that the activity was eluted at both monomeric (40±3 kDa) and dimeric (80±8 kDa) position, although more than 75% of the total activity was associated with the monomeric form. However, both forms had similar k_{cat} values. This observation was not found in the native enzyme isolated from the parasite. The dimeric property of the recombinant DHO was, however, confirmed by dimethyl suberimidate chemical cross-linking analysis. Only the cross-linked dimeric form was observed. The recombinant *Pf*DHO, demonstrated in both

studies, however, also suggest that the monofunctional enzyme may possibly active in both monomeric and dimeric forms. The dimeric form may exist under certain conditions during the enzyme preparations. Thus, oligomerization behavior remains to be explored by having its crystal structure.

The enzyme catalyzes reversible reaction of L-CA to L-DHO, the activities of the *pf*DHO were then measured in both reactions over the pH range of 4.0-11.0. In the ring cyclization reaction, very high activity appeared at acidic pH. In contrast, the rate of ring cleavage reaction was very high at alkaline pH, and lower activity was observed at acidic pH. The pH-activity profile for both reactions intersects at pH 6.6, suggesting that the enzyme catalysis involves His residues located on the active site. This observation is similar to those found in mammalian, *T. gondii*, and *C. fasciculata* enzymes. At pH 6.0, the enzyme displayed normal Michaelis-Menten saturation kinetics for the maximal ring cyclization of L-CA. K_m of L-CA and k_{cat} values were determined to be 0.285$\pm$0.012 mM and 60.2$\pm$4.4 min^{-1}, respectively. The maximal ring cleavage reaction rate of L-DHO measured at pH 9.0 has K_m of L-DHO and k_{cat} values of 0.012$\pm$0.001 mM and 89.5$\pm$10.6 min^{-1}, respectively. The k_{cat}/K_m values for the ring cyclization and cleavage reactions were 2.11×10^5 and 7.46×10^5 M^{-1} min^{-1}, respectively. Comparing to the mammalian enzyme, its catalytic efficiency in the ring cyclization reaction is 680-fold more than that of the malarial enzyme, while that in the ring cleavage reaction, only a 60-fold difference was noted. This indicates that the monofunctional DHO enzyme is generally less efficient than the DHO in the mammalian multifunctional CAD enzyme.

Since the equilibrium between L-CA and L-DHO favors the former by a ratio of 17:1 at physiological pH 7.4, inhibitor studies involving reversible reactions of the enzyme were measured at this pH. The inhibitory effect of orotate and various 5-

substituted orotate derivatives was investigated with *Pf*DHO and compared with the mammalian enzyme. All of the six compounds were competitive inhibitors of both ring cyclization and cleavage reactions. The K_i values for the inhibitors were obtained from double reciprocal plots, and are shown in **Table 1**.

Table 1 Kinetic constants of L-CA, L-DHO, OA and its 5-substituted derivatives in the ring cyclization and cleavage reactions of the recombinant *Pf*DHO

Substrate or OA derivative	Diameter of substituent in position 5 (Å)	Ring cyclization reaction			Ring cleavage reaction		
		K_m (μM)	k_{cat} (min^{-1})	K_i (μM)	K_m (μM)	k_{cat} (min^{-1})	K_i (μM)
L-CA (pH 6.0)		285±12	60.2±4.4	-			
L-DHO (pH 9.0)					12±1	89.5±10.6	-
OA (pH 7.4)				660±52			950±88
F-OA	2.7			145±11			70±5
NH_2-OA	2.9			440±43			160±17
CH_3-OA	3.5			560±65			302±40
Br-OA	3.8			1,460±140			2,600±170
I-OA	4.1			>3,500			>3,500

The most effective inhibitor was 5-fluoroorotate, while 5- bromoorotate and 5-iodoorotate were the least in both enzymatic directions. Both L-CA and L-DHO had no inhibitory effect on the enzyme at the pH 7.4. In the ring cleavage reaction, the

reported K_i values for the mammalian and *E. coli* enzymes are 6 and 32 μM, respectively. Increasing K_i values of the 5-substituted orotate derivatives of the parasite enzyme were proportional to the bulkiness in the size of the 5-substituents, but were not related to the order of electronegativity of these 5-substituents. The result suggests a steric hindrance of the bulky 5-substituent which may have positioned adjacent to the binding site of the inhibitor. Similar findings reported in the mammalian enzyme, indicates that *Pf*DHO shares some enzyme characteristics to the mammalian DHO although significant kinetic differences do exists, the mammalian enzyme is 10 to 20-fold more sensitive to the inhibitors than that of the parasite.

In summary, the malarial enzyme shares a number of characteristics to both type I (e.g., mammals) and II (e.g., *E. coli*) enzymes. Comparison of parasite and human enzymes by the overall amino acid sequence homology, structural properties, kinetic and inhibitor characteristics lent valuable insights into the differences of the active site between these two enzymes. Specific inhibitors may limit the pyrimidine nucleotide pool in the parasite, but have no significant adverse effect to humans in which the salvage pathway for nucleotide synthesis is actively functional. Thus, the parasite DHO presents a potential chemotherapeutic target for new antimalarial drug design.

2.2 Characterization of kinetic, structural and functional properties of the recombinant *P. falciparum* OPRT and OMPDC

2.2.1 Production of the recombinant *Pf*OPRT and *Pf*OMPDC in *E. coli*

In the last project, the poor production of the recombinant enzymes *Pf*OPRT and *Pf*OMPDC in *E. coli* was achieved (less than 1 mg recombinant protein per 1 L *E. coli* culture). We have improved our cloning and expression for the recombinant enzymes by changing both the expression vectors and the *E. coli* host cells to get 3-4 mg recombinant

protein per 1 L *E. coli* culture. Purification of the recombinant *Pf*OPRT and *Pf*OMPDC was performed by using the N-terminal His6-tag protein-nickel-affinity chromatography and following by gel-filtration chromatography. These produced soluble active enzymes with purity of more than 99% assessed by a dynamic light scattering. The highest purified and concentrated enzymes (~10 mg/ml) are important for crystallization.

2.2.2 Production of the N-terminally truncated *Pf*OPRT and *Pf*OMPDC

Since the amino acid sequences of both *Pf*OPRT and *Pf*OMPDC have unique properties, i.e., N-terminal extensions and insertions. *Pf*OPRT and *Pf*OMPDC have extensions of 66 and 32 amino acids from their N-termini, respectively. We propose that the extensions play a functional role on formation of the multienzyme complex. In addition to the full-length genes of both *Pf*OPRT and *Pf*OMPDC enzymes expressed in *E. coli*, we have constructed the mutant enzymes lacking the N-terminal extension for both *Pf*OPRT and *Pf*OMPDC by PCR cloning with appropriated primers. Constructions of N-terminally truncated *Pf*OPRT and *Pf*OMPDC enzymes were performed by our PhD graduates. The N-terminally truncated *Pf*OPRT was produced as the mutant enzymes, however, its very low yield was obtained. The truncated mutant *Pf*OMPDC was not expressed in *E. coli*. Our preliminary study suggests that the truncated *Pf*OPRT mutant enzyme is less active and stable than the wild type *Pf*OPRT. Further characterization of the mutant enzymes and the wild type enzymes will be studied in details (e.g., kinetic and functional properties etc.). This part will be the PhD thesis of the two students in the coming years.

2.2.3 Crystallization and crystal structure determination of either *Pf*OMPDC or *Pf*OPRT

There are two crystal structures for bacterial OPRT (*E. coli* and *S. typhimurium*) and four crystal structures for bacterial and yeast OMPDC (*E. coli*, *B. subtilis, M.*

thermoautotrophicum, and *S. cerevisiae*). By having the highly purified and concentrated proteins of both recombinant *Pf*OPRT and *Pf*OMPDC, 3-dimensional structures with/without substrate and inhibitor complexing at the active site will be elucidated by crystallization using an automated hanging-drop vapor-diffusion method, and X-ray data collection, structure determination/ refinement/analysis. Potent inhibitors will be designed based on the obtained 3-D structure of the enzymes.

At present, we are very lucky to have crystal of *Pf*OMPDC (**Fig. A & B**) and its X-ray diffraction data (**Table 2**). These experiments are being co-operated with Prof. Toshihiro Horii and Dr. Tsuyoshi Inoue (Osaka University, Japan).

Fig. A Thin crystals from sitting-drop from the first crystallization trial.

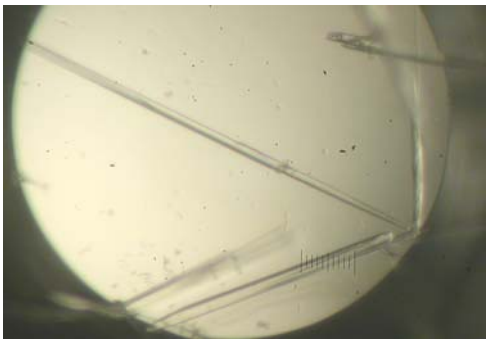


Fig. B Improved quality of crystals by the hanging-drop method.

Table 2 Statistics of crystal parameters and X-ray diffraction data collection*

Crystal system	Trigonal with hexagonal unit-cell
Space group	*R3*
Unit-cell parameters (Å)	$a = b = 201.81$, and $c = 44.03$
(°)	$\alpha = \beta = 90.0$, and $\gamma = 120.0$
Resolution range (Å)	50.0 – 2.70
No. of molecules per asymmetric unit	2
V_M (Å ^{3}/dalton)	2.3
V_{solv}(%)	46
No. of measured reflections	451,733
No. of unique reflections	18,313
$^+R_{merge}$ (%)	5.9 (27.5)
Completeness (%)	99.6 (100.0)
Average $I/\sigma(I)$	12.4 (4.0)

* Values in parentheses are for the highest resolution shell (2.85 –2.70 Å)

$^+R_{merge} = \Sigma|I(k)-I|/\Sigma I(k)$, where $I(k)$ is value of the kth measurement of the intensity of a reflection, I is the mean value of the intensity of that reflection and the summation is the over all measurements.

The results are summarized as follows: The active recombinant *P. falciparum* OMPDC (*Pf*OMPDC) was crystallized by the seeding method in a hanging-drop using PEG3000 as a precipitant. A complete set of diffraction data from the native crystal was collected to 2.7 Å resolution at 100 K by using synchrotron radiation. The crystal exhibits trigonal symmetry (space group *R*3) with hexagonal unit-cell of $a = b = 201.81$ Å, and $c = 44.03$ Å. With a dimer in the asymmetric unit, the solvent content is 46% ($V_M = 2.3$ Å^3 Da^{-1}).

Then, we have characterized the X-ray analysis of apo, substrate OMP or product UMP-complex forms of OMPDC from *Pf*OMPDC at 2.7, 2.65 and 2.6 Å, respectively. The structural analysis provides the substrate recognition mechanism with dynamic structural changes, as well as the rearrangement of the hydrogen bond array at the active site. The structural basis of substrate or product binding to *Pf*OMPDC will help to uncover the decarboxylation mechanism and facilitate structure-based optimization of antimalarial drugs. We also propose a protruding domain at the N-terminal insertion of *Pf*OMPDC is responsible for binding to *Pf*OPRT, supporting our proposal earlier that the N-terminal domain of *Pf*OMPDC binds to that of *Pf*OPRT.

The sequence alignment for 10 known crystal structures of OMPDC is shown in **Fig. 1**. This reveals the existence of 11 totally conserved amino acid residues, corresponding to Asp23, Lys102, Asp136, Lys138, Asp141, Ile142, Gly169, Pro264, Gly265, Gly293 and Arg294 in *P. falciparum.* The alanine replacement around the active site, however, shows that *Plasmodium* OMPDCs have somewhat different sequences. The most striking observation is that a large insertion is present at the N-terminal domain, in *Plasmodium* OMPDCs.

The molecular replacement calculations were performed with the program MOLREP in the CCP4 program package, using the structure of the OMPDC-PO_4 complex from *P. falciparum* (PDB code: 2f84) as the search model. A dimer molecule was located in an asymmetric unit of the crystal. Structure models of the apo form were refined against the diffraction data using CNS and REFMAC. The models were manually adjusted by electron density mapping using COO. Even after several cycles of refinement, the electron density for residues 70–74 and 268–274 for one subunit, and 68–70 and 268–272 for the other subunit, was poor. Some other parts of the side chain structure (residues 1, 35, 69, 75, 76,

79, 199, 201, 255, 269, 275, 281, 301, 306, 314, 317 and 318 for one subunit; and residues 1, 71, 96, 174, 207, 222, 231, 255, 266, 269, 273, 274, 275, 281, 306, 314, 317 and 318 for the other subunit) also had poor electron density. The models for these residues were built by using alanine. All models were refined for a few cycles of CNS with B-factor refinement until convergence. The coordinates of the apo, and OMP- or UMP-complex forms of *Pf*OMPDC are deposited in the Protein Data Bank.

2.2.4 Overall structure of *Pf*OMPDC

The 2.7 Å structure of the apo form of *Pf*OMPDC was solved with R and R_{free} values of 21.6 and 31.2, respectively, by using the MR method with the PO_4-complex OMPDC. *Pf*OMPDC was crystallized in the trigonal space group of $R3$. One dimer molecule existed in the asymmetric unit. Each OMPDC subunit of this dimeric enzyme folded as an $(\alpha/\beta)_8$ barrel, with eight central β-sheets surrounded by thirteen α helices (**Fig. 2a**). β1, residues 18–21; β2, 100–104; β3, 132–139; β4, 161–164; β5, 187–193; β6, 237–241; β7, 261–263; and β8, 288–292; and α1, residues 3–14; α2, 26–37; α3, 42–47; α4, 60–64; α5, 78–94; α6, 113–128; α7, 143–154; α8, 173–176; α9, 200–203; α10, 213–227; α11, 246–255; α12, 276–279; and α13, 302–315. There were three extra short α helices: η1, residues 51–54; η2, 105–108; and η3, 230–233.

As predicted from protein sequence analysis (Fig. 1), there was a large insertion region (48–86), forming α3, η1 and α4 helices and extended α5-helix (**Fig. 2b**). Also *Pf*OMPDC contained a small insertion region (208–217), extended α10-helix These insertions are not found in other characterized OMPDC structures, and appear to be a unique structural feature of the OMPDCs belonging to *Plasmodium*.

The active site was located at the open end of the $(\alpha/\beta)_8$ barrel, which corresponded to the carboxy-terminal of the β strands and the amino-terminal of the α helices. In the apo structure, the L1 loop comprised of residues 268–274 was disordered

around the active site, while the L1 loop structure was stabilized upon binding of substrate or product.

2.2.5 Structural changes of L1 and L2 loops upon binding of OMP/UMP of *Pf*OMPDC

The structural analysis of *Pf*OMPDC complexes has been performed with *R* and R_{free}values of 20.8 and 28.3, respectively for the 2.65 Å structure complexed with OMP, and 21.5 and 28.9, respectively, for the 2.6 Å with UMP.

Comparison between the apo and OMP- or UMP-complex of *Pf*OMPDC showed a root-mean-square deviation of 0.75 and 0.74 Å, respectively. Several notable structural differences were found around the active site near the open end of the $(\alpha/\beta)_8$ barrel upon binding of OMP or UMP.

The L1 loop which was not determined in the apo form, was stabilized upon binding of the ligands, Glu269 in the L1 loop access to the pyrimidine ring moiety of OMP or UMP formed the loop structure of L1 (**Fig. 3**). While the structure of the α9 helix was formed in the L2 loop region (194–213) in the apo form, the secondary structure unfolded in the presence of OMP or UMP, associated with the movement of Thr195. The difference in C_α position and the bond angle of C_α–C_β in Thr195 was calculated to be 3.8 Å and 70°, respectively, between the apo and OMP-complex; and 3.6 Å and 70°, respectively, between the apo and UMP-complex. It was revealed that both the L1 and L2 loops recognized the pyrimidine ring with the large structural changes. The details of the structural changes to the active site of *Pf*OMPDC are described below.

2.2.6 Change in active site structure upon binding of OMP/UMP of *Pf*OMPDC

Schematic drawings of the OMP- or UMP-complex are shown in **Fig.4a and 4b**, respectively. Both the guanidium group and the backbone amide of Arg294 interacted directly with the phosphate group of OMP or UMP, as did the amide of Gly293.

Thr145(B) and Asn104 hydrogen-bonded to the 2'- and 3'-hydroxyl group of the ribose ring, respectively. In the top part of the pyrimidine ring, comprising O4, N3 and O2, there were hydrogen bonds to Thr195 and Gln269. Thr195 accepted a hydrogen bond from N3 and donated a hydrogen bond to O4. A pocket near C5 of the pyrimidine ring was surrounded by several hydrophobic residues of Ile142(B), Leu191, Thr194, Val240 and Pro264 (not shown in Fig. 4a).

Compared with the apo form, there was a change in active site structure upon ligand binding. Arg294 and Thr195 in L2, and Gln269 in L1 exhibited a large structural movement upon binding of OMP or UMP. The movement of three nitrogen positions and the dihedral angle in the guanidium residue of Arg294 were calculated to be 2.9 Å and 48°, respectively, for the OMP-complex; and 2.9 Å and 52°, respectively, for the UMP-complex. The helix structure of α12 did not change, while the L1 and L2 loops had the large structural movements described above. The average difference in the top part of residues Thr195 and Arg294 was measured to be 1.5 and 1.6 Å, respectively for OMP- and UMP-complexes. On the other hand, in the bottom part of the active site, the C_α atom of Gln293, Asn104 and Thr145(B) adopted almost the same positions between the apo and complex forms. The average difference in the bottom part of residues Asn104, Thr145(B) and Gln293 was 0.3 Å for both the OMP- and UMP-complexes.

The binding mode of OMP was basically the same as that of UMP, except for the residue Lys102. In the OMP binding form, Lys102 hydrogen-bonded to the carboxylate oxygen of pyrimidine. After decarboxylation, Lys102 changed the hydrogen bond to O5 of ribose (**Fig. 4**).

2.2.7 Rearrangement of hydrogen bond network around Lys102 of *Pf*OMPDC

In the apo form, Lys102 formed a hydrogen bond network with Asp136, Lys138 and Asp141 from the other protomer of the dimer (**Fig. 5a**). The conserved catalytic array

of Lys102-Asp136-Lys138-Asp141(B) makes hydrogen bond network at the active site. The amino group of Lys102 recognized the carboxyl group of OMP, and then the distance between Lys102-Asp136 was lengthened from 2.7 to 3.6 Å, breaking the hydrogen bond between Lys102 and Asp136 upon binding of OMP (**Fig. 5b**). The UMP binding form (**Fig. 5c**), which mimicked the structure after decarboxylation, possessed two hydrogen bonds at Lys102-Asp136 and Lys138-Asp141(B) (**Fig. 5c**). In the UMP-complex, Lys102 hydrogen-bonded to Asp136 instead of the carboxyl group of the pyrimidine ring, breaking the hydrogen bond between Asp136 and Lys138, after decarboxylation.

The details of the reaction mechanism have been proposed by Raugei *et al*., that a very stable charged network OMP-Lys-Asp-Lys-Asp promotes transition state electrostatic stabilization, and fully conserved Lys138 (*Pf*OMPDC numbering) largely contributes to the stabilization of the transition state or equivalently, the carboanionic intermediate.

In this study we have determined the X-ray structure of the apo, substrate or product-complex forms of *Pf*OMPDC. These three kinds of structural analyses provided an insight into the rearrangement of the hydrogen network in the array of OMP-Lys-Asp-Lys-Asp, by comparison between the initial and completion stages of the decarboxyl reaction.

At the initial reaction step, the side chain of Lys102 broke a hydrogen bond with the carboxyl group of Asp136, and then formed a hydrogen bond with the carboxyl oxygen atom of the substrate, upon binding OMP. These processes increased the negative charge on the two carboxyl groups on the substrate and Asp136, which led electrostatic repulsion between the carboxyl groups. Even the short distance of 2.5 Å between the two carboxyl groups, and the positively charged residues of Lys102 and Lys138 maintained the unstable structure.

Then, the decarboxylation reaction started from the structure with electrostatic repulsion. After direct decarboxylation, the newly generated carboanion at the C6 position

of the pyrimidine ring may have been stabilized by the conserved array, and absorbed a proton from the Lys138 side chain directly. In the completion step, two pairs of hydrogen bonds, Lys102-Asp136 and Lys138-Asp141(B), were formed, and all of the electrostatic charge in the array was compensated (**Fig. 4c**). It seems to be difficult, however, to specify from our structural models which Lys residues stabilized the forming carboanion intermediate in the transition state. A structural analysis of complexes with a transition state analogue will uncover the mechanism of transition state stabilization by Lys residues.

Eukaryotes express OMPDC as a bifunctional protein, UMP synthase, which catalyzes the orotate phosphoribosyltransferase reaction before the decarboxylation reaction. On the other hand, *Pf*OMPDC forms a complex with orotate phosphoribosyltransferase (OPRT), which forms a heterotetrameric complex in *P. falciparum*. In our multiple sequence alignment, we found a unique structural feature of the OMPDCs belonging to *Plasmodium*. A large insertion was observed between β1 and α5, which formed a protruding domain consisting of three α helices, α2, α3 and α4. We propose that the unique insertion may participate in making a complex with OPRT.

In our structural observations, the top part of the active site played a role in substrate binding, with large structural movement. The average difference of 1.5 Å in the top part is twice the root-mean-square deviation calculated from all C_α atoms. In contrast, the bottom part of the active site was significantly rigid; the average difference was half of the overall root-mean-square deviation. Even a small difference, such as the rearrangement of hydrogen bonds in the array of OMP-Lys-Asp-Lys-Asp, may have played a pivotal role in catalysis. The corresponding residues for binding and catalyzing showed >80% consensus among all the OMPDCs.

In summary, we have proposed a reaction mechanism and characteristic domain for binding of *Plasmodium* OPRT, by the three kinds of structural analysis. Further structural

analyses of complexes with transition state analogues will reveal the stabilization mechanism of the carboanion intermediate. Moreover, structural analysis of the complex between *Pf*OMPDC and *Pf*OPRT will also clarify the heterotetrameric reaction mechanism, which may lead to the development of important antimalarial drugs.

Figure 1

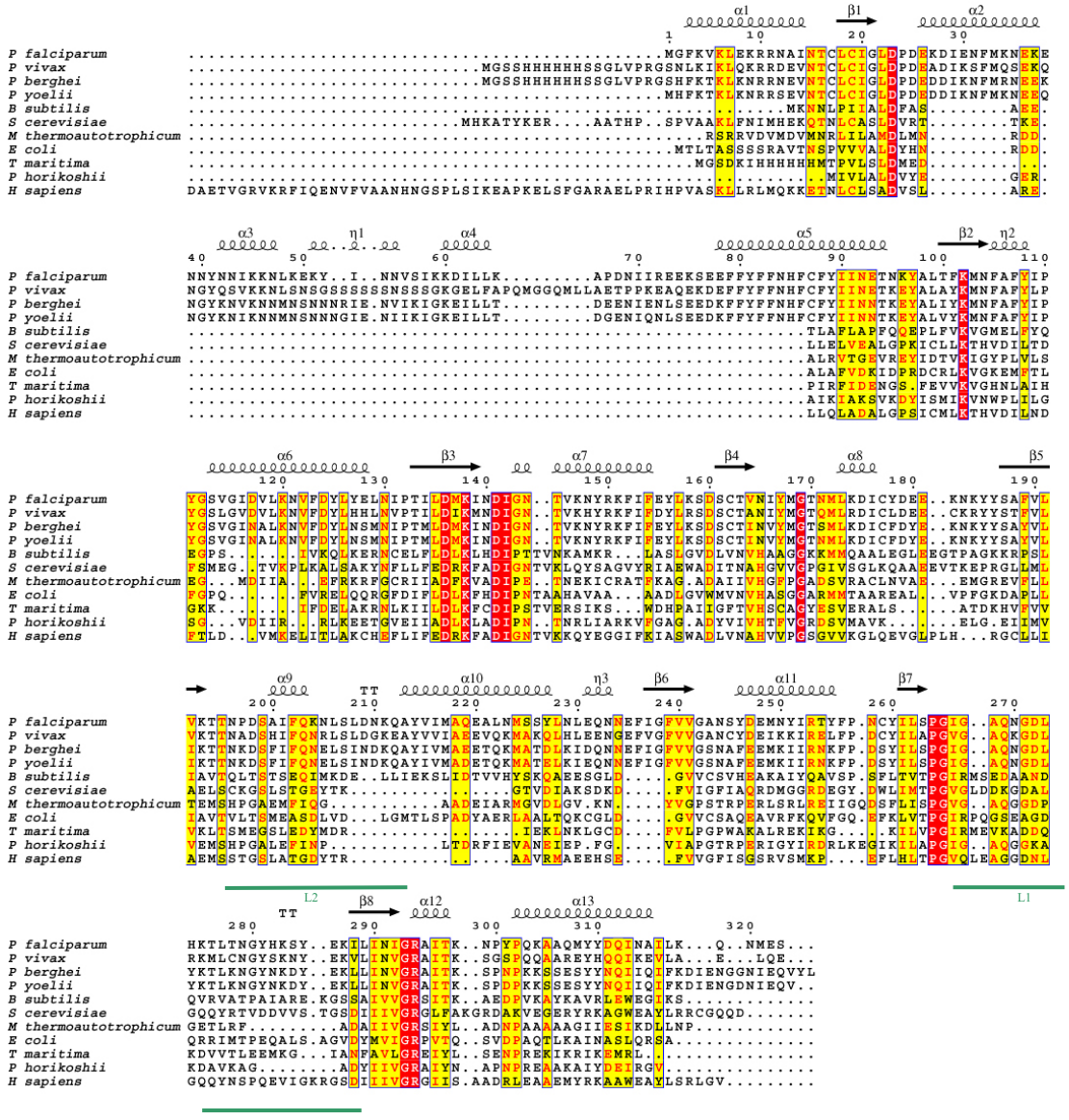


Fig. 1. Multiple sequence alignments of *Pf*OMPDC compared with those of 10 known crystal structures. PDB IDs are showed in parentheses: *Plasmodium vivax* (2FFC), *Plasmodium berghei* (2FDS), *Plasmodium yoelii* (2AQW), *Bacillus subtilis* (1DBT), *Saccharomyces cerevisiae* (1DQW), *M. thermoautotrophicum* (1DV7), *E. coli* (1L2U), *Thermotoga maritima* (1VQT), *Pyrococcus horikoshii* (2CDZ) and *Homo sapiens* (2EAW). Red shaded amino acids are conserved in all 10 sequences; yellow shaded amino acids are similar residues. Above the sequences, the secondary structural elements are shown, as identified in *Pf*OMPDC. The L1 and L2 loops are identified and marked by bottom underlines.

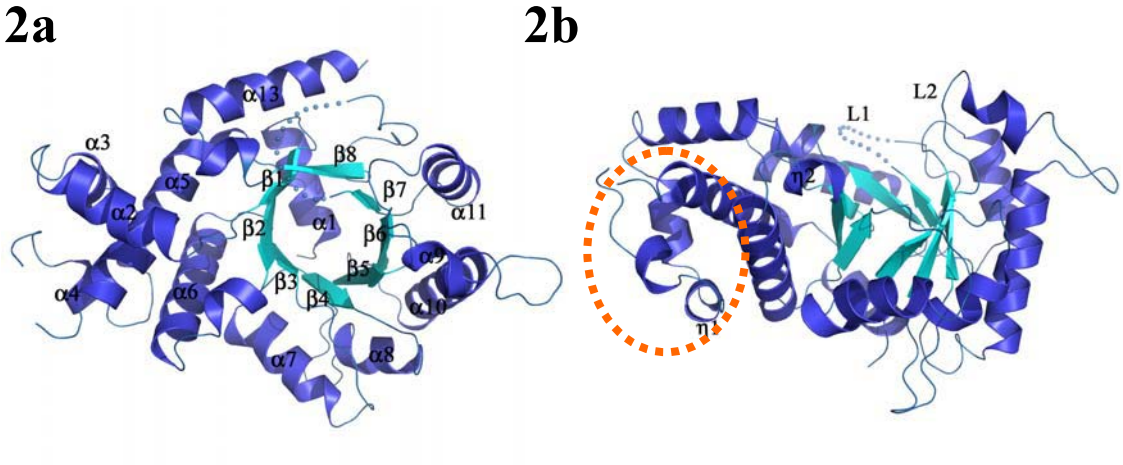


Fig. 2. Monomer of *Pf*OMPDC. The α helices (in blue) and β sheets (in cyan) are separated by loops. One subunit folds as an $(\alpha/\beta)_8$ barrel with eight central β sheets surrounded by 13 α helices. The disordered L1 loop is shown as a dotted line. (a) Viewed through α/β barrel. (b) Viewed perpendicular to the view in panel a. A large insertion region from residue 48 to 86, constructed the insertion domain containing three helices, Tand was present in dot circle (in *orange*)he figures were drawn with PYMOL.

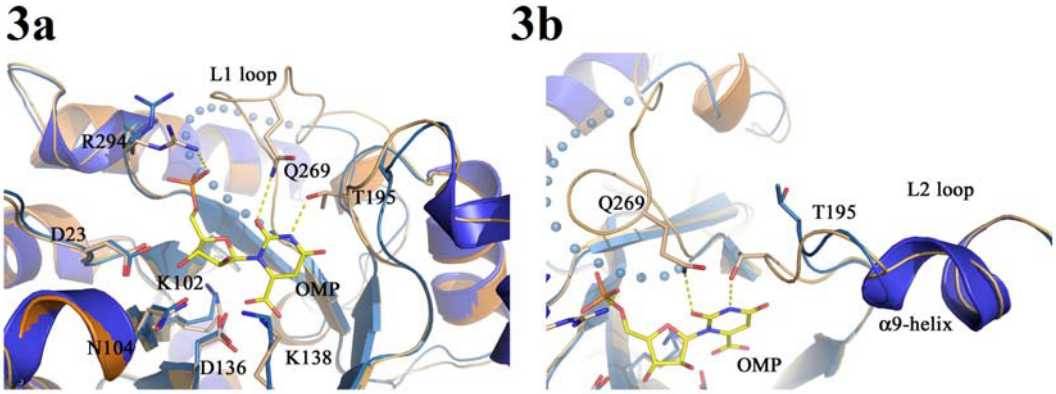


Fig. 3. Movement of L1 (a) and L2 (b) loops upon bindings of OMP. Blue and orange structures show the apo form and OMP-complex. (a) The L1 loop, which was not determined in the apo form (blue dotted line), was stabilized, and the loop structure containing Q269 was formed. (b) The α9 helix only formed in the apo from, and unfolded in association with the movement of Thr195 (C_α distance of 3.8 Å), and then access to the ligand.

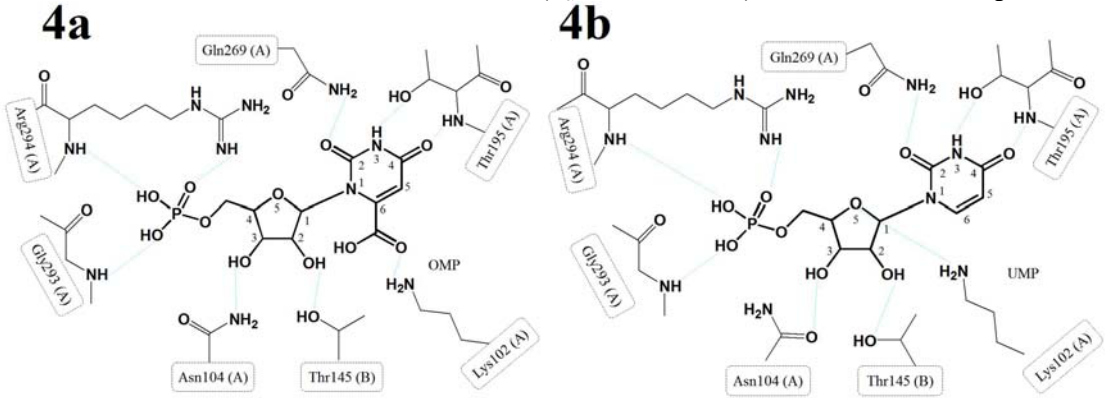


Fig. 4. Schematic drawings of the hydrogen bonding pattern around OMP (a) and UMP (b). The binding motif of OMP was basically the same as that of UMP, except for the residue Lys102.

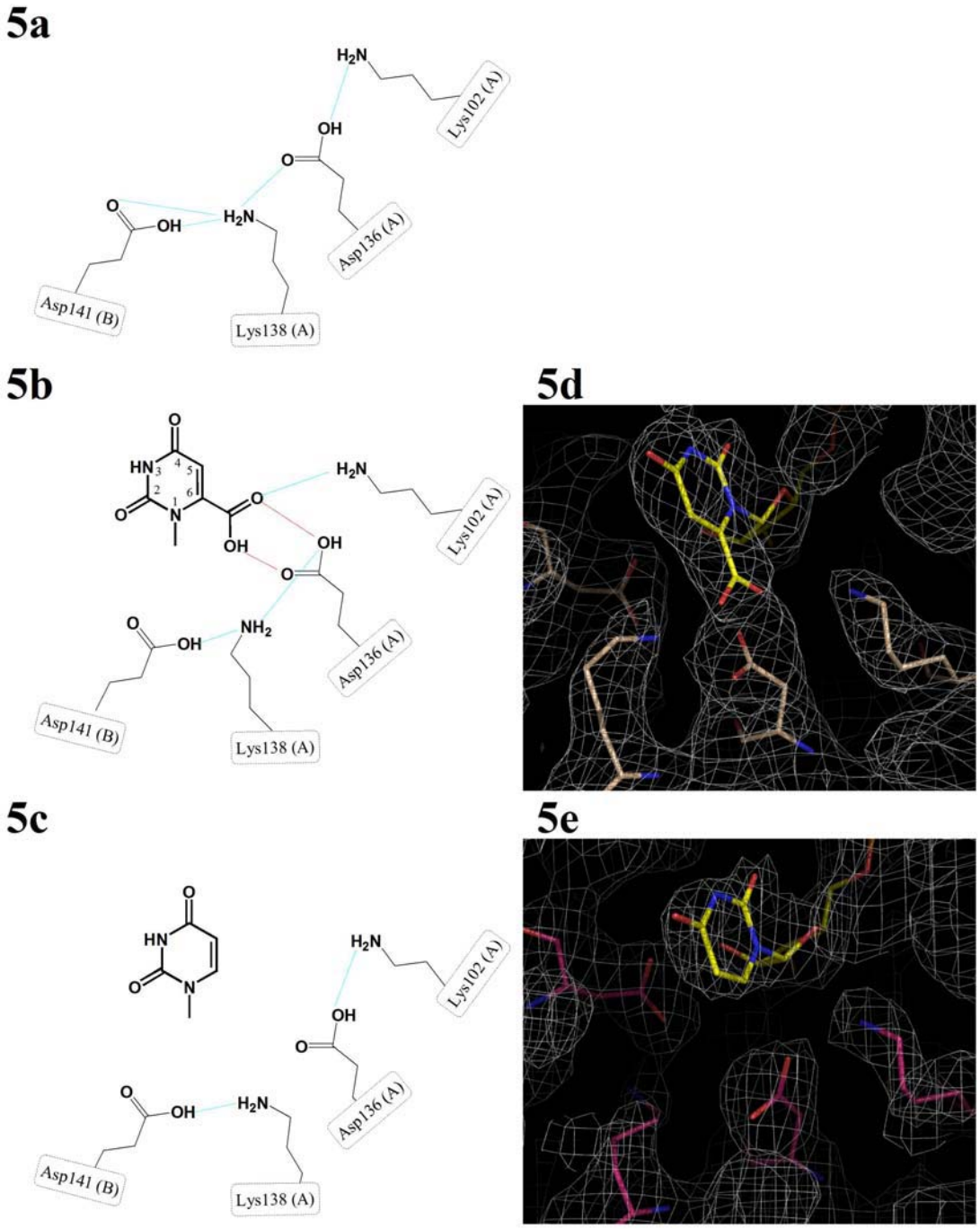


Fig. 5. Proposed reaction mechanism. Stepwise representation of the rearrangement of the hydrogen bond network, with a particular electron density map around Lys102. (a) Apo form. Lys102 formed a hydrogen bond network with Asp136, Lys138 and Asp141. (b) The substrate entered the active site. As Lys102 turned away from Asp136, charge repulsion between the two carboxylate groups initiated decarboxylation and Lys 138 donated a proton to C6. (c) After decarboxylation, Lys102 hydrogen-bonded to Asp136 instead of the carboxyl group of the pyrimidine ring. (d) Electron density maps of OMP (d) and UMP (e) binding forms. These contours show the SIGMAA-weighted 2*F*o – *F*c map at 1.5 σ. The programs COOT and PYMOL were used to make these figures.

Outputs

In the 3-year project, we have outputs for our activities as follows:

(a) There are four publications:

1) Krungkrai S.R., Kusakari, Y., Tokuoka, K., Inoue, T., Adachi, H., Matsumura, H., Takano, K., Murakami, S., Mori, Y., Kai, Y., Krungkrai, J., and Horii, T.(2006) Crystallization and preliminary crystallographic analysis of orotidine 5'-monophosphate decarboxylase from the human parasite *Plasmodium falciparum*. *Acta Crystallographica* **F62**, 542-545.

2) Tokuoka K., Kusakari Y., Krungkrai S.R, Matsumura H., Kai Y., Krungkrai J., Horii T., Inoue T. (2008) Structural basis for the decarboxylation of orotidine 5'-monophosphate (OMP) by *Plasmodium falciparum* OMP decarboxylase. *Journal of Biochemistry* **143**, 69-78.

3) Krungkrai, S.R., Wutipraditkul, N., and Krungkrai, J. (2008) Dihydroorotase of human malarial parasite *Plasmodium falciparum* differs from host enzyme. *Biochemical and Biophysical Research Communication* **366**, 821-826.

4) Krungkrai, J., and Krungkrai, S. (2006) Malaria parasite: genomics, biochemistry and drug target for antimalarial development. *Chulalongkorn Medical Journal* **50**, 127-142.

(b) There are four data deposited in databases:

1) The three crystal structures complexed with substrate or product or uncomplexed forms were deposited in ProteinDataBank with the PDB entries ID: 2ZA1, 2ZA2 and 2ZA3.

2) The nucleotide sequence of the gene *Pf*DHO was deposited in GenBank/EMBL/DDBJ databases with accession number AB373011.

(c) Graduation:

One MSc student was graduated, two PhD students are being trained.

(d) Presentation:

There was a poster presentation in "The second Thailand-Japan joint forum on infectious diseases" with topic entitled: "Functional characterization of *Plasmodium falciparum* pyrimidine enzymes orotate phosphoribosyltransferase and orotidine 5'-monophosphate decarboxylase".

(e) Collaboration:

We have solid collaborations with expert groups in Osaka University (Japan) and University of Florence (Italy).

(f) There are four publications related to the ongoing project:

1) Krungkrai, J., Krungkrai, S.R., and Supuran, C.T. (2007) Malarial parasite carbonic anhydrase and its inhibitors. *Current Topics in Medicinal Chemistry* **7**, 909-917.

2) Krungkrai, J., and Supuran, C.T. (2008) The alpha-carbonic anhydrase from the malarial parasite and its inhibition. *Current Pharmaceutical Design* **14**, 631-640.

3) Krungkrai, J., Kanchanaphum, P., Pongsabut, S., and Krungkrai, S.R. (2008) Putative metabolic roles of the mitochondria in asexual blood stages and gametocytes of the malaria parasite. *Asian Pacific Journal of Tropical Medicine* **1**, 31-49.

4) Krungkrai, J., Incharoensakdi, A., and Tungpradabkul, S. (2008) Biochemistry research in Thailand: present status and foresight studies. *ScienceAsia* **34**, 1-6.

Appendix
(ภาคผนวก)

1. บทความสำหรับการเผยแพร่

โครงการวิจัยเรื่อง

"เอนไซม์ของวิถีการสังเคราะห์เบสไพริมิดีนเป็นตำแหน่งเป้าหมายของการพัฒนายาในเชื้อมาลาเรียของคน"

ปัจจุบันมาลาเรียยังเป็นโรคติดเชื้อที่เป็นปัญหาทางสาธารณสุขที่สำคัญ โดยเชื้อมาลาเรียก่อให้ เกิดอาการอย่างรุนแรงในคน มีคนติดเชื้อทั่วโลกปีละถึง 500 ล้านคนและเสียชีวิตลงปีละ 1.5-2.7 ล้านคน การควบคุมโรคมาลาเรียทำได้ด้วยความยากลำบากขึ้น เนื่องจากมีการแพร่ระบาดของเชื้อที่ดื้อต่อยาที่ใช้ในการรักษา ยาที่ใช้มักมีผลข้างเคียงต่อผู้ป่วย การพัฒนาและการแพร่กระจายของยุงก้นปล่องที่ดื้อต่อยาฆ่าแมลง อีกทั้งยังไม่มีวัคซีนที่จะนำมาใช้ในการป้องกันโรคในขณะนี้ จึงเป็นสถานการณ์เร่งด่วนที่จะต้องพัฒนาการควบคุมโรคให้มีประสิทธิภาพ อาทิเช่น การพัฒนาวัคซีน และการพัฒนายารักษามาลาเรียชนิดใหม่ๆ ขึ้นมาทดแทนให้ทันใช้โดยอาศัยตำแหน่งเป้าหมายที่มีความจำเพาะในเชื้อมาลาเรียที่ได้จากโครงการจีโนมและความรู้ที่สะสมต่อเนื่องกันมานานหลายสิบปี ถึงกระนั้นความรู้ทางชีวเคมีของเชื้อมาลาเรียยังไม่กระจ่างนัก โดยเฉพาะการสังเคราะห์เบสไพริมิดีนซึ่งเป็นสารตั้งต้นในกระบวนการสังเคราะห์สารพันธุกรรมของเชื้อมาลาเรียของคน

เมื่อผู้ป่วยมาลาเรียมีการติดเชื้อเกิดขึ้น เชื้อมาลาเรียจะแบ่งตัวอย่างรวดเร็วและเจริญเติบโตเพื่อให้มีชีวิตรอดอยู่ได้ในผู้ป่วย เชื้อมีความจำเป็นจะต้องสร้างสารพันธุกรรมขึ้นมาเพื่อการดังกล่าว สารตั้งต้นที่สำคัญตัวหนึ่งในการสร้างสารดังกล่าวคือเบสไพริมิดีน ซึ่งมีลักษณะโครงสร้างเป็นวงแหวนรูปหกเหลี่ยม การสร้างเบสดังกล่าวต้องอาศัยเอนไซม์หลายชนิดในการเร่งปฏิกิริยาเปลี่ยนโมเลกุลขนาดเล็ก อาทิเช่น คาร์บอนไดออกไซด์ อะดีโนซีนไตรฟอสเฟต กรดอะมิโนกลูตามีนและแอสปาเตต จนได้เป็นโมเลกุลของไพริมิดีน อาศัยหลักฐานทางชีวเคมีที่ทำก่อนหน้านี้พบว่าเชื้อมาลาเรียมีเอนไซม์ต่างๆในการสร้างไพริมิดีนดังกล่าว และเรียกว่า "วิถีการสังเคราะห์เบสไพริมิดีนในเชื้อมาลาเรีย"

โครงการวิจัยเรื่อง **"เอนไซม์ของวิถีการสังเคราะห์เบสไพริมิดีนเป็นตำแหน่งเป้าหมายของการพัฒนายาในเชื้อมาลาเรียของคน"** ต้องการสร้างองค์ความรู้เกี่ยวกับวิถีการสังเคราะห์เบสไพริมิดีนในเชื้อมาลาเรียของคนให้มีความเข้าใจอย่างถ่องแท้ และนำไปใช้เป็นตำแหน่งเป้าหมายของการพัฒนายารักษาต่อไป จากผลงานวิจัยที่ผ่านมาแสดงว่าการสังเคราะห์เบสไพริมิดีนซึ่งเป็นสารตั้งต้นในกระบวนการสังเคราะห์สารพันธุกรรม มีความแตกต่างระหว่างเชื้อมาลาเรียและของคน ดังนั้นถ้ายับยั้งการทำงานของวิถีการสังเคราะห์เบสไพริ

มิดีนในเชื้อมาลาเรียได้ จะต้านการเจริญเติบโตของเชื้อมาลาเรีย สามารถนำสารยับยั้งดังกล่าวมาสู่การพัฒนายารักษามาลาเรียชนิดใหม่ได้อย่างมีประสิทธิภาพ

โครงการวิจัยนี้มีวัตถุประสงค์ดังนี้

ก.ได้สร้างความรู้เกี่ยวกับชีวเคมีและอณูชีววิทยาของคุณ ลักษณะวิถีการสังเคราะห์เบสไพริมิดีนในเชื้อมาลาเรียของคนที่เกี่ยวกับไดไฮโดรออโรเทท (DHO) เอนไซม์ตัวที่สามของวิถี และ

ข. มีความเข้าใจอย่างถ่องแท้ถึงโครงสร้างเอนไซม์ 2 ชนิดคือ ออโรเทท ฟอสโฟไรโบสิลทรานซ์เฟอเรส (OPRT) และ ออโรทิไดเลท ดีคาร์บอกซีลเลส (OMPDC) เอนไซม์ตัวที่ห้าและหกของวิถีการสังเคราะห์เบสไพริมิดีนในเชื้อมาลาเรียโดยมีเป้าหมายในการได้มาซึ่งโครงสร้างสามมิติของเอนไซม์ที่ศึกษาอยู่เป็นตำแหน่งเป้าหมายใหม่ในการออกแบบและพัฒนายารักษามาลาเรีย

ผลการวิจัยที่ได้จากโครงการในระยะเวลา 3 ปีที่ผ่านนี้โดยครอบคลุมวัตถุประสงค์ข้างต้น สรุปได้ดังนี้

1. ได้ทำการแยกเอนไซม์ DHO ซึ่งเร่งปฏิกิริยาการเปลี่ยน carbamoyl aspartate ไปเป็น dihydrorotate ได้อย่างบริสุทธิ์จากเชื้อมาลาเรียของคนที่เพาะเลี้ยงได้มากพอในห้อง ทดลอง และได้ทำการโคลนจีน DHO ซึ่งพบว่าอยู่บนโครโมโซมคู่ที่ 14 แล้วนำไปแสดงออกของจีนนี้ในแบคทีเรียอีโคไล ทำการแยกเอนไซม์รีคอมบิแนนท์ให้บริสุทธิ์และมีการทำงานได้ประสิทธิภาพสูง เอนไซม์ที่เตรียมได้ทั้งจากเชื้อมาลาเรียและรีคอมบิแนนท์ข้างต้นแสดงคุณสมบัติการเร่งปฏิกิริยาแบบโมเลกุลเชิงเดี่ยวได้เหมือนกับคุณสมบัติของเอนไซม์แบบที่ 2 ที่มีผู้ศึกษามาก่อน และยังแสดงคุณสมบัติทั้งในแง่จลนศาสตร์และผลการใช้สารยับยั้งเอนไซม์ เช่น orotate และอนุพันธ์ของมันเหมือนกับคุณสมบัติของเอนไซม์แบบที่ 1 ที่พบได้ในเอนไซม์ของสิ่งมีชีวิตชั้นสูงรวมทั้งของคนด้วย ผลการวิจัยนี้ได้รับการตีพิมพ์และเก็บข้อมูลยีนไว้ในฐาน ข้อมูลสากล

2. ได้ทำการโคลนจีน OPRT และ OMPDC จากเชื้อมาลาเรียของคน ซึ่ง OPRT เร่งปฏิกิริยาการเปลี่ยน orotate และ 5-phosphoribosyl-1-pyrophosphate ไปเป็น orotidine 5'-monophosphate (OMP) และ OMPDC เร่งปฏิกิริยาการเปลี่ยน OMP ไปเป็น uridine 5'-monophosphate (UMP) แล้วนำไปแสดงออกของจีนทั้งสองในแบคทีเรียอีโคไล ทำการแยกเอนไซม์รีคอมบิแนนท์ทั้งสองให้บริสุทธิ์ เอนไซม์ที่ได้ทำงานมีประสิทธิภาพสูง ได้ศึกษาเปรียบเทียบกับเอนไซม์ของคนที่มีการศึกษาไว้ก่อนหน้านี้แล้วพบ ว่ามีคุณสมบัติแตกต่างกันระหว่างเอนไซม์นี้ในเชื้อมาลาเรียและในคนอย่างสิ้นเชิง จากนั้นนำเอนไซม์รีคอมบิแนนท์ *Pf*OMPDC ที่บริสุทธิ์สูงและมีปริมาณมากพอ ไปทำให้เกิดผลึกโดยอาศัยสาร polyethylene glycol เป็นตัวตกผลึก นำผลึกที่ได้ไปทำให้เกิดการหักเหของ X-ray โดยใช้เครื่อง synchrotron ที่กำลังขยาย 2.7 อังสตรอม สมมาตรของ

ผลึกเป็น trigonal และ R3 space group ผลการวิจัยนี้มีการร่วมมือกับ Professor T. Horii และคณะ แห่ง Osaka University และได้รับการตีพิมพ์

3. ได้ทำการวิเคราะห์โครงสร้างสามมิติของเอนไซม์ *Pf*OMPDC ในรูปแบบของเอนไซม์อิสระ ของเอนไซม์ที่มี substrate OMP จับอยู่ และของเอนไซม์ที่มี product UMP จับอยู่แทน OMP โดยการนำผลึกของเอนไซม์ทั้ง 3 รูปแบบข้างต้นที่ได้ ไปใช้เครื่อง synchrotron ที่ Spring-8 Japan ที่กำลังขยายขนาด 2.7, 2.65 และ 2.6 อังสตรอม ตามลำดับ โครงสร้างสามมิติของเอนไซม์ที่ได้ ทำให้ทราบกลไกการเร่งปฏิกิริยาเริ่มตั้งแต่การที่ substrate OMP จับกับ active site มีการจัดเรียงใหม่ของ hydrogen network มีการเปลี่ยนแปลงของโครงสร้างสามมิติของเอนไซม์ และการกำจัดหมู่คาร์บอกซีลออกจาก OMP ให้ได้ UMP ในที่สุด โครงสร้างสามมิติของเอนไซม์ที่ได้นำไปเป็นตำแหน่งเป้าหมายใหม่ในการใช้ออกแบบและการพัฒนายารักษามาลาเรีย ซึ่งกลุ่มของเราเป็นกลุ่มแรกที่ได้โครงสร้างสามมิติของเอนไซม์ OMPDC จากเชื้อมาลาเรียของคนชนิดฟัลซิพารัม ผลการวิจัยนี้มีการร่วมมือกับ Professor T. Horii และคณะ แห่ง Osaka University และได้รับการตีพิมพ์และเก็บข้อมูลโครงสร้างสามมิติของเอนไซม์ทั้ง 3 รูปแบบไว้ในฐานข้อมูลสากล

4. ได้ทำการโคลนจีน OPRT และ OMPDC ที่ถูกตัดด้านปลายอะมิโนให้สั้นลงจากเชื้อมาลาเรียของคน ซึ่งทั้ง OPRT และ OMPDC ของเชื้อมีขนาดใหญ่กว่าเอนไซม์เดียวกันที่อยู่ในสิ่งมีชีวิตชั้นสูงรวมทั้งของคนโดยพบว่าที่ด้านปลายอะมิโนของเอนไซม์จากเชื้อมีจำนวนกรดอะมิโนเพิ่ม ขึ้น แล้วนำไปแสดงออกของยีนส์ที่สั้นลงทั้งสองในแบคทีเรียอีโคไล ซึ่งยีน truncated mutant OPRT เท่านั้นที่มีการแสดงออกและในปริมาณที่ต่ำมาก แต่ก็ทำการแยกเอนไซม์รีคอมบิแนนท์ mutant OPRT ออกมาได้และมีความเสถียรและมีประสิทธิ ภาพที่ไม่ดีเมื่อเทียบกับเอนไซม์รีคอมบิแนนท์ที่เป็น wild type OPRT ที่ได้จากข้อ 2 งานวิจัยส่วนนี้จะมีการดำเนินต่อไปโดยเป็นวิทยานิพนธ์ของนิสิตระดับปริญญาเอก 2 คน

ได้เสนอว่าเอนไซม์ที่ศึกษานี้ใช้เป็นตำแหน่งเป้าหมายใหม่ในการพัฒนายารักษามาลาเรีย และควรศึกษาเอนไซม์ที่ใช้ในการสังเคราะห์สารไพริมิดีนให้ครบทั้งระดับจีนและโปรตีนเหล่านี้ เพื่อสร้างองค์ความรู้นำไปใช้ออกแบบและพัฒนายารักษามาลาเรียตัวใหม่ โดยมีตำแหน่งเป้าหมายที่การสังเคราะห์เบสไพริมิดีนในเชื้อมาลาเรีย คุณสมบัติจำเพาะของเอนไซม์ในเชื้อมาลาเรียน่าจะมีความสำคัญในแง่การเกิดวิวัฒนาการของเอนไซม์ที่พบในเซลล์ของสัตว์ชั้นต่ำและชั้นสูง สัตว์เลี้ยงลูกด้วยนมรวมทั้งของคนด้วย นอกจากนี้ความสำคัญในการควบคุมการทำงานของเอนไซม์ทั้งสองในวิถีการสังเคราะห์เบสไพริมิดีน คงต้องศึกษาวิจัยต่อไป

ผลงานวิจัยของโครงการนี้ในช่วงเวลา 3 ปีที่ได้รับทุนสนับสนุน มีการผลิตปริญญาโท-เอก มีการเสนอผลงาน มีการร่วมมือกับต่างประเทศ มีการเก็บข้อมูลยีนและข้อมูลโครงสร้างสามมิติของเอนไซม์ไว้ในฐานข้อมูลสากล มีผลงานวิจัยจากโครงการโดยตรงได้รับการตีพิมพ์จำนวน 4 เรื่อง (ลำดับ 1-4) และจากโครงการทางอ้อมอีก 4 เรื่อง (ลำดับ 5-8) ดังนี้

1) Crystallization and preliminary crystallographic analysis of orotidine 5'-monophosphate decarboxylase from the human parasite *Plasmodium falciparum*. *Acta Crystallographica* **F62**, 542-545 (2006)

2) Structural basis for the decarboxylation of orotidine 5'-monophosphate (OMP) by *Plasmodium falciparum* OMP decarboxylase. *Journal of Biochemistry* **143**, 69-78 (2008)

3) Dihydroorotase of human malarial parasite *Plasmodium falciparum* differs from host enzyme. *Biochemical and Biophysical Research Communication* **366**, 821-826 (2008)

4) Malaria parasite: genomics, biochemistry and drug target for antimalarial development. *Chulalongkorn Medical Journal* **50**, 127-142 (2006)

5) Malarial parasite carbonic anhydrase and its inhibitors. *Current Topics in Medicinal Chemistry* **7**, 909-917 (2007)

6) The alpha-carbonic anhydrase from the malarial parasite and its inhibition. *Current Pharmaceutical Design* **14**, 631-640 (2008)

7) Putative metabolic roles of the mitochondria in asexual blood stages and gametocytes of the malaria parasite. *Asian Pacific Journal of Tropical Medicine* **1**, 31-49 (2008)

8) Biochemistry research in Thailand: present status and foresight studies. *ScienceAsia* **34**, 1-6 (2008)

Appendix
(ภาคผนวก)

2. Reprints

1) Krungkrai S.R., Kusakari, Y., Tokuoka, K., Inoue, T., Adachi, H., Matsumura, H., Takano, K., Murakami, S., Mori, Y., Kai, Y., Krungkrai, J., and Horii, T.(2006) Crystallization and preliminary crystallographic analysis of orotidine 5'-monophosphate decarboxylase from the human parasite *Plasmodium falciparum*. *Acta Crystallographica* **F62**, 542-545.

2) Tokuoka K., Kusakari Y., Krungkrai S.R, Matsumura H., Kai Y., Krungkrai J., Horii T., Inoue T. (2008) Structural basis for the decarboxylation of orotidine 5'-monophosphate (OMP) by *Plasmodium falciparum* OMP decarboxylase. *Journal of Biochemistry* **143**, 69-78.

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4) Krungkrai, J., and Krungkrai, S. (2006) Malaria parasite: genomics, biochemistry and drug target for antimalarial development. *Chulalongkorn Medical Journal* **50**, 127-142.

5) Krungkrai, J., Krungkrai, S.R., and Supuran, C.T. (2007) Malarial parasite carbonic anhydrase and its inhibitors. *Current Topics in Medicinal Chemistry* **7**, 909-917. (First page)

6) Krungkrai, J., and Supuran, C.T. (2008) The alpha-carbonic anhydrase from the malarial parasite and its inhibition. *Current Pharmaceutical Design* **14**, 631-640. (First page)

7) Krungkrai, J., Kanchanaphum, P., Pongsabut, S., and Krungkrai, S.R. (2008) Putative metabolic roles of the mitochondria in asexual blood stages and gametocytes of the malaria parasite. *Asian Pacific Journal of Tropical Medicine* **1**, 31-49. (First page)

8) Krungkrai, J., Incharoensakdi, A., and Tungpradabkul, S. (2008) Biochemistry research in Thailand: present status and foresight studies. *ScienceAsia* **34**, 1-6. (First page)

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Crystallization and preliminary crystallographic analysis of orotidine 5′-monophosphate decarboxylase from the human malaria parasite *Plasmodium falciparum*

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Crystallization and preliminary crystallographic analysis of orotidine 5′-monophosphate decarboxylase from the human malaria parasite *Plasmodium falciparum*

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Orotidine 5′-monophosphate (OMP) decarboxylase (OMPDC; EC 4.1.1.23) catalyzes the final step in the *de novo* synthesis of uridine 5′-monophosphate (UMP) and defects in the enzyme are lethal in the malaria parasite *Plasmodium falciparum*. Active recombinant *P. falciparum* OMPDC (*Pf*OMPDC) was crystallized by the seeding method in a hanging drop using PEG 3000 as a precipitant. A complete set of diffraction data from a native crystal was collected to 2.7 Å resolution at 100 K using synchrotron radiation at the Swiss Light Source. The crystal exhibits trigonal symmetry (space group *R*3), with hexagonal unit-cell parameters $a = b = 201.81$, $c = 44.03$ Å. With a dimer in the asymmetric unit, the solvent content is 46% ($V_M = 2.3$ Å^3 Da^{-1}).

1. Introduction

There are an estimated 300–500 million cases of malaria and up to three million people die from this disease annually. *Plasmodium falciparum* is the causative agent of the most lethal and severe form of human malaria (Guerin *et al.*, 2002). Chemotherapy of malaria is available, but is complicated by both adverse effects and widespread resistance to most of the currently available antimalarial drugs (Attaran *et al.*, 2004; White, 2004). More efficacious and less toxic agents that uniquely target the parasite are therefore required.

The malaria parasite depends on *de novo* synthesis of pyrimidine nucleotides, whereas the human host has the ability to synthesize them by both *de novo* and salvage pathways (Krungkrai *et al.*, 1990; Jones, 1980; Weber, 1983). The final two steps of uridine 5′-monophosphate (UMP) synthesis require the addition of ribose 5-phosphate from 5-phosphoribosyl-1-pyrophosphate (PRPP) to orotate by orotate phosphoribosyltransferase (OPRT; EC 2.4.2.10) to form orotidine 5′-monophosphate (OMP) and the subsequent decarboxylation of OMP to form UMP by OMP decarboxylase (OMPDC; EC 4.1.1.23). Both enzymes are encoded by two separate genes in most prokaryotes and the malaria parasite (Krungkrai *et al.*, 2003; Krungkrai, Aoki *et al.*, 2004), whereas their genes in most multicellular organisms, including humans, are fused into a single gene, resulting in the bifunctional UMP synthase (Livingstone & Jones, 1987; Suttle *et al.*, 1988; Suchi *et al.*, 1997). Our recent studies have demonstrated that the two enzymes exist as a heterotetrameric $(OPRT)_2(OMPDC)_2$ complex in two species of malaria parasites, *P. falciparum* and *P. berghei* (Krungkrai, Prapunwattana *et al.*, 2004; Krungkrai *et al.*, 2005). By multiple sequence-alignment analysis, the protein sequence of *P. falciparum* OMPDC was found to be less than 20% identical to both the human enzyme (Suttle *et al.*, 1988) and the four bacterial and yeast OMPDCs for which crystal structures are known, *i.e. Bacillus subtilis* (Appleby *et al.*, 2000), *Escherichia coli* (Harris *et al.*, 2000), *Methanobacterium thermoautotrophicum* (Wu *et al.*, 2000) and *Saccharomyces cerevisae* (Miller *et al.*, 2000). The low sequence identities make it difficult to build a homology model for *P. falciparum* OMPDC. However, the low similarity between the enzymes of the host and the pathogen mean that OMPDC has promise as a drug target. In addition, OMPDC is known to be an unusually proficient catalyst (Miller & Wolfenden, 2002). These facts have prompted us to perform crystallization and X-ray diffraction analysis of the monofunctional *P. falciparum* OMPDC (*Pf*OMPDC)

expressed in *E. coli*. Here, we report the conditions for expression, purification, crystallization and preliminary crystallographic analysis of the uncomplexed apo form of *Pf*OMPDC.

2. Experimental

2.1. Protein expression and purification

The recombinant OMPDC protein was prepared by cloning and expression of the gene encoding *Pf*OMPDC in *E. coli*. The oligonucleotide primers were 5′-CG**G GAT CC**A TGG GTT TTA AGG TAA AAT TA-3′ and 5′-CC**A TCG AT**T TAC GAT TCC ATA TTT TGC TTT AA-3′, which encompass *Bam*HI and *Cla*I restriction sites (in bold), respectively. The polymerase chain reaction was carried out using *P. falciparum* cDNA as a template and *Pfu* DNA polymerase (Promega) at 368 K for 5 min, followed by 40 cycles of 368 K for 1 min, 328 K for 1 min and 341 K for 3 min and finally an additional 341 K for 10 min. The PCR-derived fragment of ∼980 bp complete *Pf*OMPDC was inserted into the expression vector pTrcHis-TOPO (Invitrogen) linearized with *Bam*HI and *Cla*I. *E. coli* TOP10 (Invitrogen) cells were transformed with the recombinant plasmid and induced with 1 m*M* isopropyl β-D-thiogalactopyranoside at 291 K for 18–20 h and the cell paste was then harvested by centrifugation at 8000*g* for 10 min. The recombinant enzyme was expressed as a soluble protein and purified using an Ni^{2+}–nitrilotriacetic acid–agarose affinity column (Qiagen) with 50 m*M* Tris–HCl pH 8.0, 300 m*M* NaCl, 250 m*M* imidazole and 10% glycerol as the elution buffer. The recombinant protein was further purified by AKTA fast protein liquid chromatography using a Superdex-75 HiLoad 26/60 column (Amersham Biosciences) equilibrated with 50 m*M* Tris–HCl pH 8.0, 300 m*M* NaCl, 5 m*M* dithiothreitol. The enzyme activity was eluted as a single symmetrical peak at a position corresponding to 76 kDa. SDS–PAGE analysis (Laemmli, 1970) showed a homogenous preparation with a molecular weight of 38 kDa, suggesting that the active form of the *Pf*OMPDC is a dimer. The OMPDC activity was monitored by the decrease in the absorbance of OMP at 285 nm according to a previously described method (Yablonski *et al.*, 1996). The purified recombinant enzyme had a specific activity of approximately 10 µmol min^{-1} per milligram of protein, corresponding to a 300-fold purification and 30% yield. Up to 30 mg of the pure recombinant protein was obtained from 10 l *E. coli* cell culture. The purified protein was concentrated by Centricon ultrafiltration to 10 mg ml^{-1} and the homogeneity was confirmed by dynamic light scattering using a DynaPro-MS/X (Protein Solutions Inc.).

2.2. Crystallization of *Pf*OMPDC

Purified *Pf*OMPDC in 50 m*M* Tris–HCl pH 8.0 buffer containing 300 m*M* NaCl and 5 m*M* dithiothreitol was concentrated to 10 mg ml^{-1}. Crystallization of *Pf*OMPDC was first performed using the sitting-drop vapour-diffusion method at 293 K with the semi-automatic crystallization robot SHOZO (Adachi *et al.*, 2005). The crystallization conditions were initially screened by a sparse-matrix

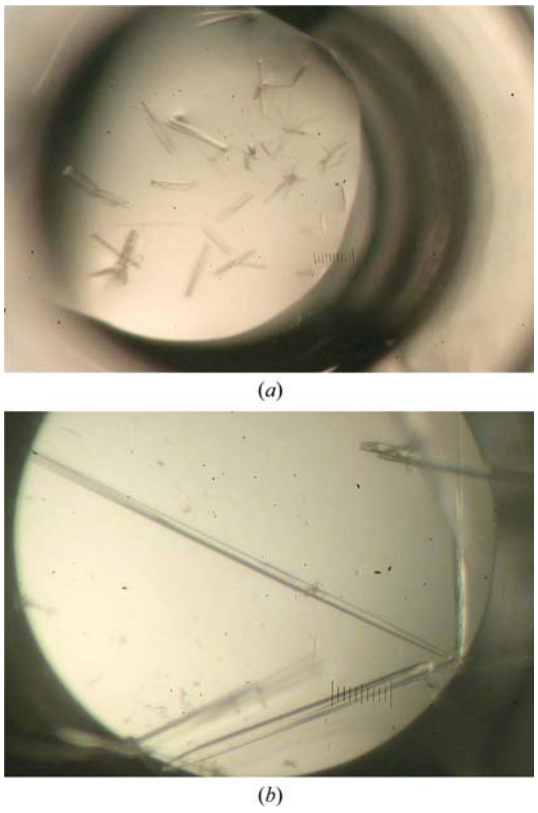

Figure 1
Crystals of OMPDC from *P. falciparum*. Thin crystals were obtained in the first crystallization trial in a sitting drop (*a*); however, the quality of the crystals was improved by using the seeding method in a hanging drop (*b*).

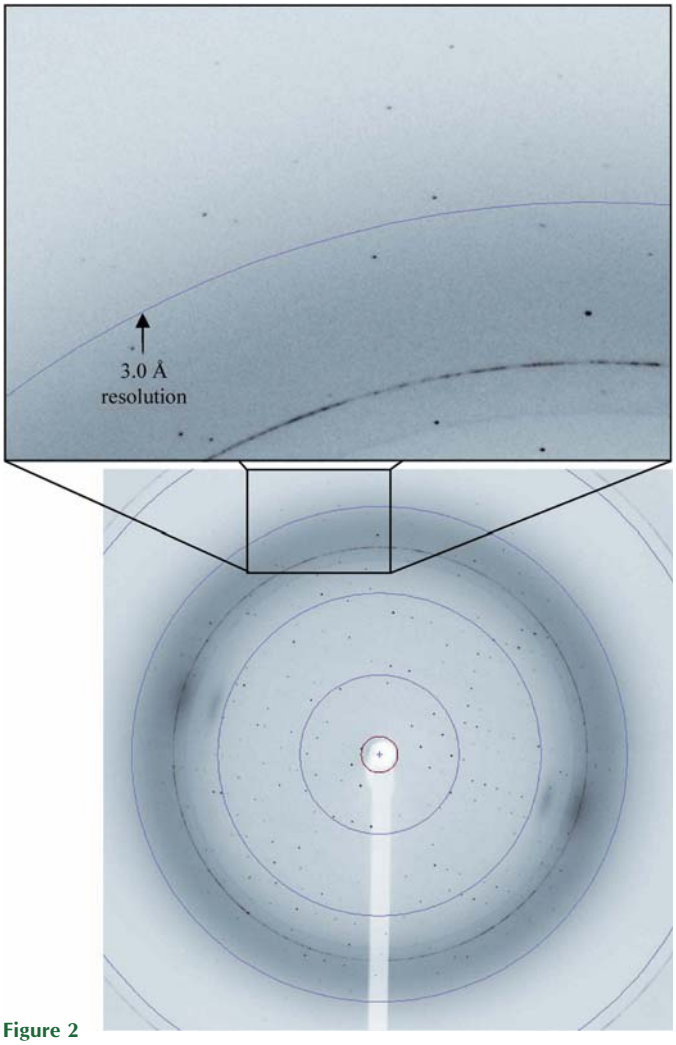

Figure 2
X-ray diffraction image from a OMPDC crystal. The frame edge in the close-up is 2.7 Å.

sampling method (Jancarik & Kim, 1991) using Crystal Screens I and II (Hampton Research) and Wizard Screens I and II (Emerald BioStructures) in 96-well sitting-drop plates (Corning) at 293 K. The drop size was 1 µl protein solution (10 mg ml^{-1}) plus 1 µl of one of the precipitating reagents. The reservoir contained 0.2 ml of the same reagent. From the 200 crystallization conditions, needle-shaped crystals with maximum dimensions of 0.03 × 0.03 × 1.2 mm appeared in the drops using Crystal Screen II solutions No. 30 [10% PEG 6K, 0.1 *M* HEPES pH 7.5, 5% 2-methyl-2,4-pentanediol (MPD)] and No. 37 (10% PEG 8K, 0.1 *M* HEPES pH 7.5, 8% ethylene glycol) and Wizard Screen I solution No. 41 (30% PEG 3K, 0.1 *M* CHES pH 9.5) within 2 d at 293 K (Fig. 1). While the crystals that appeared from Crystal Screen II solutions No. 30 and No. 37 were fragile, those from Wizard Screen II solution No. 41 were suitable for X-ray crystallographic studies after size improvement by the seeding method using 13%(*w*/*v*) and 18%(*w*/*v*) PEG 3K solutions for the sitting drop and the mother liquor, respectively.

3. Data collection and processing

X-ray diffraction data were collected from a single crystal of *Pf*OMPDC on the X06SA beamline at the Swiss Light Source (Paul Scherrer Institute, Villigen, Switzerland). Prior to data collection, the crystal of *Pf*OMPDC was soaked in a cryoprotectant solution consisting of 18%(*w*/*v*) PEG 3K, 0.1 *M* CHES pH 9.5, 8% MPD. The crystal of *Pf*OMPDC was then mounted in a standard nylon loop in a stream of cold nitrogen gas at 100 K. The diffraction patterns were recorded on a MAR CCD detector (MAR USA) at cryogenic temperature (100 K). The wavelength, crystal-to-detector distance, oscillation range and exposure time were 0.978 Å, 250 mm, 1.0° and 1 s, respectively. A complete data set was collected from 200 images covering 200° in total (Fig. 2).

The diffraction intensity data were processed and scaled using *MOSFLM* (Leslie, 1992). The crystal of *Pf*OMPDC is trigonal, belonging to space group *R*3, with unit-cell parameters $a = b = 201.81$, $c = 44.03$ Å. From the 451 733 accepted observations to 2.7 Å resolution, 18 313 unique reflections were obtained. The statistics of the diffraction data are shown in Table 1.

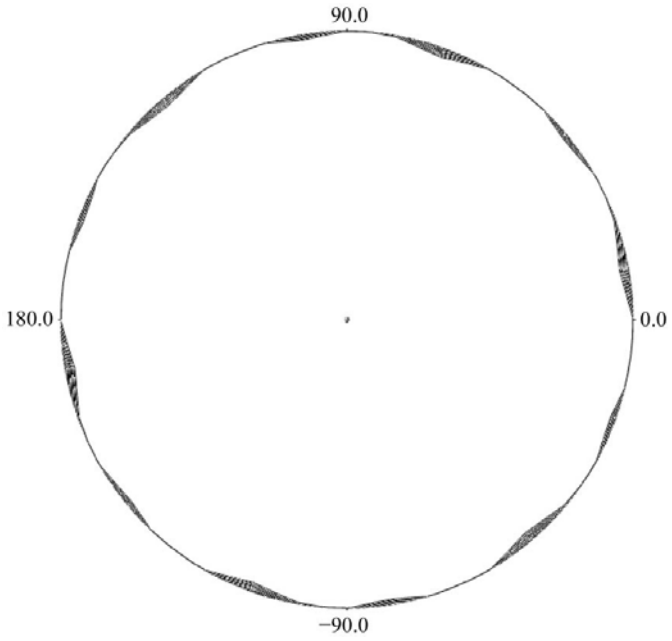

Figure 3
Stereographic projections of the self-rotation function calculated in the $\kappa = 180°$ section. The resolution of the data used was 15.0–2.7 Å. The X-ray data were reduced in a trigonal crystal system using a hexagonal cell. 12 peaks were clearly obtained in the calculated self-rotation map, suggesting that a non-crystallographic twofold axis exists in the asymmetric unit.

Table 1
Crystal parameters and X-ray diffraction data-collection statistics.

Values in parentheses are for the highest resolution shell (2.85–2.70 Å).

Crystal system	Trigonal with hexagonal unit cell
Space group	*R*3
Unit-cell parameters (Å, °)	$a = b = 201.81$, $c = 44.03$, $\alpha = \beta = 90.0$, $\gamma = 120.0$
Resolution range (Å)	50.0–2.70
No. of molecules in ASU	2
V_M (Å^3 Da^{-1})	2.3
V_{solv} (%)	46
No. of measured reflections	451733
No. of unique reflections	18313
R_{merge}† (%)	5.9 (27.5)
Completeness (%)	99.6 (100.0)
Average $I/\sigma(I)$	12.4 (4.0)

† $R_{merge} = \sum |I(k) - I| / \sum I(k)$, where $I(k)$ is value of the kth measurement of the intensity of a reflection, I is the mean value of the intensity of that reflection and the summation is over all measurements.

The self-rotation function calculated with the program *POLARRFN* from the *CCP*4 program package (Collaborative Computational Project, Number 4, 1994) suggested that two *Pf*OMPDC molecules correlated by a non-crystallographic twofold axes were present in the asymmetric unit (Fig. 3), giving a Matthews coefficient of 2.3 Å^3 Da^{-1} and a solvent content of 46%.

Preliminary molecular-replacement calculations were performed with the program *AMoRe* (Navaza, 2001) from the *CCP*4 program package (Collaborative Computational Project, Number 4, 1994) using the structure of OMPDC from *B. subtilis* (PDB code 1dbt) as the search model (Appleby *et al.*, 2000). Since the identity of the amino-acid sequences between *Pf*OMPDC and *B. subtilis* OMPDC is only 16%, MAD data collection using SeMet-derivative crystals was attempted. During the phase calculation using the anomalous dispersion signal, the crystal structure of OMPDC from *P. vivax* was deposited in the PDB (PDB code 2ffc). The deposited structure is that of a complex with UMP, demonstrating a product-binding form. The space group of the crystal is $P2_12_12$, in contrast to the crystal reported here, which belongs to space group *R*3. The molecular-replacement method using this structure and a structural comparison between the apo and holo forms are in progress.

The authors are grateful to Mr T. Tomizaki, Paul Scherrer Institute for his kind help during data collection on the X06SA beamline at the Swiss Light Source (SLS). This work was partially supported by the Thailand Research Fund (BRG 4880006) to JK and SRK. This work is also partly founded by Grant-in-Aids for Scientific Research 16017260 (to TI) and 13226058 (to TH) and CLUSTER (to TI and TH) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, by grants from the CREST Project (to YM), the Japan Science and Technology Agency and partly from the National Project on Protein Structural and Functional Analyses, Japan (to TI). One of the authors (KT) expresses his special thanks to the Center of Excellence (21st Century COE) program 'Creation of Integrated EcoChemistry of Osaka University'.

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Structural Basis for the Decarboxylation of Orotidine 5′-Monophosphate (OMP) by *Plasmodium Falciparum* OMP Decarboxylase†

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Orotidine 5′-monophoshate decarboxylase (OMPDC) catalyses the decarboxylation of orotidine 5′-monophosphate (OMP) to uridine 5′-monophosphate (UMP). Here, we report the X-ray analysis of apo, substrate or product-complex forms of OMPDC from *Plasmodium falciparum* (*Pf*OMPDC) at 2.7, 2.65 and 2.65 Å, respectively. The structural analysis provides the substrate recognition mechanism with dynamic structural changes, as well as the rearrangement of the hydrogen bond array at the active site. The structural basis of substrate or product binding to *Pf*OMPDC will help to uncover the decarboxylation mechanism and facilitate structure-based optimization of antimalarial drugs.

Key words: apo form, OMP-complex, orotidine 5′-monophoshate decarboxylase, structural comparison, UMP-complex, x-ray structural analysis.

Abbreviations: OMP, orotidine 5′-monophosphate; OMPDC, OMP decarboxylase; PfOMPDC, OMPDC from *Plasmodium falciparum*; OPRT, orotate phosphoribosyltransferase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Human malaria is caused by four species of the parasitic protozoan genus *Plasmodium*. Of these four species, *Plasmodium falciparum* is responsible for the vast majority of the 500 million episodes of malaria worldwide, and accounts for 2–3 million deaths annually (*1*). Chemotherapy of malaria is available, but is complicated by drug toxicity and widespread resistance to most of the currently available antimalarial drugs (*2*). There is an urgent need for more efficacious and less-toxic agents, particularly rational drugs that exploit pathways and targets unique to the parasite. In general, drug-screening procedures have rarely been applied to this disease, and there is a paucity of information on a number of biochemical pathways that can be exploited for chemotheraphy. The malarial parasite is totally dependent on *de novo* pyrimidine biosynthesis for its growth and development because it lacks the relevant salvage enzymes (*3–5*). The *de novo* pathway contains six reaction steps. The initial reaction catalysed by carbamoyl phosphate synthetase is the formation of carbamoyl phosphate by combination of carbonate, ATP and an amino group from glutamine. Three additional reactions are necessary to form the pyrimidine ring from carbamoyl phosphate. In the final two steps, uridine 5′-monophosphate (UMP) requires the addition of a ribose phosphate moiety from 5-phosphoribosyl-1-pyrophosphate to orotate by orotate phosphoribosyltransferase (EC 2.4.2.10, OPRT) to form orotidine 5′-monophosphate (OMP) and pyrophosphate (PP_i), and the subsequently decarboxylation of OMP to form UMP, by OMP decarboxylase (EC 4.1.1.23, OMPDC).

In most prokaryotes and yeasts (*6*, *7*), the OPRT and OMPDC enzymes are encoded by two separate genes, while in most multicellular eukaryotes, the genes for both enzymes have been joined into a single gene, resulting in the expression of a bifunctional protein, namely UMP synthase, with two different catalytic domains (*8–11*). The bifunctional UMP synthase has also been reported in kinetoplastid parasites, e.g. *Trypanosoma cruzi* and *Leishmania mexicana* (*12*, *13*). In contrast, mammalian hosts can utilize both the *de novo* and salvage pathways (*8*, *14*). Inhibitors of the *de novo* pathway have strong antimalarial activity for *in vitro P. falciparum* growth (*15–18*).

The sequence alignment for 10 known crystal structures of OMPDC is shown in Fig. 1. This reveals the existence of 11 totally conserved amino acid residues (shaded in red), corresponding to Asp23, Lys102, Asp136, Lys138, Asp141, Ile142, Gly169, Pro264, Gly265, Gly293 and Arg294 in *P. falciparum*. The alanine replacement around the active site, however, shows that *Plasmodium* OMPDCs have somewhat different sequences (*19*). The most striking observation is that a large insertion

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†Coordinates have been deposited in the Protein Data Bank (accession codes 2ZA2, 2ZA1 and 2ZA3 for the apo form, and OMP- or UMP-complex of PfOMPDC, respectively).

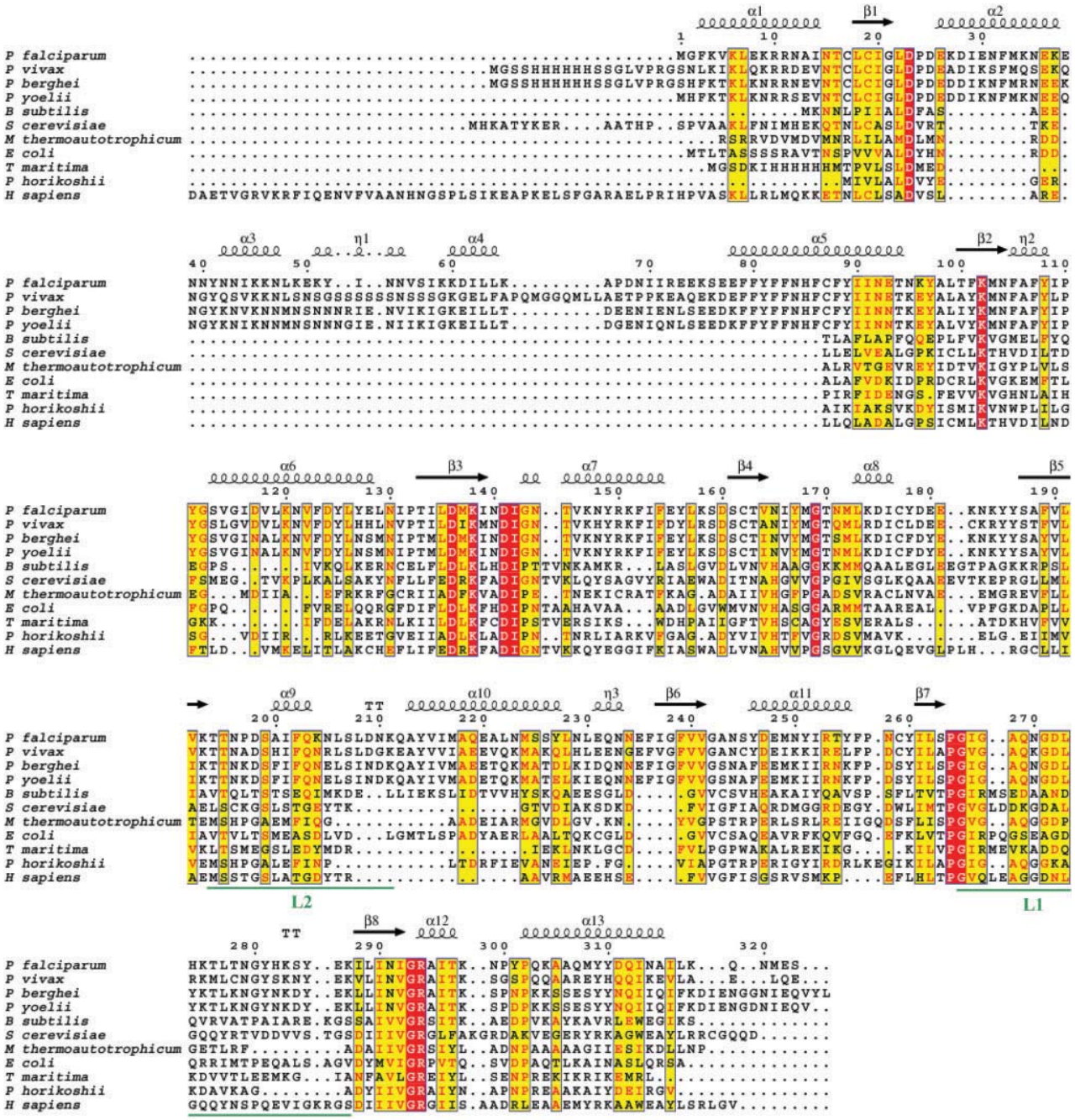

Fig. 1. **Multiple sequence alignments of *Pf*OMPDC compared with those of 10 known crystal structures.** PDB IDs are showed in parentheses: *Plasmodium vivax* (2FFC) (*48*), *Plasmodium berghei* (2FDS) (*48*), *Plasmodium yoelii* (2AQW) (*48*), *Bacillus subtilis* (1DBT) (*24*), *Saccharomyces cerevisiae* (1DQW) (*32*), *M. thermoautotrophicum* (1DV7) (*22*), *E. coli* (1L2U) (*49*), *Thermotoga maritima* (1VQT), *Pyrococcus horikoshii* (2CDZ) and *Homo sapiens* (2EAW). Red-shaded amino acids are conserved in all 10 sequences; yellow-shaded amino acids are similar residues. Above the sequences, the secondary structural elements are shown, as identified in *Pf*OMPDC. The L1 and L2 loops are identified and marked by bottom underlines.

is present at the N-terminal domain, in all *Plasmodium* OMPDCs. It has been proposed that the N-terminal domain of *Pf*OMPDC binds to that of *P. falciparum* OPRT (*Pf*OPRT) (*20*).

Currently, the most accredited mechanisms involve direct decarboxylation of OMP (*21*). This was proposed based on X-ray analysis of the structure of OMPDC–inhibitor complexes (*22–28*), and is supported by a wealth of theoretical and experimental studies (*22*, *29–31*). It has been proposed that the protonation/decarboxylation of OMP could be a concerted event at the direct decarboxylation (*23*). In contrast, some other experimental

evidence supports the formation of a carboanionic intermediate upon decarboxylation (*32*, *33*). In both cases, kinetic studies have unambiguously identified Lys72 (*Methanobacterium thermoautotrophicum* numbering) as the residue that protonates the C6 center (*21*, *34*, *35*). Another recently proposed catalytic mechanism assumes a preliminary protonation of the uracil ring far from the decarboxylation center. Kollman (*36*), assuming a protonated Asp70, has proposed as a first step in decarboxylation, an interesting mechanism involving an enamine protonation at C5 carried out by the Lys72 residue (*M. thermoautotrophicum* numbering). Furthermore, based on *ab initio* calculations, it has been proposed that O2 and O4 are protonated prior to decarboxylation (*36*, *37*). The latest proposals for this reaction mechanism strongly support direct decarboxylation of OMP, with the formation of a carboanionic intermediate via a transition-state stabilization mechanism, in which the fully conserved Lys72 (*M. thermoautotrophicum* numbering) plays a fundamental role by stabilizing the forming negative charge at C6, and consequently, the transition state (*31*).

In this study, we report the X-ray analysis of the apo, substrate- and product-complex forms of OMPDC from *P. falciparum* (*Pf*OMPDC). This structural analysis provides us with a substrate recognition mechanism with dynamic structural changes, as well as the rearrangement of the hydrogen-bond array occurring during enzymatic catalysis at the active site. We also propose a protruding domain at the N-terminal insertion of *Pf*OMPDC is responsible for binding to *Pf*OPRT.

MATERIALS AND METHODS

Protein Expression and Purification—The recombinant OMPDC protein was prepared by cloning and expression of the gene encoding *Pf*OMPDC in *Escherichia coli*. The oligonucleotide primers were 5′-CG**G GAT CC**A TGG GTT TTA AGG TAA AAT TA-3′ and 5′-CC**A TCG ATT** TAC GAT TCC ATA TTT TGC TTT AA-3′, which includes encompass *BamH*I and *Cla*I restriction sites (in bold), respectively. Polymerase chain reaction (PCR) was carried out using *Pfu* DNA polymerase (Promega) at 95°C for 5 min, followed by 40 cycles of 95°C for 1 min, 55°C for 1 min and 68°C for 3 min, with a final 68°C for 10 min. The expected 980 bp product for full-length *Pf*OMPDC was inserted into the linearized expression vector pTrcHis-TOPO (Invitrogen). *Escherichia coli* TOP10 (Invitrogen) cells were transformed with the recombinant plasmid and induced with 1 mM isopropyl β-D-thiogalactopyranoside at 37°C for 18–20 h, and the cell paste was harvested by centrifugation at 8000*g* for 10 min. The recombinant enzyme was expressed as a soluble protein, and further purified by using a Ni^{2+}-nitrilotriacetic acid-agarose affinity column (Qiagen) with 50 mM Tris–HCl, pH 8.0, 300 mM NaCl, 250 mM imidazole and 10% glycerol as the eluting buffer. Further purification was achieved using a Superdex-75 Hiload 26/60 column (GE Healthcare) on ÄKTA fast protein liquid chromatography (GE Healthcare) equilibrated with 50 mM Tris–HCl, pH 8.0, 300 mM NaCl, 5 mM dithiothreitol. The enzyme activity was eluted as a single symmetrical peak at a position of 76 kDa. SDS–PAGE analysis (*38*) showed that the homogeneous preparation had a molecular mass of 38 kDa. The active form of *Pf*OMPDC was a dimer. OMPDC activity was monitored by a decrease in absorbance of OMP at 285 nm, as described previously (*11*). The purified recombinant enzyme had a specific activity of $\sim$10 μmol min^{-1} (mg protein)$^{-1}$, 300-fold purification, and 30% yield. Up to 30 mg pure recombinant protein was obtained from 10 L of the *E. coli* cell culture. The purified protein was concentrated by Centricon ultrafiltration to 10 mg mL^{-1}, and the homogeneity was confirmed by dynamic light scattering with DynaPro-MS/X (Protein Solutions).

Data Collection and Processing—The apo form was crystallized by a previously reported method (*39*). Complex crystals with OMP or UMP were prepared by soaking in a solution containing 10 mM OMP or UMP for 2 min.

X-ray diffraction data from the apo form were collected at X06SA beamline (Swiss Light Source, Switzerland) and from OMP- or UMP-complex at BL38 beamline (SPring-8, Japan). Prior to data collection, crystals of apo form and OMP- or UMP-complex of *Pf*OMPDC were soaked in a cryoprotectant solution consisting of 23% (w/v) PEG3K, 0.1 M CHES (pH 9.5) and 8% glycerol. The apo form and OMP- or UMP-complex were then mounted in a standard nylon loop in a stream of liquid nitrogen at 100 K. The diffraction patterns were recorded on a MarCCD detector (Mar Research) for the apo form, and on an ADSC Quantum detector (Area Detector Systems Corporation) for the complexes at cryogenic temperature (100 K).

The diffraction intensity data were processed and scaled using MOSFLM (*40*) and HKL2000 (*41*). The statistics of the diffraction data collection are shown in Table 1.

Structure Determination and Refinement—The molecular replacement calculations were performed with the program MOLREP (*42*) in the CCP4 program package (*43*), using the PO_4-complex of *Pf*OMPDC (PDB code: 2f84) as the search model. A dimer molecule was located in an asymmetric unit of the crystal. Structure models of the apo form were refined against the diffraction data using CNS (*44*) and REFMAC (*45*). The models were manually adjusted by electron density map using COOT (*46*). Even after several cycles of refinement, the electron density for residues 70–74 and 268–274 for one subunit, and 68–70 and 268–272 for the other subunit, was poor. Some other parts of the side chain structure (residues 1, 35, 69, 75, 76, 79, 199, 201, 255, 269, 275, 281, 301, 306, 314, 317 and 318 for one subunit; and residues 1, 71, 96, 174, 207, 222, 231, 255, 266, 269, 273, 274, 275, 281, 306, 314, 317 and 318 for the other subunit) also had poor electron density. The models for these residues were built by using alanine. All models were refined for a few cycles of CNS with B-factor refinement until convergence. The coordinates of the apo, and OMP- or UMP-complex forms of *Pf*OMPDC was deposited as 2ZA2, 2ZA1 and 2ZA3 in the Protein Data Bank. Final refinement statistics for the refined coordinate sets for three structures are shown in Table 1.

Table 1. **Data collection and refinement statistics.**

	apo form	OMP-complex	UMP-complex
Diffraction data			
Space group cell dimensions (Å)	*R3*	*R3*	*R3*
a	201.81	201.87	202.49
b	201.81	201.87	202.49
c	44.03	44.19	44.36
Resolution (Å)	50.0–2.70 (2.85–2.70)	50.0–2.60 (2.69–2.60)	50.0–2.60 (2.69–2.60)
Measured reflections (n)	57,278	105,551	65,099
Unique reflections (n)	18,313	20,677	20,877
Completeness (%)	99.6 (100.0)	98.8 (99.2)	99.0 (99.9)
R^{a}_{merge} (%)	5.9 (27.5)	8.6 (33.9)	8.7 (31.7)
Refinement statistics			
Resolution (Å)	50–2.70	50–2.65	50–2.65
R^{b}_{cryst} (%)	21.6	20.8	21.1
R^{c}_{free} (%)	31.2	28.3	29.4
No. of water molecules	39	134	56
Root mean square deviation bond length (Å)	0.007	0.009	0.007
Root mean square deviation bond angle (°)	1.4	1.9	1.4

[a] $R_{merge} = \sum|I(k) - I|/\sum I(k)$, where $I(k)$ is value of the kth measurement of the intensity of a reflection, I is the mean value of the intensity of that reflection and the summation is the over all measurements. [b] $R_{cryst} = \sum\|F_o| - |F_c\|/\sum|F_o|$ calculated from 90% of the data, which were used during the course of the refinement. [c] $R_{free} = \sum\|F_o| - |F_c\|/\sum|F_o|$ calculated from 10% of the data, which were used during the course of the refinement.

RESULTS

Overall Structure—The 2.7 Å structure of the apo form of *Pf*OMPDC was solved with R and R_{free} values of 21.6 and 31.2, respectively, by using the MR method with the PO_4-complex of *Pf*OMPDC. *Pf*OMPDC was crystallized in the trigonal space group of $R3$. One dimer molecule existed in the asymmetric unit (Fig. 2a). The dimer interface was consisted of 10 hydrogen-bond interactions, Lys138(A)-Asp141(B), Asn140(A)-Asn165(B), Asn140(A)-Lys138(B), Asp141(A)-Lys138(B), Lys151(A)-Glu26(B), Asn165(A)-Asn140(B), Asn165(A)-Asn165(B), Asn196(A)-Met168(B), Asp198(A)-Asn171(B) and Asp198(A)-Thr170(B) (Fig. 2b). Each OMPDC subunit of this dimeric enzyme folded as an $(\alpha/\beta)_8$ barrel, with eight central β-sheets surrounded by 13 α helices (Fig. 2c). β1, residues 18–21; β2, 100–104; β3, 132–139; β4, 161–164; β5, 187–193; β6, 237–241; β7, 261–263; and β8, 288–292; and α1, residues 3–14; α2, 26–37; α3, 42–47; α4, 60–64; α5, 78–94; α6, 113–128; α7, 143–154; α8, 173–176; α9, 200–203; α10, 213–227; α11, 246–255; α12, 276–279 and α13, 302–315. There were three extra short α helices: η1, residues 51–54; η2, 105–108 and η3, 230–233 (Fig. 2d).

As predicted from protein sequence analysis (Fig. 1), there was a large insertion region (*48–86*), forming α3, η1 and α4 helices and extended α5-helix (Fig. 2b). Also *Pf*OMPDC contained a small insertion region (208–217), extended α10 helix. These insertions are not found in other characterized OMPDC structures outside of the *Plasmodium* species, and appear to be a unique structural feature of the *Plasmodium* OMPDCs.

The active site was located at the open end of the $(\alpha/\beta)_8$ barrel, which corresponded to the carboxy-terminal of the β strands and the amino-terminal of the α helices. In the apo structure, the L1 loop comprised of residues 268–274 was disordered around the active site, while the L1 loop structure was stabilized upon binding of substrate or product. The detailed structural comparison between the apo and holo form is described later.

Structural Changes of L1 and L2 Loops upon Binding of OMP/UMP—The structural analysis of *Pf*OMPDC complexes has been performed with R and R_{free} values of 20.8 and 28.3, respectively for the 2.65 Å structure complexed with OMP, and 21.1 and 29.4, respectively, for the 2.65 Å with UMP.

Comparison between the apo and OMP- or UMP-complex of *Pf*OMPDC showed a root-mean-square deviation of 0.75 and 0.74 Å, respectively, by using for 318 structurally equivalent C_α atom positions. Several notable structural differences were found around the active site near the open end of the $(\alpha/\beta)_8$ barrel upon binding of OMP or UMP.

The L1 loop which was not determined in the apo form, was stabilized upon binding of the ligands, preventing from solvent. Glu269 in the L1 loop access to the pyrimidine ring moiety of OMP or UMP formed the loop structure of L1 (Fig. 3a). While the structure of the α9 helix was formed in the L2 loop region (194–213) in the apo form, the secondary structure unfolded in the presence of OMP or UMP, associated with the movement of Thr195 (Fig. 3b). The difference in C_α position and the dihedral bond angle of C_β–C_γ in Thr195 was calculated to be 3.8 Å and 149.1°, respectively, between the apo and OMP-complex; and 3.6 Å and 150.0°, respectively, between the apo and UMP-complex. It was revealed that both the L1 and L2 loops recognized the pyrimidine ring with the large structural changes. The details of the structural changes to the active site of *Pf*OMPDC are described later.

Change in Active-Site Structure upon Binding of OMP/UMP—Schematic drawings of the OMP- or UMP-complex are shown in Fig. 4a and b, respectively. Both the guanidium group and the backbone amide of Arg294 interacted directly with the phosphate group of OMP

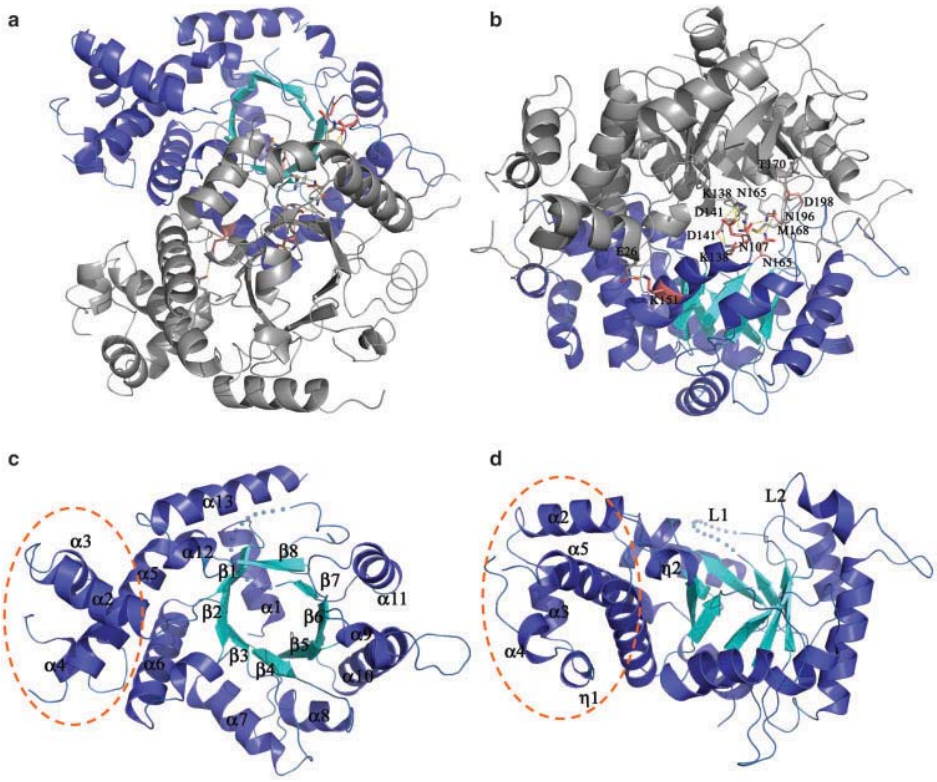

Fig. 2. **Mol-A (colour in blue) and mol-B (colour in gray) forms a dimer structure of *Pf*OMPDC.** (a) Viewed through α/β barrel. (b) Viewed perpendicular to the view in panel 2a. Monomer of *Pf*OMPDC. The α helices (in blue) and β sheets (in cyan) are separated by loops. One subunit folds as an $(\alpha/\beta)_8$ barrel with eight central β sheets surrounded by 13 α helices. The disordered L1 loop is shown as a dotted line. (c) Viewed from same direction as the mol-A in 2a. (d) Viewed from same direction as the mol-A in 2b. A large insertion region from residue 48 to 86, constructed the insertion domain containing three helices, and was presented in dot circle (in orange). The figures were drawn with PYMOL (*50*).

or UMP, as did the amide of Gly293. Thr145(B) and Asn104 hydrogen-bonded to the 2′- and 3′-hydroxyl group of the ribose ring, respectively. In the top part of the pyrimidine ring, comprising O4, N3 and O2, there were hydrogen bonds to Thr195 and Gln269. Thr195 accepted a hydrogen bond from N3 and donated a hydrogen bond to O4. A pocket near C5 of the pyrimidine ring was surrounded by several hydrophobic residues of Ile142(B), Leu191, Thr194, Val240 and Pro264 (not shown in Fig. 4a).

Compared with the apo form, there was a change in active-site structure upon ligand binding. Arg294 and Thr195 in L2, and Gln269 in L1 exhibited a large structural movement upon binding of OMP or UMP. The averaged movement of three nitrogen positions in the guanidium residue of Arg294 were both calculated to be 2.9 Å for the OMP- or UMP-complexes, and the dihedral angles of C_δ–N_ε and N_ε–C_ζ of Arg294 are 120.1° and 87.2° for OMP-, and 64.6° and 118.2° for UMP-complexes, respectively. The helix structure of α12 did not change, while the L1 and L2 loops had the large structural movements described earlier. The average difference in the top part of residues Thr195 and Arg294 was measured to be 1.5 and 1.6 Å, respectively for OMP- and UMP-complexes. On the other hand, in the bottom part of the active site, the C_α atom of Gln293, Asn104 and Thr145(B) adopted almost the same positions between the apo and complex forms. The average difference in the bottom part of residues Asn104, Thr145(B) and Gln293 was 0.3 Å for both the OMP- and UMP-complexes.

The binding mode of OMP was basically the same as that of UMP, except for the residue Lys102. In the OMP binding form, Lys102 hydrogen-bonded to the carboxylate oxygen of pyrimidine. After decarboxylation, Lys102 changed the hydrogen bond to O5 of ribose (Fig. 4).

Rearrangement of Hydrogen-Bond Network Around Lys102—In the apo form, Lys102 formed a hydrogen-bond network with Asp136, Lys138 and Asp141 from the other protomer of the dimer (Fig. 5a). The conserved catalytic array of Lys102-Asp136-Lys138-Asp141(B) makes the hydrogen-bond network at the active site. The amino group of Lys102 recognized the carboxyl group of OMP, and then the distance between Lys102-Asp136 was lengthened from 2.7 to 3.6 Å, breaking the hydrogen bond between Lys102 and Asp136 upon binding of OMP (Fig. 5b and d). The UMP binding form (Fig. 5c and e), which mimicked the structure after decarboxylation, possessed two hydrogen bonds at Lys102-Asp136 and Lys138-Asp141(B) (Fig. 5c). In the UMP-complex, Lys102 hydrogen bonded to Asp136 instead of the carboxyl group of the pyrimidine ring, breaking the hydrogen bond between Asp136 and Lys138, after decarboxylation.

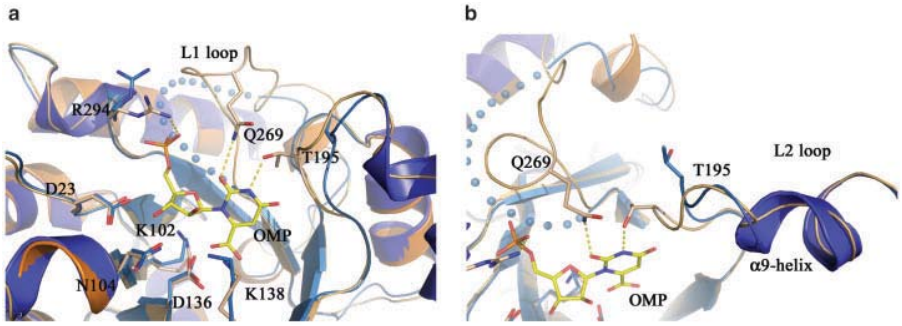

Fig. 3. **Movement of L1 (a) and L2 (b) loops upon bindings of OMP.** Blue and orange structures show the apo form and OMP-complex. (a) The L1 loop, which cannot be unequivocably determined in the apo form (blue dotted line), was stabilized, and the loop structure containing Q269 was formed. (b) The α9 helix only formed in the apo from, and unfolded in association with the movement of Thr195 (C_α distance of 3.8 Å), and then access to the ligand. The figures were drawn with PYMOL (*50*).

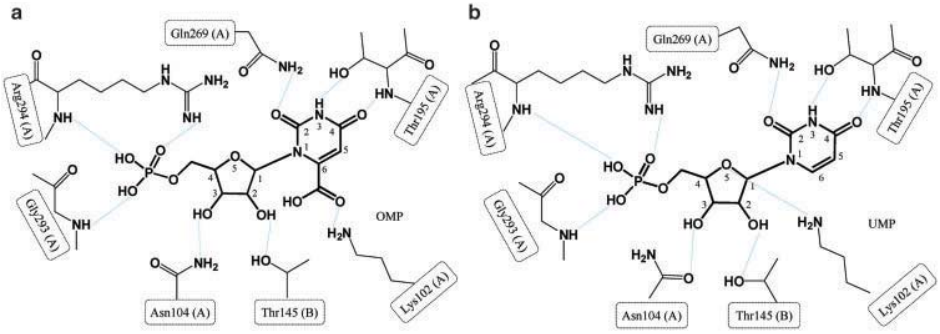

Fig. 4. **Schematic drawings of the hydrogen bonding pattern around OMP (a) and UMP (b).** The binding motif of OMP was basically the same as that of UMP, except for the residue Lys102.

The observed rearrangement of the hydrogen-bond network based on the structural determination of the apo form and OMP- and UMP-complex in *Plasmodium* OMPDCs is the first report.

DISCUSSION

The details of the reaction mechanism have been proposed by Raugei *et al*. (*31*), that a very stable charged network OMP-Lys-Asp-Lys-Asp promotes transition state electrostatic stabilization, and fully conserved Lys138 (*Pf*OMPDC numbering) largely contributes to the stabilization of the transition state or equivalently, the carboanionic intermediate.

In this study, in an attempt to provide further proof-of-principle, we have determined the X-ray structure of the apo, substrate or product-complex forms of *Pf*OMPDC. These three kinds of structural analyses provided an insight into the rearrangement of the hydrogen network in the array of OMP-Lys-Asp-Lys-Asp, by comparison between the initial and completion stages of the decarboxyl reaction. At the initial reaction step, the side chain of Lys102 broke a hydrogen bond with the carboxyl group of Asp136, and then formed a hydrogen bond with the carboxyl oxygen atom of the substrate, upon binding OMP. These processes increased the negative charge on the two carboxyl groups on the substrate and Asp136, which led to electrostatic repulsion between the carboxyl groups. Even the short distance of 2.5 Å between the two carboxyl groups, and the positively charged residues of Lys102 and Lys138 maintained the unstable structure.

Then, the decarboxylation reaction started from the structure with electrostatic repulsion. After direct decarboxylation, the newly generated carboanion at the C6 position of the pyrimidine ring may have been stabilized by the conserved array, and absorbed a proton from the Lys138 side chain directly (*31*). In the completion step, two pairs of hydrogen bonds, Lys102-Asp136 and Lys138-Asp141(B), were formed, and all of the electrostatic charge in the array was compensated (Fig. 5c). It seems to be difficult, however, to specify from our structural models which Lys residues stabilized the forming carboanion intermediate in the transition state.

Ning Wu *et al*. (*47*) reported the mutational study for this enzyme from *M. thermoautotrophicum*. Asp70 and Lys72 (corresponding to Asp136 and Lys138, respectively, in *P. falciparum*) were mutated to an alanine, however those mutants still had enzymatic activity to convert from OMP to UMP, indicating Asp70 cannot be the only driving force for decarboxylation. The same argument can be made for Lys72, and then the mechanism of protonation as well as that of decarboxylation was not specified completely.

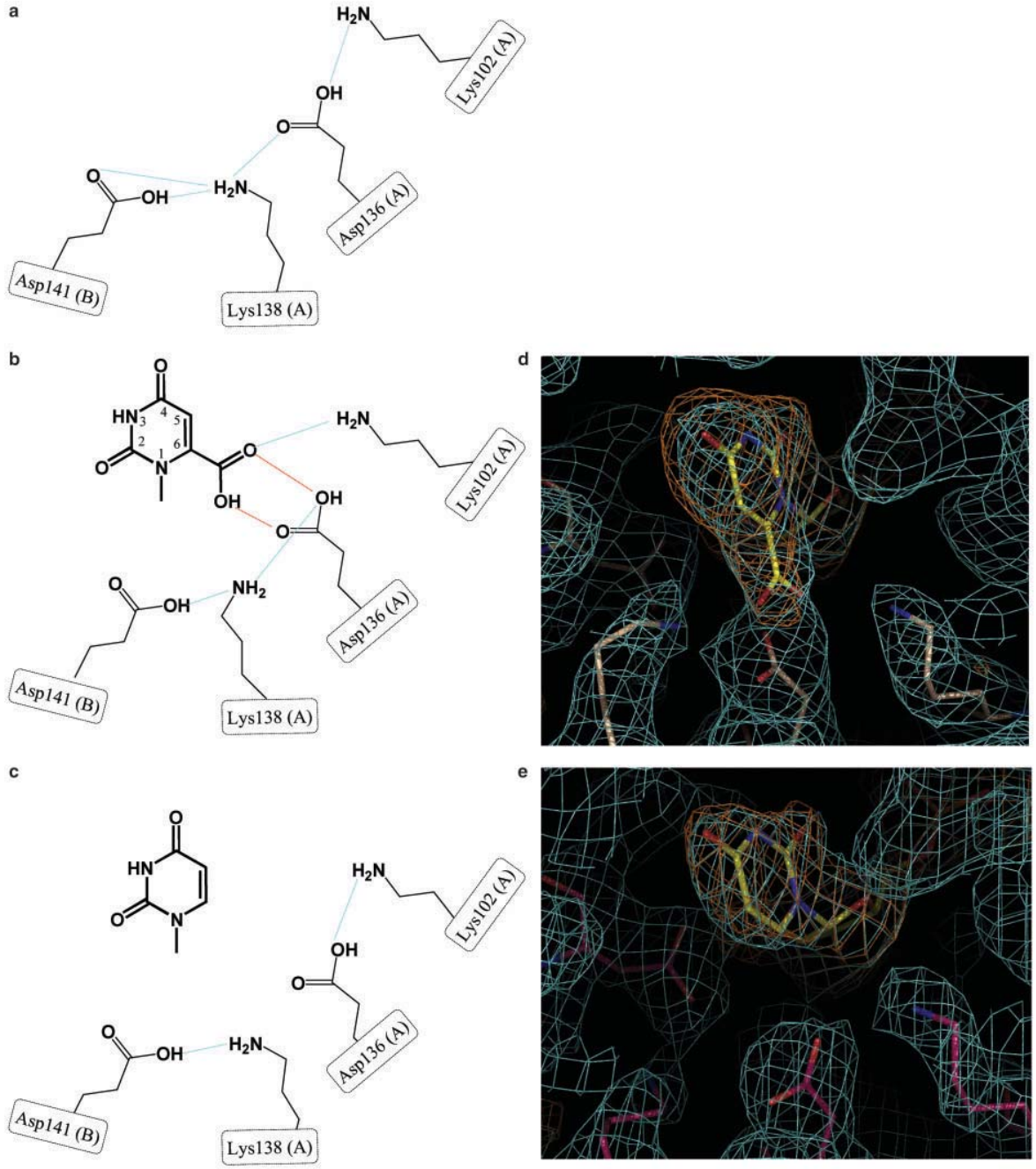

Fig. 5. **Proposed reaction mechanism.** Stepwise representation of the rearrangement of the hydrogen-bond network, with a particular electron density map around Lys102. (a) Apo form. Lys102 formed a hydrogen-bond network with Asp136, Lys138 and Asp141. (b) The substrate entered the active site. As Lys102 turned away from Asp136, charge repulsion between the two carboxylate groups initiated decarboxylation and Lys138 donated a proton to C6. (c) After decarboxylation, Lys102 hydrogen bonded to Asp136 instead of the carboxyl group of the pyrimidine ring. (d) Electron density maps of OMP (d) and UMP (e) binding forms. These contours show the SIGMAA-weighted $2F_o - F_c$ map at 1.5 σ as well as the $F_o - F_c$ omit map. The programs COOT (*46*) and PYMOL (*50*) were used to make these figures.

A structural analysis of complexes with a transition state analogue will further uncover the mechanism of transition state stabilization by Lys residues.

Eukaryotes express OMPDC as a bifunctional protein, UMP synthase, which catalyses the orotate phosphoribosyltransferase reaction before the decarboxylation reaction. On the other hand, *Pf*OMPDC forms a complex with orotate phosphoribosyltransferase (OPRT), which forms a heterotetrameric complex in *P. falciparum* (*20*). In our multiple sequence alignment, we found

a unique structural feature of the OMPDCs belonging to *Plasmodium*. A large insertion was observed between β1 and α5, which formed a protruding domain consisting of three α helices, α2, α3 and α4. We propose that the unique insertion may participate in making a complex with OPRT.

In our structural observations, the top part of the active site played a role in substrate binding, accompanied by large structural movement. The average difference of 1.5 Å in the top part is twice the root-mean-square deviation calculated from all C_α atoms.

Eleven structures of OMPDC have been reported. Among them four kind of nucleotide complex structures are available to compare with those of apo forms to date. Each of OMPDC from *M. thermoautotrophicum, P. horikoshii, E. coli and S. cerevisiae* showed a large structural change around the active site near the open end of the $(\alpha/\beta)_8$ barrel upon binding of the nucleotide. The structure of each loop which corresponds to the L1 loop in *Pf*OMPDC was stabilized by making hydrogen bond with the pyrimidine ring moiety of nucleotide ligands, which is same manner as our complex showed. On the other hand, the OMPDCs from three species except *S. cerevisiae* retained the structure of α-helix which correspond to α9 near the L2 loop region in *Pf*OMPDC, did not display any structural changes upon binding of nucleotide ligands. *Pf*OMPDC has long insertion after α9 helix which makes the L2 loop harder to access to pyrimidine moiety. Therefore α9 helix needs to be unfold upon binding OMP or UMP in *Pf*OMPDC.

On the contrary, the bottom part of the active site was significantly rigid; the average difference was half of the overall root-mean-square deviation. Even a small difference, such as the rearrangement of hydrogen bonds in the array of OMP-Lys-Asp-Lys-Asp, may have played a pivotal role in catalysis. The corresponding residues for binding and catalysing showed >80% consensus among all the OMPDCs, and the structural movements described earlier may be in common with them.

In summary, we have proposed a reaction mechanism and characteristic domain for binding of *Plasmodium* OPRT, as inferred from structural analyses of apo and holo forms. Future studies on the structural analyses of complexes with transition state analogues will unravel the stabilization mechanism of the carboanion intermediate. The structural analysis of the complex between *Pf*OMPDC and *Pf*OPRT will, likewise, clarify the hetero-tetrameric reaction mechanism which could lead to the development of important antimalarial drugs.

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Dihydroorotase of human malarial parasite *Plasmodium falciparum* differs from host enzyme

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Abstract

Plasmodium falciparum, the causative agent of human malaria, is totally dependent on *de novo* pyrimidine biosynthetic pathway. A gene encoding *P. falciparum* dihydroorotase (*pf*DHOase) was cloned and expressed in *Escherichia coli* as monofunctional enzyme. *Pf*DHOase revealed a molecular mass of 42 kDa. In gel filtration chromatography, the major enzyme activity eluted at 40 kDa, indicating that it functions in a monomeric form. This was similarly observed using the native enzyme purified from *P. falciparum*. Interestingly, kinetic parameters of the enzyme and inhibitory effect by orotate and its 5-substituted derivatives parallel that found in mammalian type I DHOase. Thus, the malarial enzyme shares characteristics of both type I and type II DHOases. This study provides the monofunctional property of the parasite DHOase lending further insights into its differences from the human enzyme which forms part of a multifunctional protein.

Keywords: Monofunctional enzyme; Dihydroorotase; Pyrimidine; *Plasmodium falciparum*; Malaria

Malaria remains a major and growing threat to the public health of the population living in the endemic areas [1]. One of the four species that infect humans, *Plasmodium falciparum* is the most lethal parasite. Resistance to antimalarial drugs and their toxicity are contributing factors to the spread of the disease. This highlights the need to develop quickly more effective and less toxic new antimalarial drugs with different mechanism of action [2].

Pyrimidine nucleotides are essential metabolites. Unlike human and other mammalian cells [3], however, *P. falciparum* cannot salvage preformed pyrimidine bases or nucleosides from human host, but relays solely on nucleotide synthesized through the *de novo* pathway [4–10]. The *de novo* pathway involves six sequential enzymes catalyzing the conversion of precursors HCO_3^-, ATP, Glu, and Asp to uridine 5′-monophosphate (UMP) [3,4,8]. There are some key differences of the enzymes in the *de novo* pathway between malarial parasites and higher eukaryotes, including human host [4–7,9–13]. The third enzyme of the pathway, dihydroorotase (DHOase), is a Zn^{2+} amidohydrolase superfamily that catalyzes reversible cyclization of *N*-carbamoyl-L-aspartate (L-CA) to form L-dihydroorotate (L-DHO). Based on known DHOase sequences and phylogenetic analysis, they are classified into two families [14]. The type I DHOase is found in higher organisms, archaebacteria, and eubacteria (e.g., *Aquifex aeolicus*) [14,15]. The type II DHOase is predominately found in eubacteria (e.g., *Escherichia coli*) [16], exhibiting significant differences in their sequences.

In this study, we report the cloning, functional expression of the *P. falciparum* gene encoding DHOase activity in *E. coli* and the purification of the recombinant DHOase to apparent homogeneity. Physical, kinetic, and inhibitory properties of the recombinant enzyme are characterized and compared with the native enzyme, purified from the *in vitro* culture of *P. falciparum*.

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Materials and methods

Materials. Cloning and expression vectors, restriction enzymes, and all molecular biological reagents were purchased from Qiagen, Invitrogen, Novagen, and Promega. Radiochemicals were from Du Pont. Other chemicals were of the highest quality commercially available. L-[^{14}C]DHO and L-[^{14}C]CA were synthesized as described previously [5].

Parasite cultivation and crude extract preparation. Asynchronous stages of *P. falciparum* (strain 3D7) were cultivated according to Trager and Jensen method [17]. Synchronous cultures were achieved using sorbitol treatment [7]. Parasites and the crude extract were prepared as essentially described [9].

Genomic DNA, total RNA, and cDNA preparation. Genomic DNA and total RNA were purified from an asynchronous culture of *P. falciparum* by DNAZOL and TRIZOL (Life Technologies), respectively. First strand cDNA was synthesized as essentially described [10].

Identification and characterization of P. falciparum DHOase homolog. Homology search of *pf*DHOase was performed with the BLAST program of the NCBI server [18]. A single open reading frame encoding DHOase homolog was annotated as putative DHOase within a sequence on chromosome 14 of *P. falciparum* in the *Plasmodium* Genome Database (http://www.PlasmoDB.org) and further characterized by the Tblastn program. Pair-wise and multiple sequence alignments of *pf*DHOase with known sequences from other organisms, including other *Plasmodium* species, were performed using the CLUSTALW program.

Cloning and sequencing of P. falciparum DHOase homolog. cDNA encoding *pf*DHOase was amplified by PCR using *Pfu* DNA polymerase (Promega) with the following forward and reverse primers 5′CGC**GGATCC**ATGAAAAATTACTTTTATATTCC3′ and 5′CCC**AAGCTT**TTAAAACTTACTTACATAATG3′ introducing BamHI and HindIII restriction sites, respectively (shown in boldface type).

The PCR cycling condition, including denaturation, annealing, and extension steps, was performed as described [10]. The PCR product was purified using gel extraction kit (Qiagen) and then ligated by using Ligafast (Toyobo) into Zero Blunt TOPO cloning vector (Invitrogen). The *pf*DHOase sequence was determined by the dideoxy chain-termination method. The *pf*DHOase plasmid was subcloned into three expression vectors: pET 15b (Novagen), pTrcHis A (Invitrogen), and pQE-30 Xa (Qiagen) plasmids.

Recombinant expression and purification of P. falciparum DHOase from E. coli. Plasmid constructs were transformed into competent *E. coli* cells as follows: pET 15b/*E. coli* BL21 (DE3); pTrcHis A/*E. coli* TOP10; pQE-30 Xa/*E. coli* M15. The cells were grown in LB medium (37 °C) to $A_{600} = 0.5$, induced with 1 mM isopropyl β-D-thiogalactopyranoside, and harvested after induction for 18 h at 18 °C. Recombinant protein purification was performed at 4 °C by a nickel-nitrilotriacetic acid (Ni-NTA) gel and a gel filtration Superose 12 column connected to a FPLC system, as described [10].

Purification of native DHOase from P. falciparum. Native DHOase from the crude extract of *P. falciparum* was purified by three sequential steps on a FPLC system using anion-exchange Mono Q, gel filtration Superose 12, and chromatofocusing Mono P columns. The purification conditions, including buffers, are reported elsewhere [5].

Enzymatic activity assay. DHOase activity in the ring cleavage reaction using L-[^{14}C]DHO was measured in the different purification steps [5]. Spectrophotometric method was used to detect both the ring cyclization and cleavage reactions of the recombinant *pf*DHOase, as described [19]. Radiochemical assay was used to confirm the authenticity of the *pf*DHOase reaction in both enzymatic directions from the spectrophotometric method.

Kinetics and inhibition studies. Kinetic analysis for both ring cyclization and cleavage reactions were determined using five different preparations of pure recombinant *pf*DHOase enzyme (50 nM per reaction). The K_m and V_{max} values were obtained from triplicate measurements of initial velocities with at least five substrate concentrations. The k_{cat} values were calculated from $V_{max}/[E]$. Catalytic efficiency (k_{cat}/K_m) values were also determined. Kinetic data of initial velocities and inhibition by orotate (OA) and its 5-substituted derivatives as double-reciprocal plots were fitted to the appropriate equations for competitive inhibitors.

Results and discussion

Recent findings have highlighted potential vulnerability of the human parasite toward agents which affect pyrimidine biosynthetic pathway [6–8,10,11,20,21]. Nevertheless, only two enzymes of the *P. falciparum* pathway have crystal structures, the fourth [11] and sixth [22] enzymes. Much importance has been given to DHOase since compounds presumed to affect the enzyme activity have been shown to be selectively toxic toward the human parasite [6,8]. However to fully exploit this target, detailed physical, kinetic, and structural characterization of *pf*DHOase is required. Here, we have prepared appropriate quantities of the recombinant enzyme for detailed characterization, as compared to limited amounts of the native enzyme obtainable from *in vitro P. falciparum* culture. The malarial enzyme is unique since it is a monofunctional protein and thus differs from the mammalian host in which the 36.7-kDa enzyme is located on the central part of the 240-kDa CAD multifunctional protein [23].

Identification and characterization of gene and amino acid sequence for P. falciparum DHOase

The gene encoding *pf*DHOase was identified as a single copy on chromosome 14 (locus: PF14_0697) in PlasmoDB. It has an intronless open reading frame (1077 bp), which encodes a 41,718-Da protein (358 residues) with a p*I* of 7.45. The malarial sequence was aligned to both type I and II DHOases, and found to be most similar to *E. coli*, having 348 residues with a molecular mass of 38 kDa [13,19]. The bacterial DHOase belongs to type II family and its crystal structure has already been obtained. The catalytic residues of the malarial DHOase were conserved to the amino acids predicted from the crystal structures in four organisms: *E. coli* (PDB code: 2EG6, type II), *A. aeolicus* (PDB code: IXRF, type I), *Porphyromonas gingivalis* (PDB code: 2GWN, type I), and *Agrobacterium tumefaciens* (PDB code: 2OGJ, type unknown) (Supplemental Figure).

In PlasmoDB, putative DHOases were found in six *Plasmodium* species. The *P. falciparum* enzyme had 63–65% identity to *P. vivax*, *P. knowlesi, P. berghei*, *P. chabaudi*, and *P. yoelii*. All malarial enzymes were most similar (46–57%) to the type II DHOase: bacteria *E. coli* (46%), plant *Arabidopsis thaliana* (48%), fungi *Saccharomyces cerevisae* (48%), and apicomplexan *Toxoplasma gondii* (57%); compared to type I DHOase of bacteria (<37%) *A. aeolicus*, and *P. gingivalis*. Interestingly, the malarial enzyme had also very low homology (<20%) to the human DHOase (334 residues, 36.7 kDa, p*I* 6.1) [23]. Phylogenetic analysis of most DHOase sequences obtained from the database indicates that the malarial sequence, as well as the fungal and plant enzymes, presumably has common proteobacterial ancestors.

In silico, the overall structure of the malarial enzyme is predicted to be a TIM-barrel model with two zinc atoms

bridged by a carboxylated Lys98 in the active site. The zinc ligand residues are well conserved between *E. coli* and *P. falciparum*. Furthermore, the bacterial flexible loop (residues 106–116), its movement in the active site which is necessary for catalysis [16], are also highly conserved in the *P. falciparum* sequence at residues 101–111. Five amino acids responsible for substrate L-CA binding are fully conserved residues. By comparison, this partly supports the general catalysis of DHOase involving loop movement in the active site, as shown in *E. coli*. The zinc ligand residues and the presence of flexible loops of *P. falciparum* are different from the type I human [23] and *A. aeolicus* enzymes [15]. Unique for *P. falciparum* is an insertion of 15 amino acids (206–220), close to the seventh α-helical region (Supplemental Figure).

Cloning, expression, and purification of P. falciparum DHOase in E. coli

The cDNA encoding *pf*DHOase was cloned into the pET 15b and pTrcHis A vectors; and then expressed in *E. coli* BL21(DE3) and TOP10 cells, respectively. The *pf*DHOase expression in these vectors and *E. coli* cells was relatively low, with limited solubility. The third vector, pQE-30 Xa, was then attempted and expression in *E. coli* M15 showed relatively good yield (1.5–2.0 mg of pure *pf*DHOase per 1 L *E. coli* culture). The recombinant enzyme was purified to apparent homogeneity, as assessed by SDS–PAGE (Fig. 1A). The *pf*DHOase molecular mass was estimated to be 42.4 ± 2.5 kDa ($n = 6$), corresponding to the predicted molecular mass of 41,718 Da. The recombinant enzyme was active with a specific activity of 970 nmol min^{-1} mg $protein^{-1}$.

Purification and physical properties of native P. falciparum DHOase

We measured the activity of DHOase from synchronized *in vitro* cultures of *P. falciparum*. The total activity in the trophozoite (87.4 ± 10.2 nmol min^{-1} per 10^8 parasites, $n = 3$) and schizont (108 ± 13 nmol min^{-1} per 10^8 parasites, $n = 3$) stages were increased ~23- and ~28-fold, respectively, as compared to the activity in the ring stage (3.8 ± 0.5 nmol min^{-1} per 10^8 parasites, $n = 3$). This finding confirms stage-dependent activity of the pyrimidine enzyme in *P. falciparum* [4]. From the crude extract preparation of the parasite to FPLC on Mono Q, Superose, and finally Mono P columns, the DHOase was purified to near homogeneity at ca. 356-fold and 26% yield (Table 1). The purified enzyme appeared as a dominant band at 42 kDa on SDS–PAGE (Fig. 1B). Gel filtration chromatography on the Superose column of the purified DHOase obtainable from the Mono P column showed that enzyme activity eluted as a single symmetric peak at 40 ± 4 kDa ($n = 3$), indicating that the native enzyme exists in the monomeric form (Fig. 1C).

Comparing the *P. falciparum* and *P. berghei* amino acid sequences with the crystal structure in *E. coli* to determine structural features revealed two salt bridges and three hydrophobic interactions on dimeric interface, responsible for the subunit–subunit interaction in the bacteria. These residues were not conserved in the *P. falciparum* and *P. berghei* sequences, partly supporting our observation that the native malarial enzyme is a monomeric protein, as we have premised based on *P. berghei* [5]. DHOases from the bacterial *A. aeolicus* [15] and the apicomplexan *T. gondii* [24] that appear to function in monomeric form, likewise, lack these conserved residues for the subunit–subunit interaction (Supplemental Figure).

Physical and kinetic characterization of recombinant P. falciparum DHOase

Gel filtration chromatography of recombinant enzyme shows that the activity was eluted at both monomeric (40 ± 3 kDa, $n = 5$) and dimeric (80 ± 8 kDa, $n = 5$) positions, although more than 75% of the total activity was associated with the monomeric form (Fig. 2A). However, both forms had similar k_{cat} values. This observation was not found in the native enzyme isolated from the parasite. The dimeric property of the recombinant DHOase was, however, confirmed by dimethyl suberimidate chemical cross-linking analysis [5]. As shown in Fig. 2B, only the cross-linked dimeric form was observed. The recombinant *pf*DHOase, demonstrated in both studies, however, also suggests that the monofunctional enzyme may possibly be active in both monomeric and dimeric forms. The dimeric form may exist under certain conditions during the enzyme preparations. Thus, oligomerization behavior remains to be explored by having its crystal structure.

Since the enzyme catalyzes reversible reaction of L-CA to L-DHO, the activities of the *pf*DHOase were then measured in both reactions over the pH range of 4.0–11.0. In the ring cyclization reaction, very high activity appeared at acidic pH. In contrast, the rate of ring cleavage reaction was very high at alkaline pH and lower activity was observed at acidic pH. The pH-activity profile for both reactions intersects at pH 6.6 (Fig. 3), suggesting that the enzyme catalysis involves His residues located on the active site. This observation is similar to that found in mammalian [25], *T. gondii* [24] and *Crithidia fasciculata* [5] enzymes. Metal chelators EDTA and 1,10-phenanthroline had no effect on the enzyme activity when tested at 2.5 mM. The *pf*DHOase activity was strongly inhibited by 10-min preincubation with 1.0 mM diethyl pyrocarbonate (DEPC) and its activity was restored >95% when the enzyme was preincubated with both 1.0 mM DEPC and 5.0 mM L-CA. Zn^{2+} inhibited competitively the ring cyclization reaction with a K_i of 2.85 mM. Co^{2+} also inhibited the enzyme with a K_i of 3.55 mM (Supplemental Table).

At pH 6.0, the enzyme displayed normal Michaelis–Menten saturation kinetics for the maximal ring cyclization of L-CA. K_m of L-CA and k_{cat} values were determined to be 0.285 ± 0.012 mM and 60.2 ± 4.4 min^{-1}, respectively. The maximal ring cleavage reaction rate of L-DHO measured

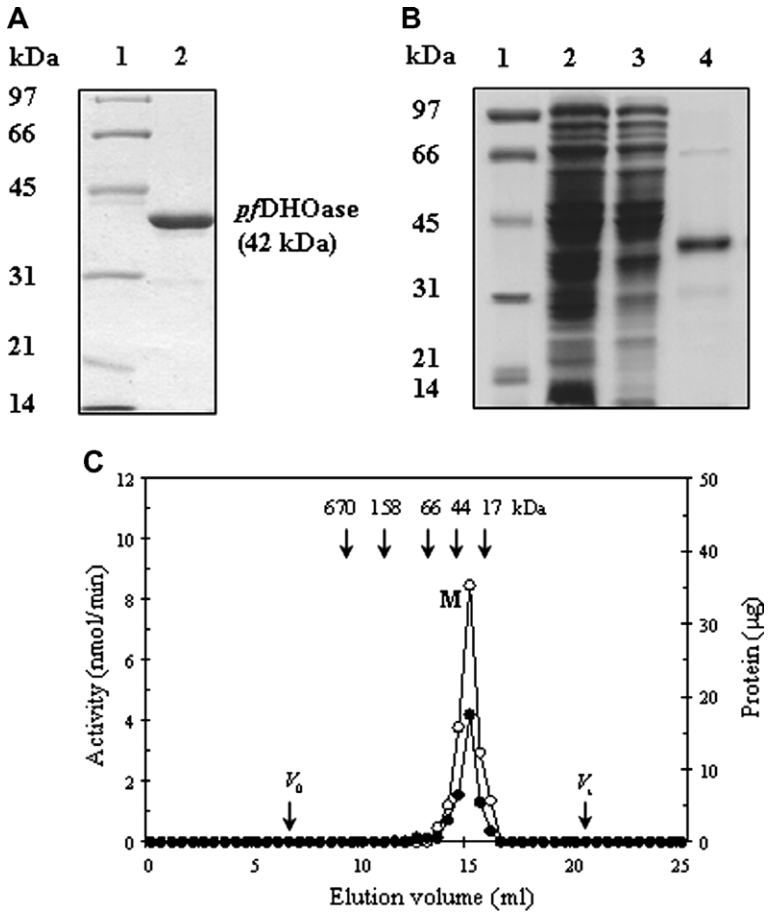

Fig. 1. SDS–PAGE and gel filtration chromatography of parasite DHOase. (A) The purity of recombinant enzyme was assessed by 12% gel of SDS–PAGE. Lane 1, the molecular mass markers are indicated in kDa; lane 2, 10 μg of the purified recombinant *pf*DHOase, a single 42-kDa band is shown. (B) SDS–PAGE of native DHOase from *in vitro P. falciparum* culture. Three purification steps of the native enzyme from *P. falciparum* were subjected to SDS–PAGE (12% gel). Lane 1, the molecular mass markers; lane 2, 80 μg protein obtained from Mono Q column; lane 3, 40 μg protein taken from Superose 12 column, lane 4, 5 μg protein obtained from Mono P column, a dominant 42-kDa protein is observed. (C) Gel filtration chromatography of native and monomeric (M) DHOase form of *P. falciparum*. The fractions (0.5 ml) were collected and assayed for both enzymatic activity (open circles) and protein concentration (closed circles).

Table 1
Purification of native DHOase from *in vitro* culture of *P. falciparum*

Step	Total protein (mg)	Total activity (nmol min^{-1})	Specific activity (nmol min^{-1} mg^{-1})	Yield (%)	*X*-fold purified
Crude extract	273.86	383.4	1.4	100	1
Mono Q FPLC	7.53	253.0	33.6	66	24
Superose 12 FPLC	1.12	172.5	154.0	45	110
Mono P FPLC	0.20	99.7	498.5	26	356

at pH 9.0 has K_m of L-DHO and k_{cat} values of 0.012 ± 0.001 mM and $89.5 \pm 10.6\ min^{-1}$, respectively. The k_{cat}/K_m values for the ring cyclization and cleavage reactions were calculated to be 2.11×10^5 and $7.46 \times 10^5\ M^{-1}\ min^{-1}$, respectively. Comparing to the mammalian enzyme, its catalytic efficiency in the ring cyclization reaction ($1.44 \times 10^8\ M^{-1}\ min^{-1}$) is 680-fold more than that of the malarial enzyme, while that in the ring cleavage reaction ($4.44 \times 10^7\ M^{-1}\ min^{-1}$) only a 60-fold difference was noted [26]. This indicates that the monofunctional DHOase enzyme is generally less efficient than the DHOase in the mammalian multifunctional CAD enzyme [5,12,19,24,26].

Since the equilibrium between L-CA and L-DHO favors the former by a ratio of 17:1 at physiological pH 7.4 [25], inhibitor studies involving reversible reactions of the enzyme were measured at this pH. The inhibitory effect of orotate (OA) and its 5-substituted derivatives; 5-fluoroorotate (F-OA), 5-aminoorotate (NH_2-OA), 5-methyloratate (CH_3-OA), 5-bromoorotate (Br-OA), and 5-iodoorotate (I-OA) was investigated with *pf*DHOase and compared with the mammalian enzyme [27]. All of the six compounds were competitive inhibitors of both ring cyclization and cleavage reactions. The K_i values for the inhibitors were obtained from double-reciprocal plots and are shown in Table 2. The most effective inhibitor was 5-fluoroorotate, while 5-bromoorotate and 5-iodoorotate

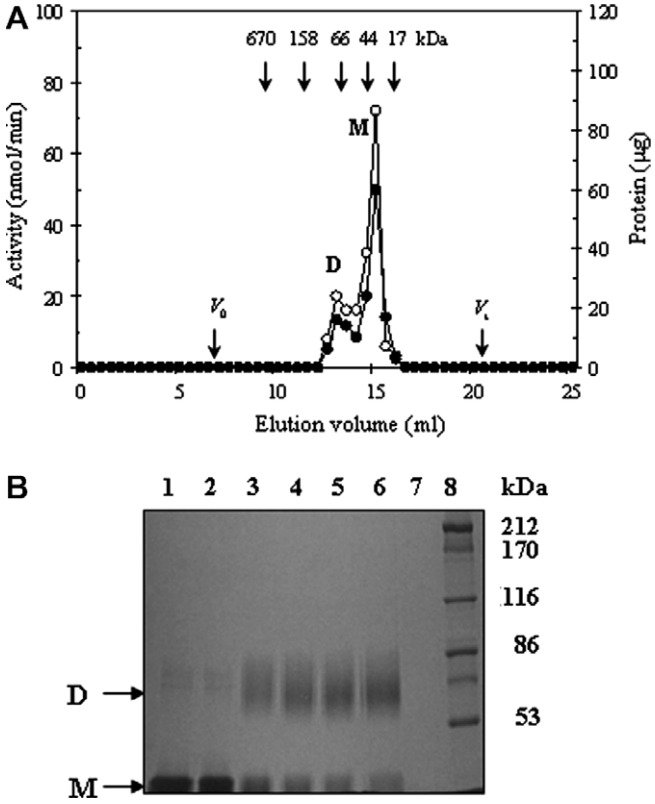

Fig. 2. Gel filtration chromatography of monomeric and dimeric forms, and SDS–PAGE of chemically cross-linked dimer of recombinant *pf*DHOase. (A) A gel filtration chromatographic profile demonstrates monomeric (M) and dimeric (D) forms of the *pf*DHOase enzyme. The 0.5-ml fractions were collected and assayed for both enzymatic activity (open circles) and protein concentration (closed circles). Molecular mass markers, void volume (V_0), and total eluting volume (V_t) are indicated with arrows. (B) Dimethyl suberimidate cross-linking of the *pf*DHOase pattern was analyzed by SDS–PAGE (8% gel). Lane 1, a non cross-linking monomeric (M) form (20 μg protein); lanes 2–6, cross-linking products of dimeric (D) form *pf*DHOase at 5, 15, 30, 45, and 60 min incubation, respectively; lane 7, sample buffer (blank); lane 8, the molecular mass markers are given in kDa.

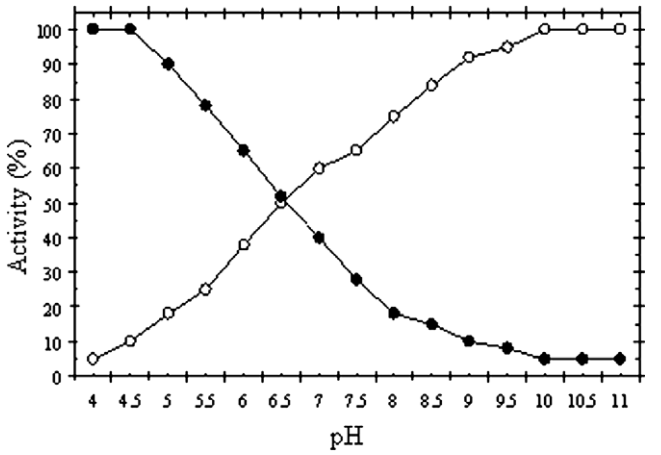

Fig. 3. Effect of pH on the ring cyclization and cleavage activities of recombinant *pf*DHOase. Incubation mixtures contained 50 nM enzyme, 50 mM buffer, MES–KOH for pH 4.0–5.5, Hepes–KOH for pH 6.0–8.0, and Tris–HCl for pH 8.5–11.0), 150 mM KCl, and 2.5 mM L-[^{14}C]CA or 0.25 mM L-[^{14}C]DHO. The maximal activities of the ring cyclization and cleavage reactions were 1.47 and 1.86 μmol min^{-1} mg protein^{-1}, respectively, and were calculated to 100% enzyme activities. The ring cyclization reaction profile is indicated by closed circles and the cleavage reaction by open circles.

were the least in both enzymatic directions. Both L-CA and L-DHO had no inhibitory effect on the enzyme at the pH 7.4. K_i values of these inhibitors for dimeric *pf*DHOase isolated from the gel filtration column (Fig. 2A) were also determined and found to be similar to values obtained from the monomeric DHOase. In the ring cleavage reaction, the K_i values reported for 5-fluoroorotate of the mammalian [27] and *E. coli* [16] enzymes are 6 and 32 μM, respectively. Increasing K_i values of the 5-substituted orotate derivatives of the parasite enzyme were proportional to the bulkiness in the size of the 5-substituents, but were not related to the order of electronegativity of these 5-substituents. The result suggests a steric hindrance of the bulky 5-substituent which may have positioned adjacent to the binding site of the inhibitor. Similar findings for the five 5-substituted compounds reported in the mammalian enzyme [27] indicate that *pf*DHOase shares some enzyme characteristics to the mammalian DHOase although significant kinetic differences do exists, the mammalian enzyme is 10- to 20-fold more sensitive to the inhibitors than that of the parasite.

In summary, the malarial enzyme shares a number of characteristics to both type I (e.g., mammals) and II (e.g., *E. coli*) enzymes. Comparison of parasite and human enzymes by the overall amino acid sequence homology, structural properties, and kinetic and inhibitor characteris-

Table 2
Kinetic constants of L-CA, L-DHO, OA, and its 5-substituted derivatives in the ring cyclization and cleavage reactions of the recombinant *pf*DHOase

Substrate or OA derivative	Diameter of substituent in position 5 (Å)	Ring cyclization reaction			Ring cleavage reaction		
		K_m (μM)	k_{cat} (min^{-1})	K_i (μM)	K_m (μM)	k_{cat} (min^{-1})	K_i (μM)
L-CA (pH 6.0)		285 ± 12	60.2 ± 4.4	–			
L-DHO (pH 9.0)					12 ± 1	89.5 ± 10.6	–
OA (pH 7.4)				660 ± 52			950 ± 88
F-OA	2.7			145 ± 11			70 ± 5
NH_2-OA	2.9			440 ± 43			160 ± 17
CH_3-OA	3.5			560 ± 65			302 ± 40
Br-OA	3.8			1460 ± 140			2600 ± 170
I-OA	4.1			>3500			>3500

tics lent valuable insights into the differences of the active site between these two enzymes. Specific inhibitors may limit the pyrimidine nucleotide pool in the parasite, but have no significant adverse effect to humans in which the salvage pathway for nucleotide synthesis is actively functional [28].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.12.025.

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เชื้อมาลาเรีย: จีโนมิกส์ และ ชีวเคมี กับตำแหน่งเป้าหมายในการพัฒนายารักษาโรคมาลาเรีย

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Malaria remains a global health problem attributed by its major cause of morbidity and mortality in developing and tropical countries. Of these, 400-500 million people are infected with the parasite, and two million die each year. Plasmodium falciparum, the etiologic agent of the most lethal and severe form of the four species that infect humans, is resistant to most of the currently available antimalarial drugs. The need of more efficacious agents — particularly rational drugs that exploit metabolic pathways and targets unique to the malaria parasite — is therefore urgent. The basic knowledge of the current genomics and biochemistry of the parasite are essential to the design and development of new antimalarial drugs. This paper reviews the most recent information on P. falciparum genomics and metabolomics, and will apply the data for drug development, and also identify the molecular targets of the drug.

Keywords: *Malaria, Plasmodium falciparum, Genomics, Biochemistry, Drug target, Antimalarial drug.*

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จิระพันธ์ กรึงไกร, สุดารัตน์ กรึงไกร. เชื้อมาลาเรีย: จีโนมิกส์ และ ชีวเคมี กับตำแหน่งเป้าหมายในการพัฒนายารักษาโรคมาลาเรีย. จุฬาลงกรณ์เวชสาร 2549 ก.พ; 50(2): 127 – 42

มาลาเรียเป็นโรคที่มีอุบัติการณ์ และอัตราการเสียชีวิตสูงในประชากรตามภูมิภาคร้อนชื้นทั่วโลก มีการติดเชื้อมาลาเรียประมาณ 400-500 ล้านคน และเสียชีวิตด้วยโรคนี้ประมาณ 2 ล้านคนต่อปี ปัญหาที่สำคัญในการรักษาคือ เชื้อมาลาเรียชนิด Plasmodium falciparum มีการดื้อต่อยารักษาที่ใช้อยู่ในปัจจุบัน จึงมีความจำเป็นในการพัฒนายาชนิดใหม่โดยอาศัยองค์ความรู้เกี่ยวกับข้อมูลจีโนมิกส์ และชีวเคมีของเชื้อมาลาเรีย บทความนี้จะสรุปความรู้ก้าวหน้าของจีโนมิกส์ และการนำองค์ความรู้ดังกล่าวของเชื้อที่มีอยู่ในขณะนี้ไปประยุกต์ใช้ในการพัฒนายาชนิดใหม่ รวมทั้งจะแสดงตำแหน่งโมเลกุลเป้าหมายในวิถีเมตาบอลิสมต่าง ๆ ที่คาดว่าจะได้ยาชนิดใหม่ไปยับยั้งที่ตำแหน่งเหล่านี้ได้และไปทำลายเชื้อมาลาเรียที่มีการดื้อต่อยาได้

คำสำคัญ : *เชื้อมาลาเรีย, Plasmodium falciparum, จีโนมิกส์, ชีวเคมี, ตำแหน่งเป้าหมายของยา, ยารักษาโรคมาลาเรีย*

วัตถุประสงค์ :

1. เพื่อให้ทราบความรู้ความก้าวหน้าด้านจีโนมิกส์ของเชื้อมาลาเรียที่ทำให้เกิดโรคในคน
2. เพื่อให้เข้าใจถึงความรู้พื้นฐานชีวเคมีของเชื้อมาลาเรีย
3. เพื่อให้ทราบตำแหน่งเป้าหมายใหม่ในการพัฒนายารักษาโรคมาลาเรียในอนาคต
4. เพื่อให้เข้าใจแนวทางในการพัฒนายารักษาโรคมาลาเรีย โดยอาศัยความรู้ด้านจีโนมิกส์และชีวเคมีของเชื้อมาลาเรีย

มาลาเรียเป็นโรคที่มีอุบัติการณ์และอัตราการเสียชีวิตสูงในประชากรตามภูมิภาคที่มีอากาศร้อนชื้นทั่วโลก รองจากการติดเชื้อนิวโมเนีย (pneumococcal acute respiratory infections) และวัณโรค (tuberculosis) จากข้อมูลขององค์การอนามัยโลก [1] พบว่ามีการติดเชื้อมาลาเรียประมาณ 400-500 ล้านคนในแต่ละปี และเสียชีวิตด้วยโรคนี้ประมาณ 2 ล้านคนต่อปี โดยมีสาเหตุจากการติดเชื้อ *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae* หรือ *Plasmodium ovale* ชนิดใดชนิดหนึ่งหรือ 2 ชนิดปนกัน [2,3] สำหรับโรคนี้ในประเทศไทย จากสถิติปี 1998 ของกระทรวงสาธารณสุข พบว่ามีอัตราการติดเชื้อประมาณ 100 ราย และการเสียชีวิตประมาณ 1.26 รายต่อประชากร 1 แสนคน [4]

การติดเชื้อชนิด *P. falciparum* ทำให้เกิดโรคมาลาเรียชนิดรุนแรง (severe malaria) ผู้ป่วยบางรายอาจเป็นมาลาเรียขึ้นสมอง (cerebral malaria) มีอาการแทรกซ้อนที่รุนแรง อาทิเช่น ภาวะเลือดจางรุนแรง (severe anemia) ไตวาย (renal failure) ปอดบวมน้ำ (pulmonary edema) ระดับน้ำตาลในเลือดต่ำ (hypoglycemia) มีเลือดออก (bleeding) ชักรุนแรง (repeated generalized convulsion) ภาวะกรดสูง (acidemia/acidosis) และปัสสาวะสีดำ (malaria hemoglobinuria) เป็นต้น ซึ่งเป็นสาเหตุทำให้ผู้ป่วยเสียชีวิตได้ [5] ปัญหาที่สำคัญในการรักษาคือ เชื้อมาลาเรีย ดังกล่าวมีการดื้อต่อยารักษาที่ใช้อยู่ในปัจจุบัน รวมทั้งยาหลายชนิดมีข้อจำกัดในการใช้ อาทิเช่น ความเป็นพิษ คุณภาพไม่ดี และราคาแพง เป็นต้น (ตารางที่ 1) ในช่วงปี 1975 -1997 ได้มีการพัฒนายาทั่ว ๆ ไปถึง 1,223 ชนิด แต่มียาใหม่นำมาใช้สำหรับรักษาโรคมาลาเรียเพียง 3 ชนิดคือ halofantrine, mefloquine และ malarone และในระยะ 3-4 ปีที่ผ่านมาได้มีความพยายามมากขึ้นของหลายองค์กรที่ให้ทุนสำหรับการพัฒนายารักษาโรคมาลาเรียชนิดใหม่ โดยอาศัยองค์ความรู้เกี่ยวกับข้อมูลจีโนมิกส์ (genomics) และชีวเคมีของเชื้อมาลาเรียมาใช้

Table 1. Overview of antimalarial drugs.

Drug	Target	Main limitation[1]
Chloroquine	food vacuole	resistance
Quinine	not known	compliance/safety/resistance
Amodiaquine	not known	safety/resistance
Mefloquine	not known	(safety)/resistance/(cost)
Primaquine	not known	safety
Halofantrine	not known	safety/resistance/cost
Artemisinins (artemether,arteether,artesunate)	food vacuole	compliance/(safety)/(GMP)[2]/(cost)
Sulfadoxine-pyrimethamine (Fansidar^{R})	folate pathway (DHPS-DHFR)[3]	resistance
Atovaquone-proguanil (Malarone^{R})	mitochondrion-folate	resistance / potential/cost
Lumefantrine-artemether (Coartem^{R})	not known-food vacuole	(compliance)/resistance/potential(cost)
Antibiotics used in combination	apicoplast	(safety)/(cost)

[1] Liabilities placed in brackets refer to issues that are less serious for the drug in question than those liabilities not placed in brackets.

[2] GMP= good manufacture practice.

[3] DHPS-DHFR= dihydropteroate synthase-dihydrofolate reductase .

ในปัจจุบัน Malaria Genome Project สำหรับเชื้อ *P. falciparum* ได้สำเร็จลงแล้ว [6, 7] และใกล้จะสำเร็จแล้วสำหรับเชื้อ *P. yoelii* ที่ติดเชื้อเฉพาะในหนูถีบจักร [8] ส่วนข้อมูลสำหรับเชื้อ *P. vivax* คาดว่าจะสำเร็จเร็ว ๆ นี้ [9] ข้อมูลจีโนมิกส์ ที่ได้ดังกล่าวทำให้ความรู้พื้นฐานด้านชีวเคมีของเชื้อ *P. falciparum* มีความกระจ่างชัดขึ้น ถึงแม้จะต้องรอการศึกษาต่อไปอีกในยุคหลังจีโนมิกส์ (post-genomics era) ซึ่งเป็นการวิจัยที่เกี่ยวข้องกับหน้าที่การทำงานของ genome เหล่านี้ อาทิเช่น gene knockout, transcriptomics, DNA microarrays, RNA interference, proteomics, metabolomics เป็นต้น เพื่อให้ข้อมูลพื้นฐานมีความสมบูรณ์ขึ้นและประยุกต์ใช้งานได้

บทความนี้จะเน้นการนำองค์ความรู้ทางจีโนมิกส์และชีวเคมีของเชื้อไปประยุกต์ใช้ในการพัฒนายารักษามาลาเรียชนิดใหม่ได้ และจะยกตัวอย่างตำแหน่งโมเลกุลเป้าหมาย (molecular drug targets) ในวิถีเมตาบอลิสมต่าง ๆ ที่คาดว่าจะได้ยาใหม่ไปยับยั้งที่ตำแหน่งเหล่านี้ได้ และไปทำลายเชื้อมาลาเรียที่มีการดื้อต่อยาได้ในที่สุด

จีโนมิกส์และชีวเคมีของเชื้อมาลาเรีย

(Genomics and biochemistry of malaria parasite)

ตั้งแต่ปี 1996 The Institute for Genomic Research, The Wellcome Trust Sanger Institute และ Stanford Genome Technology Center ได้ร่วมกันทำ

Table 2. Malaria parasites have three genomes: one chromosomal and two organellar genomes.

Genome		Size	Numbers of Genes&protein	RNA genes	Protein targeting
Chromosomal	DNAs	2.28 Mb	5,268	43	4,471 (cytosol/membrane)
Chromosome	1	0.64 Mb	143	0	ND[1]
	2	0.95 Mb	223	1	ND
	3	1.06 Mb	239	2	ND
	4	1.20 Mb	237	5	ND
	5	1.34 Mb	312	5	ND
	6	1.38 Mb	312	3	ND
	7	1.35 Mb	277	7	ND
	8	1.32 Mb	295	0	ND
	9	1.54 Mb	365	0	ND
	10	1.69 Mb	403	0	ND
	11	2.04 Mb	492	2	ND
	12	2.27 Mb	526	3	ND
	13	2.75 Mb	672	5	ND
	14	3.29 Mb	769	2	ND
Extrachromosomal (organellar) DNAs					
Mitochondrial DNA		6 kb[2]	3		246
Apicoplast DNA		35 kb[3]	30		551

[1]ND= not determined.

[2] linear DNA with A+T = 69 %

[3] circular DNA with A+T= 86 %

Malaria Genome Project โดยมีจุดหมายในการหาลำดับนิวคลีโอไทด์ของ genomeในเชื้อ *P. falciparum* ซึ่งมี chromosomes อยู่ทั้งสิ้น 14 คู่ที่เรียงขนาดจากเล็กไปใหญ่ (chromosome 1 มีขนาด 0.64 Mb → chromosome 14 มีขนาด 3.29 Mb) โดยอาศัยความช่วยเหลือจากบริษัท Cerela Genomics ในการจัดเรียงลำดับของแต่ละ gene ลงบนแต่ละ chromosome และกำหนดว่าเป็น gene ที่ code สำหรับ protein และ gene สำหรับ RNA อะไรบ้าง รวมทั้งไปทำหน้าที่ที่ไหนบ้าง (protein targeting) อาทิ เช่น ไซโตซอล (cytosol) เยื่อหุ้มเซลล์ (cell membrane) ไมโตคอนเดรีย (mitochondria) หรือ apicoplast (ตารางที่ 2) เชื้อ *P. falciparum* genome มีประมาณ 5,300 genes (ทั้ง nuclear genomes และ extrachromosomal genomes ซึ่งก็ได้แก่ genome ในmitochondria และ apicoplast) และได้ประกาศความสำเร็จเมื่อเดือนตุลาคม ปี 2002 ที่ผ่านมา [6]

Genome ของเชื้อ *P. falciparum* มีลักษณะสำคัญต่าง ๆ ได้นำมาเปรียบเทียบกับ genomes ของ *P. vivax*, ยุงพาหะ *Anopheles* spp. รวมทั้งของมนุษย์ [6, 10-14] นอกจากนี้เชื้อมาลาเรียยังมี genome อันที่ 2 อยู่ที่ออร์กาเนล ไมโตคอนเดรีย ซึ่งมีขนาด 6 kb และเป็นเส้นตรง (linear DNA) จะมี genes เพียง 3 ชนิด และมี genome อันที่ 3 อยู่ที่ออร์กาเนล apicoplast ซึ่งมีขนาด 35 kb และเป็นวงกลม (circular DNA) จะมี genes ประมาณ 30 ชนิด (ตารางที่ 3) ออร์กาเนลไมโต-คอนเดรีย และ apicoplast มีระบบ transcription และ translation เป็นแบบเซลล์โปรคารีโอต (prokaryotic cell) และแบคทีเรีย จึงเป็นตำแหน่งเป้าหมายของยา antibiotics (ตารางที่ 1) ขณะนี้ยังได้ใช้ประโยชน์ของ 6kb-DNA จากไมโตคอนเดรีย บอกถึงการวิวัฒนาการซึ่งเชื่อว่า *P. falciparum* แยกมาจาก *P. reichenowi* ของลิงชิมแปนซีเมื่อประมาณ 5 -10 ล้านปีก่อน และมีจุดกำเนิดที่ทวีปอัฟริกา และกระจายไปสู่ภูมิภาคต่าง ๆ ทั่วโลกเมื่อประมาณ 5 หมื่นปีที่ผ่านมา [15] ซึ่งสอดคล้องกับข้อมูลการวิเคราะห์ single nucleotide polymorphisms (SNPs) บน chromosome 3 ที่บอกจุดกำเนิดของ *P. falciparum* ว่ามีอายุ ประมาณ 1 แสนปีกว่า [16]

Table 3. Unique characteristics of genomes in *P. falciparum*, *P. vivax*, *Anopheles* spp. and human.

Characteristic	*P. falciparum*[1]	*P. vivax*	*Anopheles*	Human
Size (Mb)	23	24	280	2,900
(A+T) content (%)	81	60	ND[2]	59
Number of genes	5,268	5,126	15,000	31,000
Hypothetical protein (%)	60	ND	ND	ND
Gene density (kb per gene)	4.3	4.4	ND	ND
Genes with introns (%)	54	ND	ND	>90
Percent coding	53	ND	ND	<5
Number of exons per gene	2.4	ND	ND	ND
Microsattelites frequency	++	+	ND	++++
SNPs (site)	10,000[3]	ND	400,000	1,420,000

[1] Comparative genomics of *P. falciparum* and *Arabidopsis thaliana* shows the most similarity.[6, 12]

[2] ND= not determined.

[3] SNPs (single nucleotide polymorphisms) of *P. falciparum* is only determined in chromosome 3 (~403 sites), the value is based on our calculation for all 14 chromosomes, assuming 1 SNP site per 2.3 kb).

ในการศึกษาชีวเคมีของเชื้อ *P. falciparum* ในขณะนี้ทราบว่ามีโปรตีนที่ทำหน้าที่ต่าง ๆ แล้ว 39% ของจำนวนโปรตีนทั้งหมด อาทิเช่น structural proteins, cell adhesion, chaperone, defense/immunity, carrier, transporter, โปรตีนควบคุมกระบวนการการ transcription และ translation และเอนไซม์ เป็นต้น (ตารางที่ 4) มีโปรตีนที่คาดไว้แต่ยังไม่ทราบหน้าที่อีก 61 % ของจำนวนโปรตีน นอกจากนี้ยังได้มีการจัดทำ website metabolomics ของ metabolic pathways ต่าง ๆ ของเชื้อ *P. falciparum* [17] โดยใช้แผนที่ภาพของ Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway [18] และความรู้จากการศึกษาทางชีวเคมีในช่วง 30-40 ปีที่ผ่านมา รวมทั้งข้อมูลจากการวิเคราะห์ลำดับนิวคลีโอไทด์ใน Malaria Genome Project [6, 8, 9] สามารถเชื่อมโยงฐานข้อมูลต่าง ๆ เช่น Entrez, Swiss-protein database, KEGG เป็นต้น

ภาพรวมเมตาบอลิสมของเชื้อมาลาเรีย (Overview of malaria parasite metabolism)

ข้อมูลทันสมัยจาก Malaria Genome Project พบว่า เชื้อ *P. falciparum* มีเอนไซม์ 733 ชนิด (ประมาณ 14 % ของจำนวนโปรตีนทั้งหมด 5,268 ชนิด, ตารางที่ 4) สามารถสร้างวิถีเมตาบอลิสม (metabolic pathways) ต่าง ๆ อาทิเช่น glycolysis, tricarboxylic acid cycle (Krebs cycle), electron transport pathway, pentose phosphate pathway, fatty acid biosynthetic pathway, heme biosynthetic pathway, coenzyme Q biosynthesis, shikimic acid pathway, amino acid metabolism, purine และ pyrimidine pathway, folate metabolism และ hemoglobin catabolic pathway (รูปที่ 1)

วิถีเมตาบอลิสมเหล่านี้อาจถูกจัดแบ่งแยกเป็นส่วนต่าง ๆ ที่บางวิถีเมตาบอลิสมไม่พบในเซลล์ของมนุษย์ อาทิเช่น ออร์กาเนล apicoplast หรือบางวิถีเมตาบอลิสมอาจพบได้ในเซลล์ของมนุษย์แต่มีลักษณะต่าง ๆ ของเอนไซม์ในวิถีนั้น ๆ แตกต่างไป เช่น การสลายน้ำตาลกลูโคส จะเป็นแบบไม่ใช้ O_2 (anaerobic glycolysis) หรือบางวิถีเมตาบอลิสมมีความเหมือนกันกับวิถีที่พบในเซลล์แบคทีเรีย อาทิเช่นวิถีการสังเคราะห์โคเอนไซม์โฟเลต ซึ่งใช้เป็นตำแหน่งเป้าหมายของยารักษามาลาเรียกลุ่ม antifolates ตัวอย่างเช่น pyrimethamine, cycloguanil, sulfonamides เป็นต้น ความรู้ความเข้าใจลักษณะที่เป็นเอกลักษณ์จำเพาะของวิถีเมตาบอลิสมเหล่านี้ที่พบได้ในเชื้อ *P. falciparum* โดยเฉพาะเอนไซม์ที่เร่งปฏิกิริยา

Table 4. Possibly molecular functions predicted from *P. falciparum* genomics.

Function	% of total protein
Structural molecule	3
Cell adhesion molecule	2
Chaperone	2
Defense/Immunity protein	2
Enzyme	14
Enzyme regulator	1
Ligand binding or carrier	10
Transporter	2
Transcription regulator	1
Translation regulator	1
Other	1
No assignment	61

จำเพาะอาจจะเป็นตำแหน่งโมเลกุลเป้าหมาย สำหรับการพัฒนายารักษามาลาเรียชนิดใหม่ รวมทั้งเป็นเอนไซม์ตำแหน่งเป้าหมายของยารักษาที่ใช้ในปัจจุบันได้ (รูปที่ 2)

เมื่อปี 2002 Robert Ridley ผู้เชี่ยวชาญขององค์การอนามัยโลกได้สรุปตำแหน่งเป้าหมายไว้ในวิถีเมตาบอลิสมต่าง ๆ ที่พบทั้งในส่วนไซโตซอล ถุงอาหาร (food vacuole) ไมโตคอนเดรีย apicoplast รวมทั้งเยื่อหุ้มเซลล์ โดยอาศัยองค์ความรู้เกี่ยวกับชีวเคมีของเชื้อมาลาเรียและแนวทางการพัฒนายารักษามาลาเรียชนิดใหม่ [19]

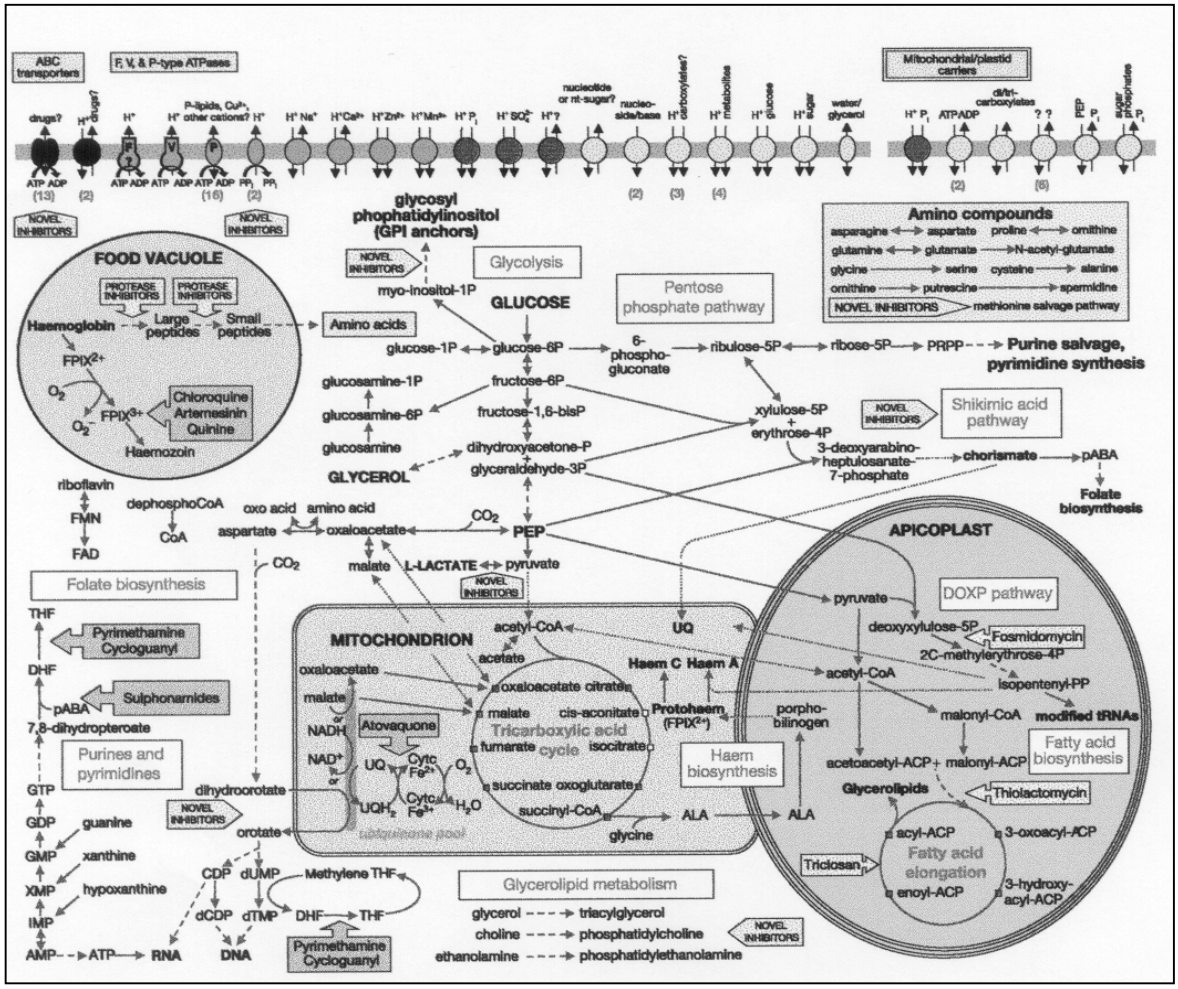

Figure 1. Overview of metabolism in *P. falciparum*. Glucose and glycerol are carbon sources. Broken lines indicate several omitted steps of a metabolic pathway. In various metabolic pathways, anaerobic glycolysis to generate ATP operates in cytosol, Krebs cycle exists in a mitochondrion and operates only in sexual gametocytes but not in asexual stages, heme and fatty acid synthesis operate in apicoplast, hemoglobin catabolic pathway operates in food vacuole. The known antimalarial drug targets are also illustrated (Ref. 6).

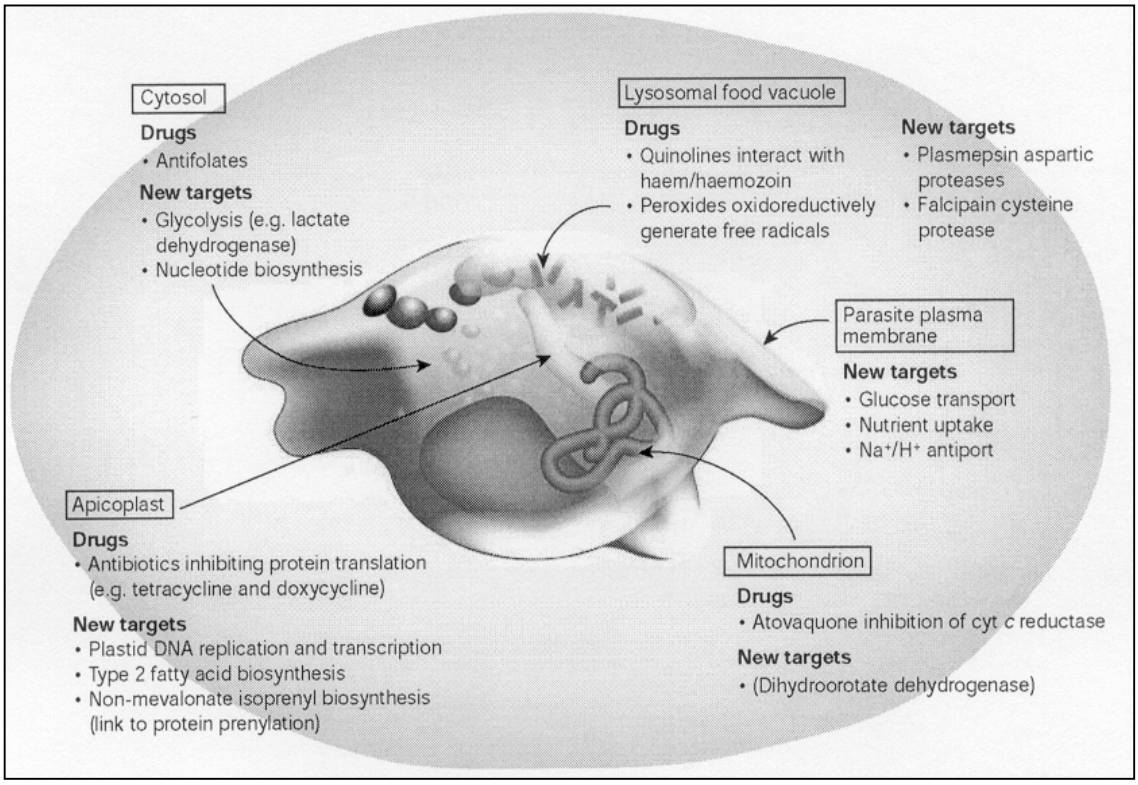

Figure 2. Growing trophozoite stage of *P. falciparum* in a human red cell. The organellar (mitochondrion, apicoplast, food vacuole) and cytosolic metabolic pathways are possible targets for new antimalarial development. Lists of about 20 enzymes in the pathways are also proposed (Ref. 19).

เมตาบอลิสมของโคเอนไซม์โฟเลต (Metabolism of coenzyme folate)

โคเอนไซม์โฟเลตทำหน้าที่เป็นตัวให้และ/หรือตัวรับหน่วยคาร์บอนในปฏิกิริยาต่าง ๆ ในวิถีเมตาบอลิสมของเพียวรีน, ไพริมิดีน, กรดอะมิโน รวมทั้งในขั้นตอนแรก ๆ ของการสังเคราะห์โปรตีน เชื้อมาลาเรียจะสังเคราะห์โฟเลตขึ้นใช้เองโดยเริ่มจากสารตั้งต้น guanosine 5'-triphosphate (GTP) เหมือนกับในเซลล์แบคทีเรียที่เป็น *de novo* pathway ในขณะที่เซลล์ของมนุษย์ไม่สามารถสังเคราะห์ได้เอง [20] นอกจากนี้เชื้อยังมี shikimic acid pathway ที่จะสังเคราะห์ p-aminobenzoic acid (pABA) ให้กับวิถีสังเคราะห์โฟเลตด้วย [22] ขณะนี้พบว่ามี gene สำหรับเอนไซม์ในวิถีโฟเลตครบยกเว้น dihydroneopterin aldolase ที่ยังตรวจสอบไม่ได้ [23] ในวิถีนี้มีเอนไซม์ dihydropteroate synthase (DHPS) เป็นตำแหน่งเป้าหมายของยา sulfonamides และเอนไซม์ dihydrofolate reductase (DHFR) เป็นตำแหน่งเป้าหมายของยา pyrimethamine และ cycloguanil การเกิดผ่าเหล่าของ genes ที่ให้เอนไซม์ทั้ง 2 ทำให้มีการสังเคราะห์เอนไซม์ที่ผิดปกติบริเวณ active site จะไม่สามารถจับกับยาดังกล่าวได้ดี เกิดมีภาวะการดื้อยาของเชื้อที่พบได้ในทุกภูมิภาคของโลกที่มีการระบาดของมาลาเรีย [21]

เมตาบอลิสมของเพียวรีนและไพริมิดีน (Metabolism of purine and pyrimidine)

วิถีการสังเคราะห์นิวคลีโอไทด์ของทั้งเพียวรีนและไพริมิดีน เป็นตำแหน่งเป้าหมายในการพัฒนายารักษามาลาเรียกลุ่มใหม่ (รูปที่ 1 และ 2) เชื้อมาลาเรียจะ

สังเคราะห์เพียวรีนนิวคลีโอไทด์โดยอาศัยการดึงเบสมาจากเซลล์เจ้าบ้าน (salvage pathway) และไม่มีความสามารถสังเคราะห์ขึ้นได้เอง ซึ่งต่างจากเซลล์ของมนุษย์ ดังนั้นถ้ายับยั้งเอนไซม์ในการดึงเบสมาใช้ จะสามารถฆ่าเชื้อมาลาเรียได้ เอนไซม์ที่มีการศึกษากันมากคือ purine nucleoside phosphorylase (PNP) และ hypoxanthine guanine xanthine phosphoribosyltransferase (HGXPRT) เอนไซม์ PNP จะเร่งปฏิกิริยา phosphorolysis ของ inosine ให้ hypoxanthine จากนั้นเอนไซม์ HGXPRT จะเติมหมู่ ribosyl phosphate ให้ hypoxanthine, xanthine หรือ guanine ได้เป็น inosine 5'-monophosphate (IMP), xanthosine 5'-monophosphate (XMP) และ guanosine 5'-monophosphate (GMP) ตามลำดับที่ต่างจากเอนไซม์นี้ของมนุษย์ (รูปที่ 1) genes ของทั้งเอนไซม์ HGXPRT และ PNP ของเชื้อ *P. falciparum* ได้ถูกโคลนและแสดงออกได้ดีในเชื้อแบคทีเรีย *E. coli* จึงทำให้มีการศึกษาถึงสารยับยั้งที่มีโครงสร้างเหมือนกับ transition state ของเอนไซม์ทั้ง 2 ได้ คือ สาร immucillin GP [24] และสาร immucillin G [25, 26] โดยอาศัยโครงสร้าง 3 มิติที่ได้จาก x-ray crystallography ของเอนไซม์ HGXPRT ในเชื้อ *P. falciparum* [27] สาร immucillin G จับกับเอนไซม์ PNP ของเชื้อ (K_i เท่ากับ 0.03 nM) ได้ดีกว่าเอนไซม์ของมนุษย์ถึง 30 เท่า และมีฤทธิ์ฆ่าเชื้อมาลาเรียได้ที่ IC_{50} (ระดับความเข้มข้นที่ทำให้เชื้อตายไปครึ่งหนึ่ง) เท่ากับ 50 nM [26] ส่วนสาร immucillin GP และสาร immucillin HP ซึ่งมีหมู่ฟอสเฟตที่ปลาย 5' ของน้ำตาลไรโบส จับกับเอนไซม์ HGXPRT ของเชื้อและเอนไซม์ HGPRT ของมนุษย์ได้ดีพอ ๆ กัน และไม่มีรายงานการทดสอบฤทธิ์ฆ่าเชื้อมาลาเรียของสารทั้งสอง [24, 27]

เชื้อ *P. falciparum* สามารถสังเคราะห์ไพริมิดีนนิวคลีโอไทด์ได้เอง (de novo) และไม่ใช้การดึงไพริมิดีนจากเซลล์เจ้าบ้าน ซึ่งแตกต่างไปจากเซลล์ของมนุษย์ [28] ดังนั้นถ้ายับยั้งเอนไซม์ในการสังเคราะห์ดังกล่าว ก็จะสามารถฆ่าเชื้อมาลาเรียได้โดยไม่ทำอันตรายต่อเซลล์ของมนุษย์ กลุ่มวิจัยของเราได้ศึกษาเอนไซม์ในวิถีการสังเคราะห์ไพริมิดีนอย่างต่อเนื่อง เอนไซม์ dihydroorotate dehydrogenase (DHOD) ของเชื้อมีความแตกต่างโดยเฉพาะที่ active site จากเอนไซม์ของมนุษย์ และเป็นตำแหน่งเป้าหมายใหม่ที่จะใช้พัฒนายารักษามาลาเรีย [28-31]

เมตาบอลิสมของการย่อยสลายฮีโมโกลบิน (Metabolism of hemoglobin breakdown)

เชื้อ *P. falciparum* มีความสามารถอย่างจำกัดในการสังเคราะห์กรดอะมิโนขึ้นเอง ถึงแม้ว่าจะมี shikimic acid pathway ช่วยสังเคราะห์กรดอะมิโนชนิด aromatic ได้บ้าง รวมทั้งมีความสามารถในการเปลี่ยนกรดอะมิโนชนิดต่าง ๆ กลับไปมาได้บ้าง (รูปที่ 1) เป็นผลทำให้เชื้อมีการดึงกรดอะมิโนเกือบทั้งหมดมาใช้โดยตรงจากเซลล์เจ้าบ้านหรือการสลายฮีโมโกลบินของเม็ดเลือดแดงที่ถูกกินเข้าไปในถุงอาหารของตัวเชื้อ (ประมาณ 60 -70 % ของฮีโมโกลบินในเม็ดเลือดแดง) เพื่อให้ได้กรดอะมิโนไปใช้ในวิถีเมตาบอลิสมต่าง ๆ และฮีมที่เกิดขึ้นอาจเป็นที่จับของยา chloroquine รวมทั้งยาสมุนไพรจีน artemisinin และอนุพันธ์ของมัน ในระหว่างปี 1988-1990 Daniel Goldberg และคณะ [32] ได้เริ่มศึกษากระบวนการสลายโกลบินในถุงอาหารของเชื้อ *P. falciparum* และค้นพบวิถีการสลายโกลบินที่จัดเป็นระเบียบโดยอาศัยเอนไซม์ที่เป็น acidic proteases หลายชนิดทั้งที่เป็น aspartate proteases เรียกชื่อเฉพาะว่า “plasmepsin” I, II, IV และ cysteine proteases ซึ่งเรียกว่า “falcipains” [33]

เอนไซม์ plasmepsin I (PM I) จะสลายโกลบินสายแอลฟาก่อนที่จำเพาะตำแหน่งเดียว จากนั้น PM II, PM IV, falcipains, facilysins จะสลายให้โกลบินเส้นสั้น ๆ แล้วจึงถูกเอนไซม์อื่น ๆ เช่น aminopeptidases สลายต่อให้กรดอะมิโนอิสระ โครงสร้างของเอนไซม์ PM II ของเชื้อมาลาเรียถือเป็นโครงสร้างแรกของเอนไซม์ในเชื้อที่มีการค้นพบ [34, 35] เอนไซม์ PM II เป็นตำแหน่งเป้าหมายใหม่ในการพัฒนายารักษามาลาเรีย [36-38] โดยจะมีบริเวณ active site ต่างจากเอนไซม์ aspartate

proteases ของมนุษย์เช่น pepsin, cathepsin D เป็นต้น สารยับยั้งที่จับเอนไซม์ PM II ของเชื้อ (K_i เท่ากับ 0.07 μM) ได้ดีกว่าเอนไซม์ cathepsin ของมนุษย์ประมาณ 22 เท่า สารยับยั้งดังกล่าวอาทิเช่น allophenylnorstatine KNI-727 มีฤทธิ์ฆ่าเชื้อมาลาเรีย *P. falciparum* ได้ที่ IC_{50} เท่ากับ 10 μM. [37, 38]

เมตาบอลิสมของลิปิด (Metabolism of lipid)

เชื้อมาลาเรีย *P. falciparum* ไม่สามารถสังเคราะห์ cholesterol ได้เอง จะอาศัยดึงจากเซลล์เจ้าบ้านมาใช้ แต่มีความสามารถในการสังเคราะห์ phospholipids (PL) บางชนิดขึ้นได้เอง เช่น phosphatidylethanolamine (PE) และ phosphatidylcholine (PC) โดยผ่าน Kennedy pathway เหมือนกับในเซลล์ของสัตว์เลี้ยงลูกด้วยนม ในเชื้อมาลาเรียพบว่าทั้ง PE และ PC จะเป็นส่วนประกอบส่วนใหญ่ถึง 90 % ของ PL ทั้งหมด สำหรับ phosphatidylserine (PS) ซึ่งเป็นส่วนประกอบประมาณ 5 % ของ PL จะถูกสังเคราะห์จากเอนไซม์ PS synthase (PSS) ที่มีคุณสมบัติเหมือนกับในเซลล์แบคทีเรีย นอกจากนี้ยังมีการเปลี่ยน serine ไปเป็น ethanolanine โดยเอนไซม์ serine decarboxylase (SDn) ที่ต่างไปจากเซลล์ของมนุษย์ (รูปที่ 3) สำหรับการสังเคราะห์ phosphatidylinositol (PI) ในเชื้อจะคล้ายกับในเซลล์ของมนุษย์ [39, 40]

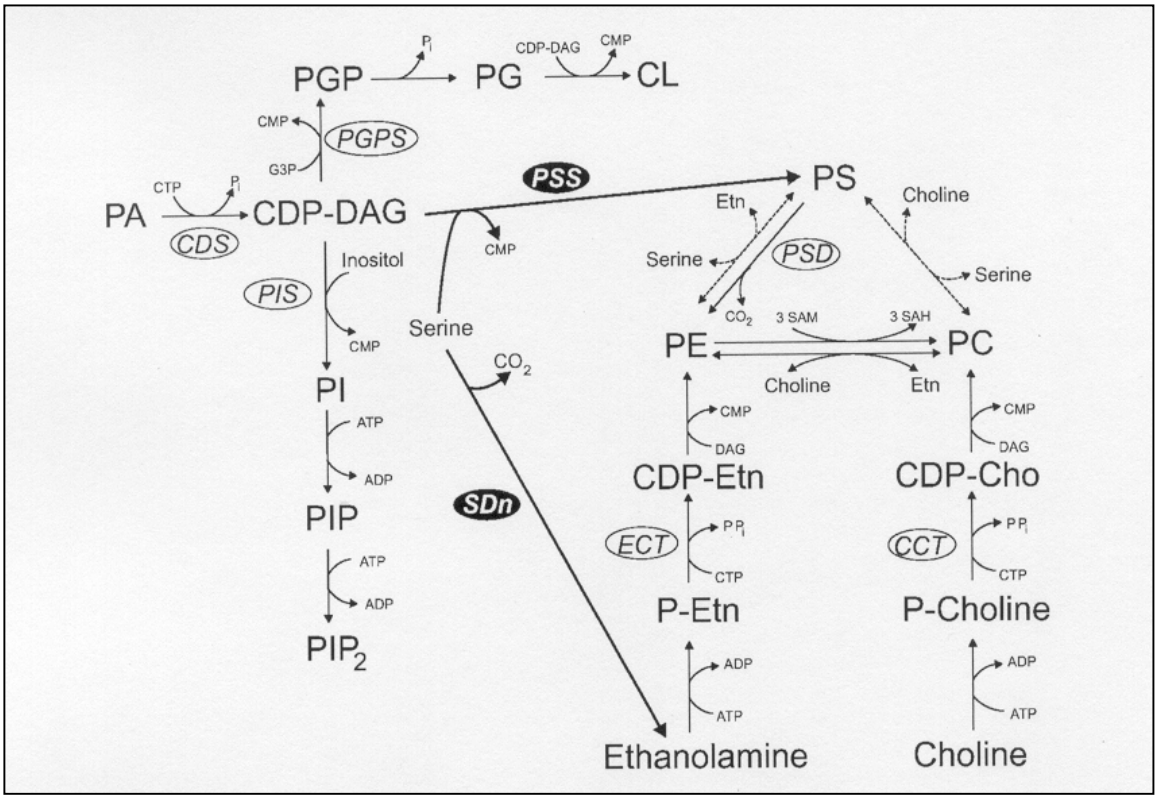

Figure 3. Biosynthesis of phospholipid (PL) in *P. falciparum*. Phosphatidylethanolamine (PE) and phhosphatidylcholine (PC) synthetic reactions are typically Kennedy pathway. Bold lines indicate the reactions operating in the malaria parasite, but absence from human cell: CDP-DAG → PS and serine → ethanolamine. Broken lines show the reactions existing in human cell, but no significance in the malaria parasite. Most abbreviations are found in the text (Ref. 39).

เนื่องจากเชื้อมาลาเรียมี apicoplast ซึ่งเป็นออร์กาเนลที่ได้จาก secondary endosymbiosis จาก algae ชนิดหนึ่งแต่ไม่สามารถทำหน้าที่สังเคราะห์แสงได้ [41, 42] ใน apicoplast มีการสังเคราะห์สาร isoprenoid เช่น isopentenyl diphosphate โดย deoxyxylulose 5-phosphate (DOXP) pathway ซึ่งเป็นแบบ non-mevalonate pathway (รูปที่ 1) โดยจะไม่พบ pathway แบบนี้ในเซลล์มนุษย์ สาร isoprenoid ดังกล่าวไปจับกับโปรตีนหลายชนิดเกิดกระบวนการ farnesylation ขึ้น DOXP pathway ก็เป็นตำแหน่งเป้าหมายหนึ่งในการพัฒนายารักษามาลาเรียกลุ่มใหม่ เช่น fosmidomycin (เป็น herbicide ชนิดหนึ่ง) ที่สามารถยับยั้งการทำงานของเอนไซม์ 2-C-methyl-D-erythrose 4-phosphate synthase ในปฏิกิริยาการสังเคราะห์ methylerythrose phosphate จาก DOXP [6, 19, 41, 42]

ออร์กาเนล apicoplast ยังมีระบบการสังเคราะห์ mRNA และโปรตีนเป็นแบบของเซลล์แบคทีเรีย จึงเป็นตำแหน่งของยา antibiotics ที่ใช้ในการรักษาร่วมกับ

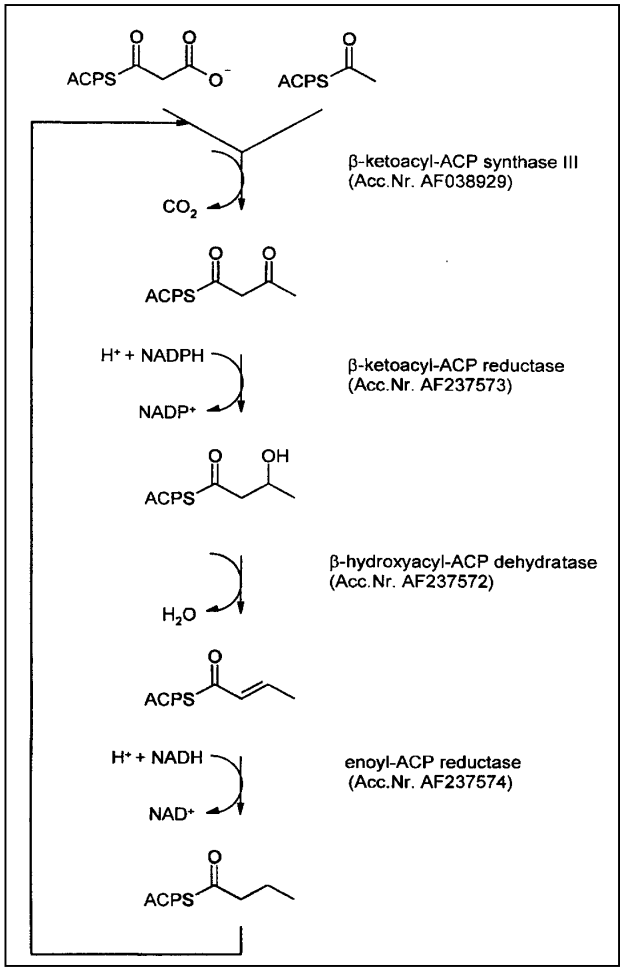

Figure 4. Four enzymatic steps in elongation of fatty acid biosynthesis in *P. falciparum*. The enzymes are shown with the GenBank database accession numbers (in brackets). Enzyme NADH-dependent enoyl-ACP reductase, catalyzes conversion of *trans*-2-enoyl-ACP into Acyl-ACP, serves as molecular drug target of antibacterial tricosan, Genz-8575 and Genz-10850.

ยารักษามาลาเรียตัวอื่น (quinine) ที่ใช้อยู่ อาทิเช่น tetracycline, doxycycline และ clindamycin เป็นต้น (ตารางที่ 1, รูปที่ 2) [41, 42] นอกจากนี้ apicoplast จะมีการสังเคราะห์กรดไขมันเป็นแบบที่ II ซึ่งต่างจากของสัตว์ทั่วไปและของมนุษย์ซึ่งเป็นแบบที่ I การสังเคราะห์กรดไขมันแบบที่ II จะพบได้ใน plastid ของพืช ใน algae และในเซลล์แบคทีเรีย เอนไซม์ในการสังเคราะห์กรดไขมันแบบที่ II นี้มีคุณสมบัติจำเพาะคือ เอนไซม์ทั้ง 7 ชนิดที่ใช้สำหรับการสังเคราะห์กรดไขมันจะไม่รวมกันเป็นกลุ่มเอนไซม์ 7 ชนิดบนโปรตีนเดียวกัน (multifunctional single polypeptide) แตกต่างไปจากเอนไซม์นี้ ที่พบในแบบที่ I (รูปที่ 4)

เอนไซม์ NADH-dependent enoyl-ACP reductase ของเชื้อมาลาเรียเป็นตำแหน่งเป้าหมายโดยสาร tricosan (ซึ่งเป็นยาฆ่าเชื้อแบคทีเรียชนิดหนึ่ง) จะยับยั้งการทำงานของเอนไซม์ดังกล่าว โดยไปจับกับเอนไซม์ได้ดีที่ K_i ประมาณ 0.05 μM สามารถฆ่าเชื้อมาลาเรีย *P. falciparum* สายพันธ์ต่าง ๆ ที่ IC_{50} ในระดับ 0.7-1.5 μM [43, 44] และฆ่าเชื้อมาลาเรีย *P. berghei* ในหนูทดลองได้หมดที่ระดับ 38 mg/kg [44] ได้มีการศึกษาโครงสร้าง 3 มิติของเอนไซม์จากเชื้อมาลาเรียแล้ว รวมทั้งสาร tricosan และสาร Genz-8575 และ Genz-10850 (ได้จากการทำ high-throughput screening) ที่ไปจับกับบริเวณ active site [43, 45] ความรู้ความเข้าใจดังกล่าวทำให้เอนไซม์ enoyl-ACP reductase ที่ใช้สังเคราะห์กรดไขมันแบบที่ II ในเชื้อมาลาเรียเป็นตำแหน่งเป้าหมายที่มีความเป็นไปได้สูงที่จะพัฒนายารักษามาลาเรียชนิดใหม่ได้

บทสรุปและแนวโน้มการศึกษาวิจัย (Conclusion and research trend)

ในระยะ 2-3 ปีที่ผ่านมาหลังจากที่มีองค์ความรู้ด้านจีโนมิกส์ของเชื้อมาลาเรียแล้ว [46] ทำให้องค์ความรู้พื้นฐานด้านชีววิทยา [47] และด้านชีวเคมีที่ศึกษาต่อเนื่องเป็นเวลานาน [48] มีความเข้าใจกระจ่างชัดยิ่งขึ้นในวิถีเมตาบอลิสมต่าง ๆ ของเชื้อมาลาเรีย ทั้งที่บางวิถีมีความเหมือนและมีความแตกต่างจากเซลล์ของมนุษย์ที่เป็นเจ้าบ้าน สามารถนำไปประยุกต์ในการพัฒนายารักษามาลาเรียชนิดใหม่ที่อาศัยตำแหน่งเป้าหมายที่เป็นเอนไซม์ในวิถีเมตาบอลิสมที่มีเอกลักษณ์จำเพาะต่อเชื้อ และไม่ทำอันตรายต่อเซลล์ของมนุษย์ จะเป็นวิธีการหนึ่งที่จะได้ยาที่ใช้ในการควบคุมโรคมาลาเรียต่อไปได้ [48-51]

ในระยะทุก ๆ 5 ปี ข้างหน้า มีการคาดหมายว่าจะมียาใหม่เพิ่มขึ้น 1 ชนิดให้ได้ ขณะนี้ องค์กรต่าง ๆ ที่ให้ความสนใจมีมากขึ้นอาทิเช่น Global Fund to Fight AIDS, Tuberculosis and Malaria [52], the Multilateral Initiative on Malaria in Africa [53, 54], the Medicines for Malaria Venture [19, 55] และ the Roll Back Malaria [56] โดยมีองค์การอนามัยโลกเป็นผู้ประสานการให้ทุนการวิจัยและพัฒนายารักษามาลาเรียตัวใหม่ อย่างไรก็ตามกระบวนการในการพัฒนายาตัวหนึ่ง ๆ จะต้องอาศัยสหศาสตร์ (multi-disciplinary) และสหทรัพยากร (multi-resources) มาร่วมกันใช้เพื่อสร้างความสำเร็จ โดยบทบาทของชีวเคมีรวมทั้งข้อมูลทางจีโนมิกส์ จะให้ตำแหน่งเป้าหมายที่คาดว่าจะพัฒนาสารยับยั้งหรือสารนำ (lead compounds) ที่ไปสู่ยารักษามาลาเรียชนิดใหม่ ๆ ได้ [57-61]

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กิจกรรมการศึกษาต่อเนื่องสำหรับแพทย์

ท่านสามารถได้รับการรับรองอย่างเป็นทางการสำหรับกิจกรรมการศึกษาต่อเนื่องสำหรับแพทย์ กลุ่มที่ 3 ประเภทที่ 23 (ศึกษาด้วยตนเอง) โดยศูนย์การศึกษาต่อเนื่องของแพทย์ จุฬาลงกรณ์มหาวิทยาลัย ตามเกณฑ์ของศูนย์การศึกษาต่อเนื่องของแพทย์แห่งแพทยสภา (ศนพ.) จากการอ่านบทความเรื่อง **"เชื้อมาลาเรีย จีโนมิกส์ และชีวเคมี กับตำแหน่งเป้าหมายในการพัฒนายารักษาโรคมาลาเรีย"** โดยตอบคำถามข้างล่างนี้ ที่ท่านคิดว่าถูกต้องโดยใช้แบบฟอร์มคำตอบท้ายคำถาม โดยสามารถตรวจจำนวนเครดิตได้จาก http://www.ccme.or.th

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คำถาม - คำตอบ

1. เชื้อมาลาเรียที่ทำให้เกิด severe and cerebral malaria คือ
   - ก. *Plasmodium vivax*
   - ข. *Plasmodium falciparum*
   - ค. *Plasmodium malariae*
   - ง. *Plasmodium ovale*
   - จ. All of above
2. ข้อความต่อไปนี้ถูกต้องเกี่ยวกับจีโนมิกส์ของเชื้อมาลาเรีย
   - ก. Mitochondrion genome มีขนาด 16 kb เท่ากับของมนุษย์
   - ข. Apicoplast genome มีขนาด 100 kb เท่ากับ chloroplast ของพืช
   - ค. Nucleus genome มีขนาด 23 Mb เล็กกว่าของยุงก้นปล่องและมนุษย์
   - ง. มี single nucleotide polymorphisms จำนวนเท่า ๆ กับของมนุษย์
   - จ. มี genes ทั้งหมด 31,000 genes
3. Metablic pathway ที่พบเฉพาะในเชื้อมาลาเรีย และไม่พบในมนุษย์ได้แก่
   - ก. Folate biosynthesis
   - ข. Heme biosynthesis
   - ค. Anaerobic glycolysis
   - ง. Fatty acid biosynthesis
   - จ. เฉพาะข้อ ก และ ข

✂.....

คำตอบ สำหรับบทความเรื่อง "**เชื้อมาลาเรีย จีโนมิกส์ และชีวเคมี กับตำแหน่งเป้าหมายในการพัฒนายารักษาโรคมาลาเรีย**"

จุฬาลงกรณ์เวชสาร ปีที่ 50 ฉบับที่ 2 เดือนกุมภาพันธ์ พ.ศ. 2549

รหัสสื่อการศึกษาต่อเนื่อง 3-23-201-9010/0602(1004)

ชื่อ - นามสกุลผู้ขอ CME credit.....เลขที่ใบประกอบวิชาชีพเวชกรรม.....

ที่อยู่.....

1. (ก) (ข) (ค) (ง) (จ)
2. (ก) (ข) (ค) (ง) (จ)
3. (ก) (ข) (ค) (ง) (จ)
4. (ก) (ข) (ค) (ง) (จ)
5. (ก) (ข) (ค) (ง) (จ)

4. คู่ของเอนไซม์และยาหรือสารยับยั้งที่ทำงานใน hemoglobin catabolic pathway คือ
    ก. Heme synthetase/chloroquine
    ข. Lactate dehydrogenase/NADH
    ค. Plasmepsin/allophenylnorstatine
    ง. Dihydrofolate reductase/pyrimethamine
    จ. Purine nucleoside phosphorylase/immucillin
5. ข้อใดต่อไปนี้ถูกต้องเกี่ยวกับยารักษามาลาเรีย และตำแหน่งเป้าหมายของยา
    ก. Fansidar ®/pentose phosphate pathway
    ข. Chloroquine/glycolysis
    ค. Coartem ®/fatty acid biosynthesis
    ง. Halofantrine/purine biosynthesis
    จ. Malarone ®/mitochondrion and folate biosynthesis

ท่านที่ประสงค์จะได้รับเครดิตการศึกษาต่อเนื่อง (CME credit)
กรุณาส่งคำตอบพร้อมรายละเอียดของท่านตามแบบฟอร์มด้านหน้า

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ประธานคณะกรรมการการศึกษาต่อเนื่อง
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Review article

Putative metabolic roles of the mitochondria in asexual blood stages and gametocytes of *Plasmodium falciparum*

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Abstract

Upon infection into human red cell, *Plasmodium falciparum* differentiates into asexual and sexual (gametocyte) stages. The mitochondrion is a tubular-cristate organelle, functionally and structurally different between the two stages. Genes and proteins involving metabolic and functional roles, protein targeting and import to this organelle, are comprehensively reviewed. The genes and proteins of the electron transport system are identified, partially characterized in human and rodent malaria parasites consisting of a single subunit of NADH dehydrogenase, two subunits of succinate dehydrogenase, cytochrome C reductase and cytochrome Coxidase. One of the primary functional roles of the mitochondrion in the parasite is the coordination of pyrimidine biosynthesis, the electron transport system and oxygen utilization through dihydroorotate dehydrogenase. All enzymes of tricarboxylic acid cycle, pyruvate dehydrogenase complex and some enzymes of ATP synthase, are identified and partially characterized using the completed *P. falciparum* genome. Some metabolic and functional roles of the organelle include oxidative phosphorylation, ubiquinone and heme biosynthesis, antioxidant defense and redox balance. Recent physiological studies involve membrane potential maintenance, cellular signaling and cation homeostasis. The organelle is a target for antimalarial drug, i.e. atovaquone. Based on the lines of evidence, we hypothesize that the parasite exhibits metabolic adaptation of the underdeveloped mitochondrial organelle to life in the mosquito vector and the human host.

Keywords: malaria; mitochondrion; metabolism; electron transport system; oxygen utilization; pyrimidine biosynthesis; tricarboxylic acid cycle; heme biosynthesis; ubiquinone biosynthesis; protein synthesis; antimalarial drug target

INTRODUCTION

Malaria remains one of the most important diseases of the world, causing annual infections to at least 515 million people from the developing countries and 1.5-2.7 million deaths mainly in sub-Saharan Africa[1-3]. Of the four *Plasmodium* species of blood-borne apicomplexan parasite, *P. falciparum* is responsible for the most severe form of human malaria. Disease symptoms include fever, chills, prostration, anemia, delirium, metabolic acidosis, cerebral malaria, multi-organ system failure, coma and death[2,4]. To combat the disease, there is an urgent need to develop new drugs due to the increasing prevalence of drug resistant parasites to currently use antimalarials, chloroquine and sulfadoxine-pyrimethamine, which consequently accounts for increase morbidity and mortality[5,6].

Upon blood stage infection, *P. falciparum* grows and differentiates into various asexual stages, i.e., ring, trophozoite and schizont, as well as into infectious sexual stages (gametocytes) which are taken up during the mosquito blood meal. In the *Anopheles* mosquito, sporozoite-stage parasites develop after fertilization of male and female gametes

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role in the transfer of electrons between the mtETS complexes and acts as a proton carrier that supports ATP synthesis [80]. Thus the inhibition of the electron transfer at this site represents an important target for chemotherapeutic attack.

One class of compounds extensively investigated over fifty years for antimalarial activity is the quinone analog, hydroxynaphthoquinone. One of the compounds, atovaquone which is 2-(trans-4-(4′-chlorophenyl) cyclohexyl) -3-hydroxy-1, 4-naphthoquinone, is currently used as a potent antimalarial [113]. Atovaquone affects multiple sites in which Q_{8-9} plays a significant catalytic role in the target enzyme, e. g. complex Ⅲ[111], and DHODase [39]. The malarial parasites synthesize their own ubiquinones and cannot salvage this from the host. Furthermore, all genes necessary for a complete ubiquinone biosynthetic pathway, starting from chorismate to ubiquinone, are also identified. We have found that atovaquone at micromolar levels strongly inhibits ubiquinone biosynthesis in *P. falciparum*, similar to its effect on *Pneumocystis carinii* [114].

Many derivatives of 5-hydroxy-2-methyl-1, 4-naphthoquinone have been synthesized and tested for their antimalarial activities on the in vitro growth of *P. falciparum* [115]. These include fifteen quinoline quinones[40]. 5-Hydroxy-2-methyl-1, 4-naphthoquinone exhibits a strong effect on the malarial mtETS complex I, II and mitochondrial oxygen consumption [74, 76]. Thus, quinoline quinones may represent a new class of compounds with potent antimalarial activity.

Proteomics

There are 246 (~4.6% of total parasite proteins) proteins targeted to the mitochondrion after translation using the TargetP and MitoProtII methods as originally identified [34, 61]. Using recently developed PlasMit program for prediction of mitochondrial transit peptides, Bender *et al* [62] have predicted 381 (~7.1%) mitochondrial proteins, based on 5334 annotated genes in the *P. falciparum* genome, close to the numbers of the mitochondrial proteins in other organisms. However, the human mitochondrial proteomics indicate about 1000 proteins involved for bioenergetic function, carbohydrate and lipid metabolisms, and regulatory functions in apoptosis and cell death, and homeostasis [116]. Most of the parasite mitochondrial protein sequences identified to date are hypothetical proteins. Nevertheless, homology searching using bioinformatics approaches have identified some components of metabolic pathways, transporter and import proteins (Table 1).

Import mechanism

Basic knowledge on mitochondrial import mechanism has been limited[117]. A long N-terminal sequence having a typical feature of a mitochondrial targeting signal has been identified in the de novo pyrimidine synthetic DHODase gene homologue for the first time in *P. falciparum* [118]. The DHODase is then verified as the mitochondrial protein by using a polyclonal antibody raised against the purified protein from *P. falciparum* and immunogold-labeled electron microscopy [26].

Recently, Wilson's group has used the GFP chimeric protein targeted to the mitochondrion and identified the import signal within a region of 68 amino acids in the mitochondrial protein HSP-60 (Table 1) which may play a role as a molecular chaperone[93-96]. Most recently, the transferring protein orthologue (translocase of outer membrane) has also been identified in *P. falciparum*[119]. The proteins contain N-terminal sequences with homologies to mitochondrial transfer peptides which use to enter the organelle, similar to higher eukaryotes[62]. Sienkiewicz *et al*[102], in contrast, recently demonstrates that the mitochondrial iron superoxide dismutase (FeSOD) has a 70-residue long N-terminal extension that shows a typical bipartite apicoplast organelle targeting sequence[120], but the FeSOD protein targets the GFP fusion into the parasite's mitochondrion. The verification of the proposed mitochondrial proteins will, therefore, be necessary.

Comparing mitochondrial and apicoplast organelles

A non-photosynthetic chloroplast, or apicoplast, has been demonstrated in malaria parasites since eleven years ago[121]. The apicoplast, as well as the mitochondrion, is considered to be a target for drug development [122]. The apicoplast retains a circular

Table 1 *P. falciparum* mitochondrial proteins and their biochemical functions

No	Name	Function
1	Coq4	CoQ biosynthesis
2	Lon protease homologue	Chaperone/protease
3	Prohibitin/BAP37 (PHB2 homologue)	Respiratory chain assembly
4	Valyl-tRNA synthetase	Translation
5	Elongation factor g (EF-G)	Translation
6	50s RPL24	Translation
7	50s RPL17	Translation
8	DNA-directed RNA polymerase	Transcription
9	EF-Tu	Translation
10	50S RPL2	Translation
11	Citrate synthase	TCA cycle
12	Isocitrate dehydrogenase (NADP-dependent)	TCA cycle
13	Succinyl-CoA sythetase beta subunit	TCA cycle
14	Succinate dehydrogenase Fe-S subunit	TCA cycle/complex II
15	Fumerate hydratase class I	TCA cycle
16	NADH dehydrogenase	Complex I
17	Rieske Fe-S protein 3	Complex III
18	Cytochrome c1	Complex III
19	ATP synthase beta subunit	Complex V
20	ATP synthase gamma subunit	Complex V
21	ATP synthase alpha subunit	Complex V
22	ATP synthase delta subunit	Complex V
23	Dihydroxy hexaprenylbenzoate methyltransferase	CoQ biosynthesis
24	Geranyl diphosphate synthase/prenyl transferase (Coq1)	CoQ biosynthesis
25	Coq2	CoQ biosynthesis
26	Coq5 (CoQ synthesis methyltransferase)	CoQ biosynthesis
27	Coq8 (ubiquinol-cytochrome c reductase assembly protein, ABC1)	CoQ biosynthesis
28	Branched-chain alpha keto-acid DH E1 alpha subunit	Amino acid degradation
29	Branched-chain alpha keto-acid DH E1 beta subunit	Amino acid degradation
30	Mitochondrial serine hydroxymethyltransferase	One carbon metabolism
31	Rhodanese	Amino acid degradation/ Cyanide detoxification
32	Mitochondrial intermediate processing peptidase (MPP)	Import
33	MPP alpha subunit	Import
34	MPP beta subunit	Import/complex III
35	HSP-60/CPN-60	Import
36	Mitochondrial phosphate carrier	Transport
37	Delta aminolevulinate synthase	Heme synthesis
38	HSP-70/DnaK	Protein folding
39	Dihydroorotate dehydrogenase	Pyrimidine biosynthesis
40	GrpE	Protein folding

Table 2 Structure, genomics and proteomics of mitochondrial and apicoplast organelles in the asexual stage of *P. falciparum*

Property	Mitochondrion	Apicoplast
Size (in trophozoite stage)	1.0x0.2 μm	1.6x0.3 μm
Membrane layer	2	3-4
Number per cell	single	single
DNA:		
-size	6 kb	35 kb
-shape	linear	circular
-copy number	>20	>15
-% A + T content	69%	86%
- gene encoding protein	3	23
tRNA	import	import
Protein identified´	381	551
Replication:		
-mechanism	phage-like	bi-directional theta, rolling circle
-replicating enzyme	unknown	multienzyme complex
Transcription/Translation	bacterial-type, 70S ribosome	bacterial-type, 70S ribosome

Representing ~7.1% and ~10% of the total proteins encoded by the parasite genome.

Table 3 Functional/metabolic roles between mitochondrial and apicoplast organelles in the asexual bloog stage *P. falciparum*

Property	Mitochondrion	Apicoplast
Electron transport system	present	absent
Oxygen consumption	present	absent
Oxidative phosporylation/ ATP synthesis	absent	absent
Pyrimidine synthesis	involved	not involved
Tricarboxylic acid cycle	present	absent
Ubiquinone biosynthesis	present	absent
Heme biosynthesis	involved	involved
Fatty acid biosynthesis´	absent	present
Isoprenoid biosynthesis	absent	present
Redox and antioxidant system	present	possibly present
Cellular signaling/homeostasis	present	unknown

The operating fatty acid biosynthesis requires the utilization of NADH as an electron donor and the production of fatty acid as an electron acceptor, however, it is presently unknown for an antioxidant defense system in the apicoplast.

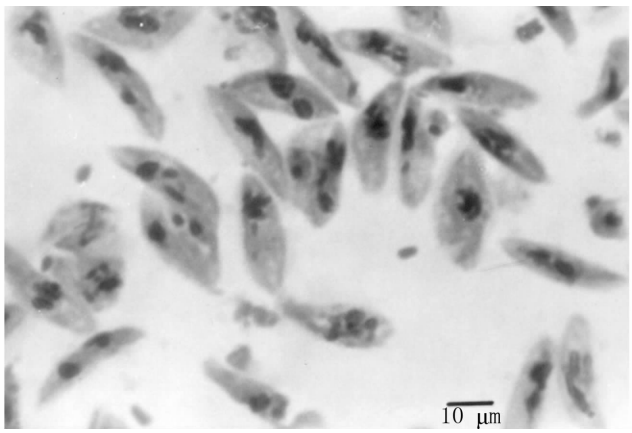

Figure 1 Maturing sexual satge of *Plasmodium falciparum* from an *in vitro* culture. (Giemsa)

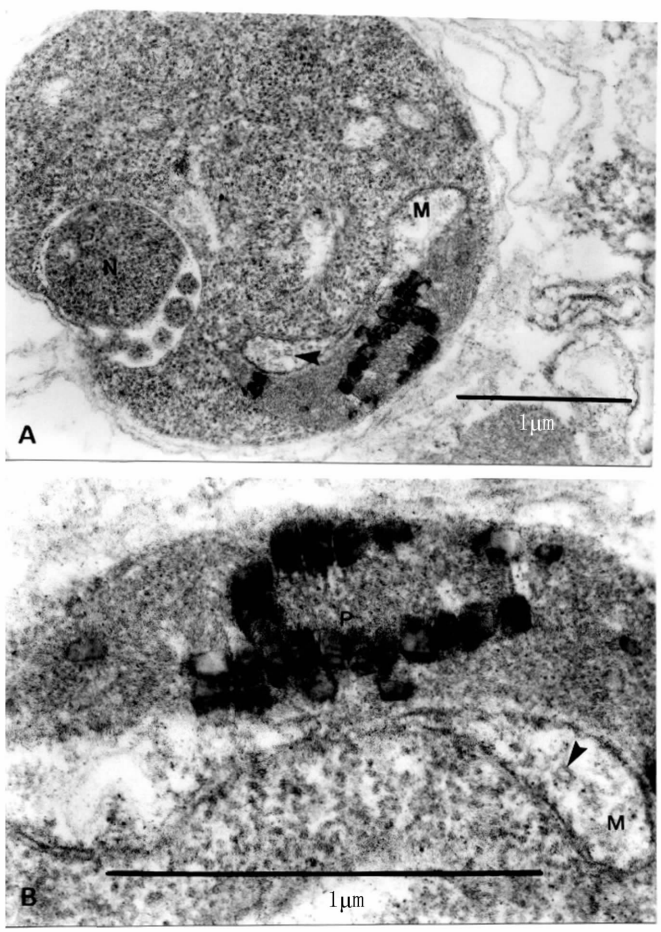

Figure 2 Asexual stage of *Plasmodium falciparum* (trophozoite stage) polymerized from catabolized heme in a food vacuole (TEM) . A: Single mitochondrion with a clearly double membrane organelle, and an elongated form, preparing for binary fission; B: Single tubular cristate structure(arrowhead) in the organelle at higher magnification, classified as type I mitochondrion . N: Nucleus; M: Mitochondria; P: Crystalline hemozoin pigment.

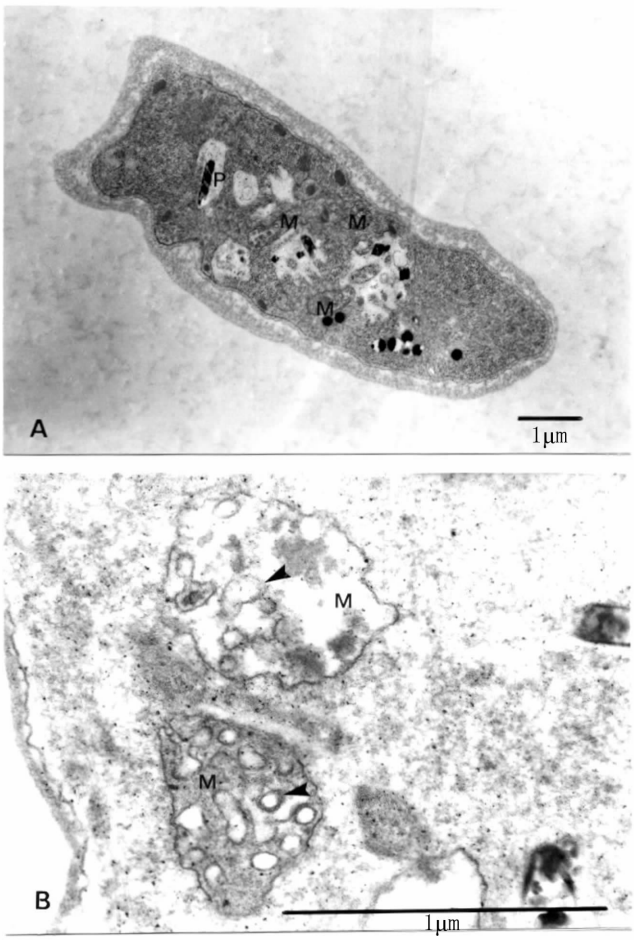

Figure 3 Sexual stage of *Plasmodium falciparum* (gametocyte stage IV) (TEM). A: Many mitochondria in a single parasite B: Two organelles containing several cristae (arrowheads) clssified as type II mitochondrion. M: Mitochondria; P: Pigment.

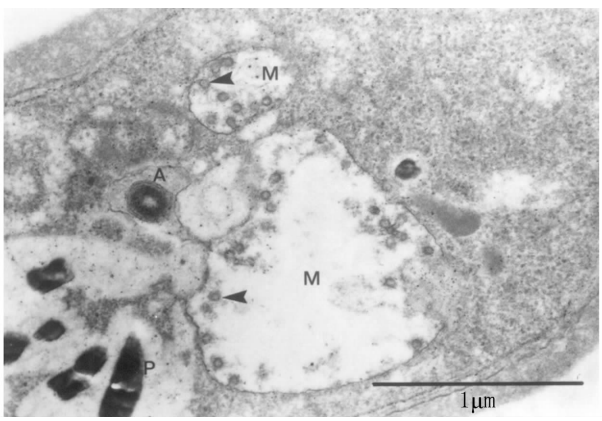

Figure 4 Type III mitochondria in the maturing sexual stage of *Plasmodium falciparum* (TEM). Electron-dense and finely compact of the tubular cristae (arrowheads) are typically found with this type of the mitochondrion. An apicoplast, associated to one of the mitochondria, was observed with a multi-membraneous organelle, containing electron-dense matrix and absence of internal cristae. M: Mitochondria; P: Pigment; A: Apicoplast. It is noted that the mitochondrial localization is closely associated to the food vacuole, a place where hemoglobin is degraded to free amino acids and hemozoin pigment is formed.

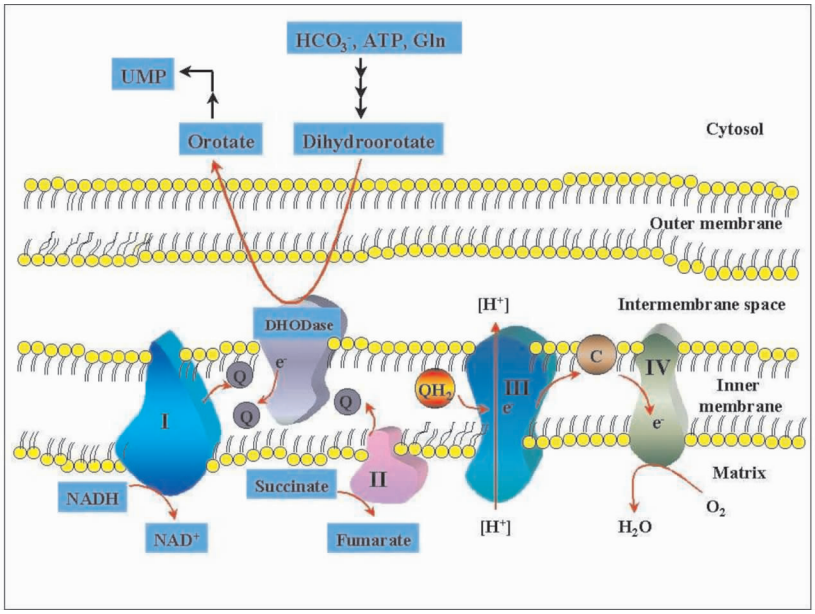

Figure 5 A diagram of the proposed arrangement of the mitochondrial electron transport system and its link with pyrimidine biosynthesis *via* dihydroorotate dehydrogenase in the asexual and sexual blood stages of *Plasmodium falciparum*. Dihydroorotate dehydrogenase (DHODase), an inner membrane protein of the pyrimidine pathway, generates electrons to the mitochondrial electron transporting complexes, containing NADH – ubiquinol reductase (a single component of complex I, NADH dehydrogenase), succinate – ubiquinone oxidoreductase (succunate dehydrogenase, complex II), cytochrome c reductase (complex III) and cytochrome c oxidase (complex IV) which is the final electron acceptor. Q, oxidized ubiquinone; QH_2, reduced ubiquinone; C, cytochrome C.

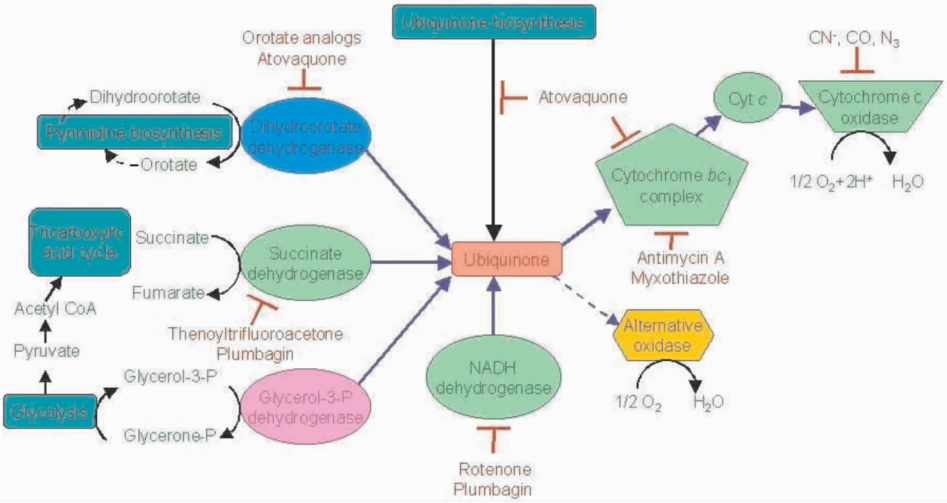

Figure 6 A diagram of proposed mitochondrial metabolic networks of *Plasmodium falciparum*. The mitochondrial electron transport complexes contain a single polypeptide NADH dehydrogenase, two – subunits succinate dehydrogenase, cytochrome c reductase (cytochrome bc_1) and cytochrome c oxidase. Alternative oxidase as a cyanide (CN^-) insensitive – branched pathway (shown in a broken line) and glycerol – 3 – phosphate (glycerol – 3 – P) dehydrogenase are also included in the respiratory chain. A part of the pyrimidine biosynthesis is shown and linked to coenzyme ubiquinone. The ubiquinone plays a central role for electron and proton transferring coenzyme. Cytochrome c (Cyt c, a component of cytochrome bc_1 complex) is also shown serving an electron transferring protein. Glycolysis, possibly links to the TCA cycle, is operating in the cytosol. The tree – bars indicate known active sites of inhibitors and the antimalarial atvaquone, plumbagin. The diagram is adapted from web site: http://sites.huji.ac.il/malaria.

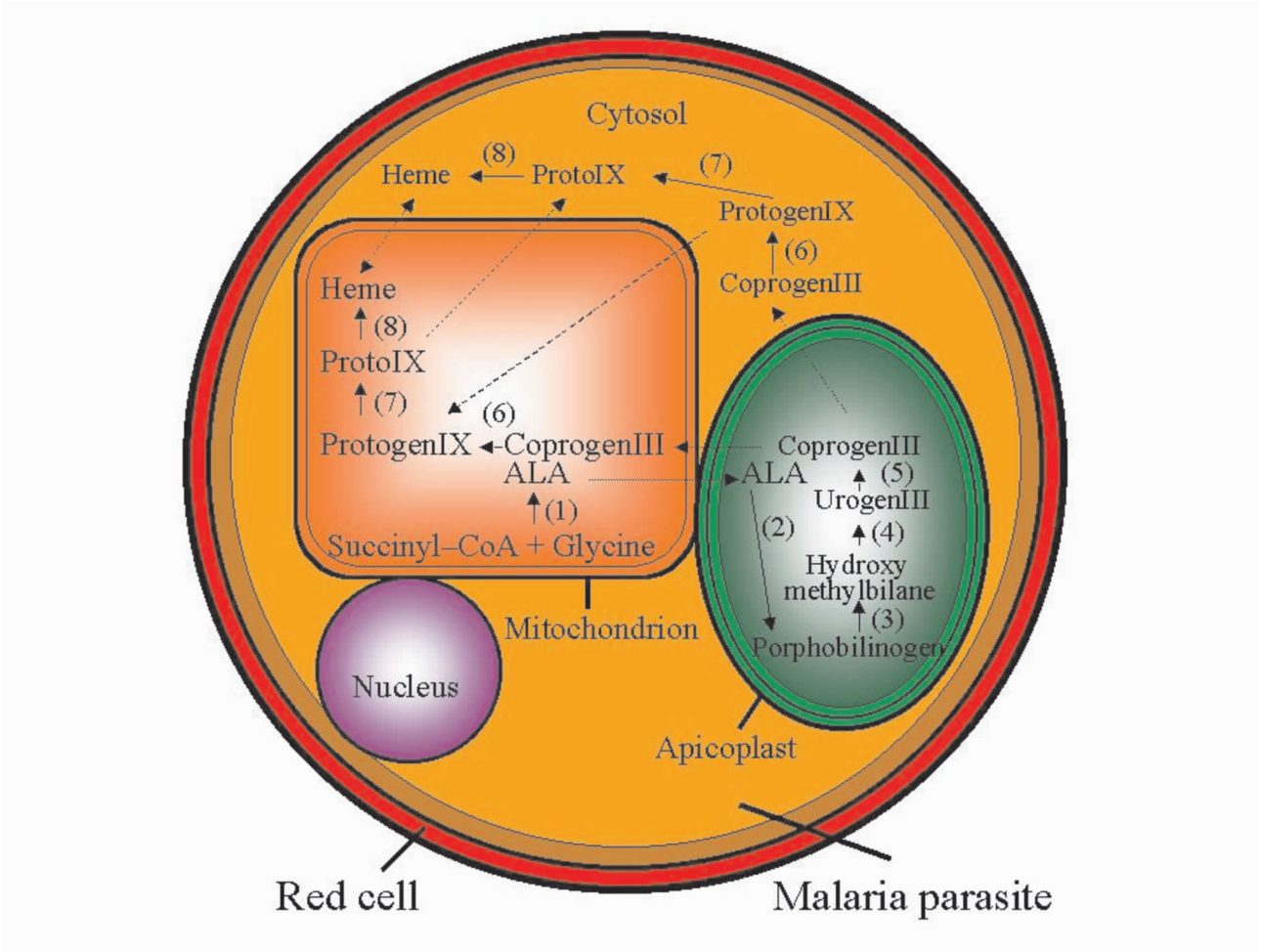

Figure 7 A schematic representation of *de novo* heme biosynthetic pathway of *Plasmodium falciparum*. The parasite's heme pathway operates in three possible compartmentations: mitochondrion, apicoplast and cytosol. Solid and broken indicate eight enzymatic Reactions (1→8) and proposed exchange of metabolites among three subcellular compartments, respectively. δ-aminolevulenic acid synthase catalyzes the condensation of succinyl – CoA and glycine (Reaction 1) to produce δ-aminolevulenic acid (ALA) in the mitochondrion, porphobilinogen synthase and hydroxylmethylbilane synthase catalyze ALA to prophobilinogen (Reaction 2) and then to hydroxymethylbilane (Reaction 3) in the apicoplast. The fourth and the fifth enzymes, uroporphyrinogenIII (UrogenIII) synthase (Reaction 4) catalyzing the production of UrogenIII, and UrogenIII decarboxylase (Reaction 5) converting UrogenIII to coproporphyrinogenIII (CoprogenIII), occur also in the apicoplast. The last three enzymes of the pathway, CoprogenIII oxidase (Reaction 6) catalyzing CoprogenIII to protoporphyrinogenIX (Protogen IX), ProtogenIX oxidase (Reaction 7) converting ProtogenIX to protoporphyrin IX (ProtoIX), and ferrochelatase (Reaction 8) producing heme from ProtoXI, are possibly operating in either cytosol or mitochondrion. The hypothetical pathway for the functional heme biosynthesis in the parasite is adapted from Sato *et al* [94].

35 kb of its original algal plastid genome and synthesizes 23 proteins[123]. Apicoplast DNA is inherited only through the female gamete [55], and the organelle DNA replication occurs by a unique enzyme complex synthesized by an open reading frame encoding contiguous DNA polymerase, DNA primer and DNA helicase components [124]. More than 500 proteins predicted to function in the apicoplast have been identified using the bioinformatic approaches and experimental evidences, leading to rapid advancement in metabolic maps and functional determinations [125]. We have summarized the present knowledge on properties, stuctures, genomics, transcriptomics, proteomics, and metabolic/functional roles in the mitochondrion and apicoplast of *P. falciparum* (Table 2, 3).

Aided by the recent progress of the malarial genome database, we now have a better understanding of several metabolic networks confined with the apicoplast. The primary ones are type II fatty acid biosynthesis (acetyl-CoA ---> fatty acids) and isoprenoid biosynthesis (non-mevalonate pathway). The type II *de novo* fatty acid synthetic pathway is catalyzed by separate enzymes as demonstrated in bacteria and plants, unlike the type I fatty acid pathway in mammals which were shown to be multifunctional enzymes [125]. Detailed characterization of lipoylation pathways involving pyruvate dehydrogenase complex indicates that the apicoplast can function in converting pyruvate to acetyl-CoA for use in fatty acid biosynthesis which it is absent in the mitochondrion [126, 127], but the other lipoylation pathway involving α-keto acid dehydrogenase complex is located in the parasite´s mitochondrion[106]. This is an unusual property of the mitochondrion, named as the strange organelle [128]. The organelle cannot produce acetyl-CoA, but takes it up from the apicoplast for the TCA cycle to generate NADH and other metabolites. In the isoprenoid biosynthesis using isopentenyl diphosphate as the precursor, the apicoplast operates the non-mevalonate pathway using the enzymes 1-deoxy-D-xylulose-5-phosphate (DOXP) synthase and DOXP reductoisomerase, similar to those of bacteria and algae [123].

It has been proposed earlier that the malaria parasite imports a nearly complete set of host-cell heme biosynthetic enzymes to use for its own machinery apparatus to produce heme [129]. Later, all eight enzymes required for the heme *de novo* pathway have been identified in the nuclear genome of the parasite [61]. The malaria parasite, unlikes any other organisms, has the ability to synthesize heme *de novo* by sharing the pathway within the boundaries of the apicoplast and the mitochondrial organelle (Figure 7) [94, 125, 130]. The localization of the first three enzymes, δ-aminolevulenic acid synthase (ALAS), porphobilinogen synthase (PBGS) and hydroxylmethylbilane synthase (HMBS) operating in the pathway has been verified using a GFP reporter in live transfected *P. falciparum* [94]. ALAS is targeted to the mitochondrion, but PBGS and HMBS are targeted to the apicoplast. The fourth and the fifth enzymes, uroporphyrinogen III synthase (UROS, Reaction 4) and uroporphyrinogen III decarboxylase (UROD, Reaction 5), have apparent apicoplast targeting sequences at their N-terminus. The last three enzymes of the pathway, coproporphyrinogen III oxidase (CPO, Reaction 6), protoporphyrinogen IX (PPO, Reaction 7) and ferrochelatase (FC, Reaction 8), lack the bipartite sub-structures at their N-terminus [94]. Hence enzymes UROS and UORD are predicted to be apicoplastic proteins, and enzymes CPO, PPO and FC are either cytosolic or mitochondrial proteins. This suggests a mechanistic model for multiple intracellular localization of the parasite organelle proteins, especially in the *de novo* heme biosynthesis. Some human mitochondrial proteins have also multiple subcellular compartments, their detailed targeting mechanisms recently reviewed[131]. Compartmentation of all heme enzymes in the parasite remains to be verified. It has been hypothesized that an exchange of metabolites in the pathway to produce heme between the two organelles ensues, including organelle attachment[94, 125]. The proposed contact/attachment of both organelles is evident by visualizing their close apposition on subcellular fractionations and electron micrographs using either *P. falciparum* (Figure 4) or other malaria parasites [30, 132].

CONCLUSIONS AND FUTURE PROSPECTS

Based on the morphological, biochemical and genetic findings in the asexual and sexual stages of the human malaria parasite *P. falciparum*, mitochondrial heterogeneity may have functional significance for growth and development and completion of life cycle. The mitochondrial structures and functions may

reflect an evolution of *Plasmodium* spp. in which they are living in relatively low oxygen environments of the human host to maintain their redox balance, and also an organelle metabolic adaptation to life in the mosquito vector. It is necessary to study the biochemistry and physiology of the mitochondrion in more detail, for instance, membrane potential, differences in the mechanism of energy metabolism, functionality of the tricarboxylic acid cycle, oxidative phosphorylation and ATP synthesis, functional properties of the electron transport complexes, roles of ubiquinone and heme biosynthesis, and oxygen tension on the survival of the parasites circulating in the human blood[74, 107, 133].

Regulation of the tricarboxylic acid cycle, the electron transport system and the oxidative phosphorylation for energy metabolism needs to be considered [77-79, 134]. Understanding of the parasite´s organelle biogenesis is still requiring, including the involvement of cellular signaling essential for the process [71]. In addition, detailed internal organization of the organelle related to its metabolic adaptation and heterogeneity should be further elucidated using novel electron microscopic tomography [45, 135].

Special thanks to the malarial genome database [61], about 250-380 proteins are predicted to target to the mitochondrion post-translationally. These include some enzymes of the pyruvate dehydrogenase complex, the complete tricarboxylic acid cycle enzymes, many electron transport complexes and ATP synthase[46-47, 51, 61-63, 128]. Functional analyses remain to be elucidated using techniques such as gene knock-out, RNA interference, microarray and metabolomics [32, 35, 42]. The mitochondrion is a chemotherapeutic target for antimalarial drug development, for example, the enzyme dihydroorotate dehydrogenase [136]. In our post-genomics era, proteomics should be performed with mitochondria from all stages of the human parasite, in the presence or absence of any novel compounds affecting biogenesis and functions of the organelle. Identification of genes/proteins responsible for the mitochondrial heterogeneity throughout the life cycle of the parasite is, likewise, necessary.

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OPINION ARTICLE
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Biochemistry Research in Thailand: Present Status and Foresight Studies

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ABSTRACT: Here, we present the status of biochemistry in Thai universities in many aspects such as departmental infrastructures, academic staffs, budgets for academic and research affairs, research activities including publications, mapping and directions, and finally foresight and policy prospects. This study provides a linkage of personnel and research activities, and lends further insights into the capability of Thai biochemists in terms of the publication in high impact journals. The data indicated that the average impact factor of the journal published by Thai biochemists was comparable to the average impact factor of the journals listed in biochemistry and molecular biology category in the Institute for Scientific Information-Web of Science (ISI-WOS). We hope that this study may serve as a model for other disciplines of science and technology in Thailand and help to enhance the capability of Thai researchers.

KEYWORDS: Thailand Biochemistry, Publications, Research mapping, Policy, Foresight.

INTRODUCTION

Biochemistry is a cross-discipline integrating chemistry and life in our world. The subject is linked to various biological sciences, e.g., medicine, para-medicine (e.g., pharmaceutical sciences, dentistry, veterinary medicine, medical technology, nurse, public health, etc.), agriculture, and also to other natural sciences, as well as to industry. The development of biochemistry in Thai universities started with the establishment of the Department of Biochemistry at the Faculty of Science at Mahidol University to train graduate students in 1964. Five years later, the Department of Biochemistry at the Faculty of Science at Chulalongkorn University started offering a Bachelor of Science degree in biochemistry. All departments responsible for teaching biochemistry in faculties of medicine were originally formed in combination with the Department of Physiology. For example, the Department of Biochemistry at the Faculty of Medicine at Chulalongkorn University, was initially established in 1947 and became independent in 1964.

At present, there are 15 departments of biochemistry distributed among nine state universities. Interestingly, only five departments offer undergraduate curricula in biochemistry, whereas up to 10 departments train graduate students in biochemistry and related subjects, e.g., molecular biology, genetic engineering and bioinformatics. Exceptionally, only the Department of Chemistry at Chiang Mai University offers a Bachelor of Science in biochemistry program, which is taught by staff in biochemistry. Although biochemistry research in Thailand originated more than 40 years ago as evidenced by the first biochemistry publication from Thailand in an international database in 1967 [1], the status of academic and research activities are not known. To our knowledge, there are very few attempts to analyze research performance and publication status of the Thai scientists both internationally [2-4] and nationally [5-7].

Here, we present our findings on the survey and analysis of biochemistry status in Thai universities in many aspects such as infrastructure, personnel, budgets for academic and research affairs, research activities, as well as publications, research mapping and directions, and finally foresight and policy prospects. The impact of research publications on biochemistry by Thai biochemists is also compared to that in the ISI-WOS database under the same category. This communication is a part of the final report on foresight project on physical and biological sciences at state universities during the period of 1998-2004 [8].

DEPARTMENTAL INFRASTRUCTURE OF BIOCHEMISTRY IN THAILAND

In the research project financially supported by Commission on Higher Education, Ministry of Education, we focused mainly on eight state universities. Among these eight universities, only one university had no Department of Biochemistry (Table 1). Totally, there are 13 departments of biochemistry in the Faculty of Science, Faculty of Medicine, Faculty of Pharmacy and

Table 1. Department of Biochemistry: infrastructure, academic staff and curricula in biochemistry and related subjects (data 2005).

University	Faculty	Department	Staff	Curriculum[1]		
				B.Sc.	M.Sc.	Ph.D.
Chulalongkorn (CU)	Science	CU SCI	23	+	+	+
	Medicine	CU MED	10	-	+	-
	Pharmacy	CU PHARM	10	-	-	-
	Dentistry	CU DENT	6	-	-	-
Chiang Mai (CMU)	Science	NA[2]	NA[2]	+	-	-
	Medicine	CMU MED	19	-	+	+
Kasetsart (KU)	Science	KU SCI	11	+	+	+
Khon Kaen (KKU)	Science	KKU SCI	11	+	+	-
	Medicine	KKU MED	18	-	+	+
Mahidol (MU)	Science	MU SCI	21	-	+	+
	Medicine	MU MED	11	-	+	+
	Pharmacy	MU PHARM	5	-	-	-
Prince of Songklanakarin (PSU)	Science	PSU SCI	15	+	+	+
Suranaree University of Technology (SUT)	Science	NA[2]		-	+	+
Srinakarinwirot (SWU)	Science	NA[2]		-	-	-
	Medicine	SWU MED	8	-	+	+
Burapha (BU)	Science	ND[3]		+	-	-
Naresuan (NU)	Medical Science	ND[3]		-	+	+

[1] + and – indicate the existence and non-existence of the curriculum, respectively.
[2]Data are not available.
[3]BU and NU are not included in the research project.

Faculty of Dentistry of seven universities, namely Chulalongkorn University (CU), Chiang Mai University (CMU), Kasetsart University (KU), Khon Kaen University (KKU), Mahidol University (MU), Prince of Songkla University (PSU) and Srinakarinwirot University (SWU). The only university that had no Department of Biochemistry is Suranaree University of Technology (SUT). There are also two Departments of Biochemistry which are not included in this research: at the Faculty of Science, Burapha University (BU) and Faculty of Medical Science, Naresuan University (NU).

Personnel: Number, Education, Academic Position and Age

From all 13 departments, there were 168 staff and 51% of these were at CU and MU. The percentage of their educational background can be described as follows: Ph.D. (77%), M.Sc. (20%), B.Sc. (1.5%) and Certificate or Medical Board (1.5%). Their academic positions were Professor (5%), Associate Professor (39%), Assistant Professor (24%) and Lecturer (32%). The personnel categorized as teaching staff with different age range were 21-35 years old (young or new generation, 20%), 36-50 years old (middle-age generation, 47%) and 51-65 years old (senior generation, 33%). Interestingly, an analysis of the number of staff among these three generations of age ranges yielded a normal distribution profile with the majority of staff in the age range 41-55 years old. Notably, two-thirds of staff in the CU departments were of senior generation. It is also noted that academic staff with academic position of Assistant Professor and Lecturer constitute a major portion (56%) of the whole staff. Approximately 70% of the senior generation were Associate Professors, whereas 40% of the middle-age generation were Assistant Professors. Eighty five percent of the young generation were still Lecturers. Interestingly, 75% of both middle-age and senior generations were Ph.D. holders, whereas about 85% of the young generation were Ph.D.s. Despite the limited numbers in all departments, the academic staff had very good educational background with some having had postdoctoral training abroad. This is an important asset for strengthening the biochemical research and postgraduate study in these departments.

Budget for Academic and Research Affairs

The total budget and income from all departments of biochemistry in seven universities was valued at 162.54 million Baht for the period 1998-2004 and as average per person per year was valued at 153,000 Baht for expenses in teaching activities for bachelor degree, master degree and doctoral degree, both in the field of biochemistry and other related programs.

All Departments of Biochemistry received research funding from both the universities themselves and outside the university totaling 289 million Baht. This can be divided into 218 million Baht (external source) and 71 million Baht (internal source), i.e. the ratio of external source to internal source was 3:1. The

average amount of research funding that faculty members from seven universities received externally and internally was 191,000 Baht and 61,800 Baht per person each year, respectively. It is worth mentioning that MU and CU received support from external research fund amounting to 61% of the research fund received by all seven universities. In particular, the Department of Biochemistry, Faculty of Science at MU ranked first in the amount of external research funding. The ability to obtain research funds, especially from external sources, is somewhat related to the productivity in terms of the number of publications, i.e. MU and CU contributed about 65% of total publications from all seven universities produced during the same period (see later section).

Research Activity: Past Records, Present Status and Foresight

Research output

There were totally 323 research articles published by seven state universities (the ISI-WOS database, 1998-2004) [9] or 93% of total publications from Department of Biochemistry in all universities nationwide (346 articles). MU had the highest number accounting for 1.6 fold of that from CU, the second-ranked in publication output. As a whole, each Thai biochemist published approximately 0.28 international research publications per year, which was double the number published by the physical science researchers [10]. The first three Departments of Biochemistry having the highest productivity in terms of the number of research publications per person per year, were MU SCI, CU MED, KKU MED with values of 0.88, 0.53 and 0.40, respectively. With regard to the research funding mentioned in the previous section, the research expense for one paper to be published averaged about 900,000 Baht.

Research system and individual interests: strength and weakness of biochemistry in Thailand

Research in the field of biochemistry depends on the individual rather than on the system. There are some reasons/characteristics for the strength of biochemistry research in Thailand:

(1).There was a very high percentage of faculty members with Ph.D. degree (77% of total faculty members in department of biochemistry). These Ph.D.s were also the most productive (0.34 article/person/year) compared to those without Ph.D. degree, i.e., M.Sc. (0.17 article/person/year) and B.Sc. (no productivity).

(2).The middle-age generation of staff was the most productive (0.39 article/person/year) when compared to the senior (0.36 article/person/year) and young (0.05 article/person/year) generation staff. In addition, the number of middle-age generation staff represented about a half of the total staff.

(3). Even though faculty members having Professor position represent only about 5 % of total members, they were the most productive (2.1 articles/person/year) compared to those having other academic positions, i.e.Associate Professor (0.9 article/person/year), Assistant Professor and Lecturer (0.36 article/person/year).

(4). Thai biochemists published their papers in journals listed in the ISI-WOS database (1998-2004) with the average impact factor (IFa) of 2.141 which is comparable to the international standard using the ISI-WOS database (IFa = 2.292, an average for 261 journals in biochemistry and molecular biology discipline). Furthermore, these publications by Thai biochemists were cited on the average of 5.5 citations per article, and more importantly 37% of these papers were cited more than five times per article.

(5). Up to 68% of total articles involved the cooperation of at least two institutes. Two institutional collaborators comprised 55%. Co-authorships with foreign universities accounted for 37%.

(6). Most departments had Ph.D. curricula in biochemistry and related fields. This facilitates the production of critical mass of graduate students resulting in high output of publications.

(7). The academic staff in some departments combine to form research units/centers, which can be upgraded to Center of Excellence. This is an effective strategy to produce high quality publications.

(8). Biochemistry academic staff had high potential to obtain research funds from external organizations, including some prestigious overseas funding agencies such as Rockefeller Foundation, World Health Organization, NIH USA, and USAID, etc.

(9). Four out of 37 outstanding scientists, announced by Foundation for the Promotion of Science and Technology under the Patronage of His Majesty the King during 1982-2007, are Thai biochemists belonging to one department [11].

On the other hand, there are reasons/characteristics for the research weakness in our biochemistry community:

(1). The number of faculty members having publications in journals listed in the ISI-WOS database (1998-2004) represented only 40% of total members. This means that the majority of our staff had no such publications, reflecting weak research performance in these departments.

(2). Surprisingly, two-thirds of the research publications were from only nineteen academic staff, six of whom published ≥ 2 articles per person per year.

(3). The new generation staff, comprising 21% of total staff and having more percentage of Ph.D. background, produced only 5% of total publications in the ISI-WOS database (around 0.05 article per person each year).

These analyses indicate that the research strength and weakness in our biochemistry community relies upon individual interests. However, our study provides a linkage between strong academic staff and research activities, and lends further insights into the impact of Thai biochemists, which is comparable to the average impact of biochemistry and molecular biology category on the ISI-WOS database. In addition, the young generation should be encouraged to engage in active research with more publication output.

Research mapping and direction

As mentioned earlier, research depends on the individual Thai biochemist. Using the information on all publications, citations and particular keywords for mapping research areas, the top ten categories (% distribution) in research mapping were analyzed and found to be: (1) protein (23% of total papers), (2) human, (3) enzyme, (4) polymerase chain reaction, (5) cancer, (6) drugs, (7) liver, (8) malaria, (9) virus, (10) shrimp (7% of total papers). Analyzing the citations per paper in each category provides the impact or strength of each research map as shown in Table 2. Drugs, shrimp and malaria are the three top ranked research directions and areas of expertise for our Thai biochemistry community (Table 2).

In comparison with research publication topics in the Journal of Biological Chemistry (*J. Biol. Chem.*), the top ranked journal in biochemistry with the highest citations and number of publications in biochemistry and molecular biology category during 2002-2005 based on the Science Citation Index database[9], the topics of publication are categorized as shown in Table 3[12]. The top five ranks are summarized: (1) signal transduction, (2) membrane, (3) protein structure, (4) gene structure and regulation, and (5) cell and development biology. The international topics and trend of researches here are not related to research mapping of biochemistry in Thailand found in the present study (Table 2).

Table 3. Research publication topics classified in *J. Biol. Chem.* during 2003-2005 (data updated in 2006).

Rank	Topics	%
1	Signal transduction	20
2	Membrane (transport, functions, structure, biogenesis)	15
3	Protein structure	13
4	Gene structure and regulation	10
5	Cell and Development Biology	9
6	Protein synthesis	7
6	Enzyme catalysis	7
7	DNA replication, repair, recombination	6
8	Metabolism and Bioenergetics	4
9	RNA structure, catalysis	3
9	Glycoprotein	3
10	Lipids	2
11	Genomics, proteomics, bioinformatics	1

Research foresight on production of publications

During the past 10 years, researches in Departments of Biochemistry have been published in international journals indexed by the PubMed MEDLINE and the ISI-WOS databases. Table 4 shows number of publications searched by name of academic staff and research work addressed by the Thai departments of biochemistry in the ISI database during 1998-2004. The productivity of research output is calculated as number of papers per person per year. It should be noted that the efficiency and the productivity increased about twice every 5 years.

In the next 3-4 years, it can be expected that the productivity will be at least 150 articles per year published (Figure 1). This will enable, in 2007-2008, Departments of Biochemistry to publish 0.85-1 article per person per year, which is comparable to department

Table 2. Department of Biochemistry: research mapping, citations per paper of the top ten research category of articles published during 1998-2004 with 2,012 total citations (data updated in 2006).

Rank	Citations per paper	Category	Papers	%of total papers	Citations	% of total citations
1	10.4	Drugs	45	12	467	23
2	9.5	Shrimp	26	7	247	12
3	9.1	Malaria	39	11	353	18
4	8.9	Virus	33	9	293	15
5	6.2	Enzyme	71	19	439	22
6	5.8	PCR	65	18	375	19
7	5.3	Protein	85	23	451	22
8	4.8	Liver	43	12	208	10
9	4.5	Human	72	20	321	16
10	3.8	Cancer	47	13	158	8

Table 4. Departments of Biochemistry: number of articles published during 1998-2005 in the SCI ISI database (data update on 2006) and the expected output during 2006-2010 (italic numbers).

Year	Papers	Paper/person/year
1998	35	0.21
1999	42	0.25
2000	39	0.23
2001	43	0.26
2002	56	0.33
2003	75	0.45
2004	79	0.47
2005	84	0.50
2006	*100*	*0.60*
2007	*118*	*0.70*
2008	*134*	*0.80*
2009	*151*	*0.80*
2010	*168*	*1.0*

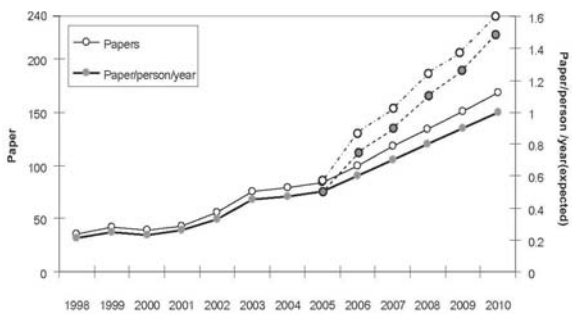

Fig 1. Department of Biochemistry: research foresight on publications. Progressive curve illustrating the output rate (total papers/year) and the productivity rate (paper/person/year) of papers published during 1998-2010 by Department of Biochemistry, Thailand. Solid lines= the forecast; broken lines = the foresight.

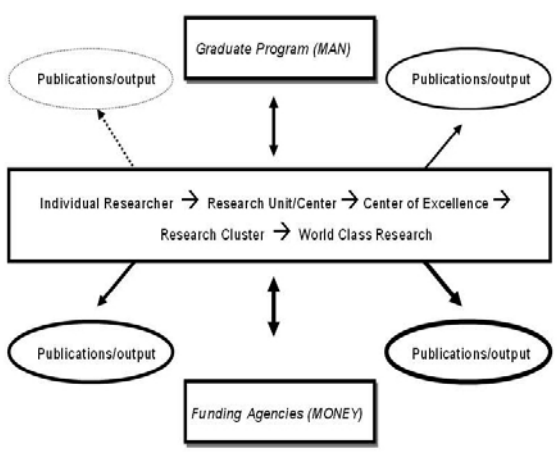

Fig 2. Strategic planning for development of biochemistry research in Thailand: integration of academic staff, graduate training and financial support to world class research in the near future.

MU SCI. This expectation is also dependent on the trend of research funding which is expected to increase, i.e., the Thailand Research Fund [13], the Commission on Higher Education [14] etc. Based on the information in 2005-2006 revealing that currently, departments MU SCI, CU MED and KKU MED have published more than one article/person/year, the above expectation can be met. Moreover, unless there are some unforeseen factors such as a decrease in research funding and graduate students [15], it is anticipated that MU may have a rate of publication in 2009-2010 equal to 0.85-1.0 article/person/year.

Research: Future Direction and Policy

By making site-visits, interviewing the chairperson, researchers and other staff (having research activity or not) in all departments, making research mapping of areas and expertise, we overview the research direction and the strategic planning for the future of Biochemistry in Thailand, as shown in Figure 2.

The strategies will be achieved by having both more research funding [13,14] and more graduate students training/programs [14,15] than in the past. This will generate novel research output, both in terms of publications and patents, for national development in terms of academic-industry co-operation and also for international competitiveness. This will be strengthened by the impact of national researchers in the biological and medical sciences [16]. In 2006, Thailand was ranked 46th (having 1,072 articles per year, ~0.13% of global publications) among selected 61 countries (mean = 11,788 articles per year), whereas Singapore was ranked 33rd (3,122 articles per year), being the first among the ASEAN countries. These results were ranked by the Institute for Management Development (IMD), Switzerland [17] which was obtained from the world competitiveness using number of scientific articles published by origin of author as an indicator, collected in year 2003 from the US National Science Foundation. USA was ranked at first in the world with 211,233 articles per year, accounting for ~25% of international publications. The results indicate that Thai researchers do not have high productivity, and have relatively low competitiveness in making contribution to scientific research.

Concluding Remarks

In general, Departments of Biochemistry in Thailand have high impact in research in some particular areas, e.g., drugs, protein, enzyme, etc., as measured by their individual publications in the ISI-WOS database. Publications by Thai biochemists have high impact comparable to the average impact factor of publication

in international journals of biochemistry and molecular biology category. There are many factors governing strength of their research; academic staff, graduate student training and funding support are predominant parameters. Weaknesses in research need further improvement with particular emphasis on more output from young generation staff. This study may serve as a model for other disciplines especially in science and technology.

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