รายงานวิจัยฉบับสมบูรณ์

การโคลนและการแสดงออกของยีนคาร์โบนิค แอนไฮเดรส ของเชื้อพลาสโมเดียม ฟัลซิปารัม

โดย ดร. สุธารทิพย์ เรืองประภาวุฒิ

มิถุนายน 2547

สัญญาเลขที่ TRG 4580036

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ผู้วิจัย ดร. สุธารทิพย์ เรืองประภาวุฒิ

หมวดวิชาชีวเคมี ภาควิชาวิทยาศาสตร์การแพทย์
คณะวิทยาศาสตร์ มหาวิทยาลัยรังสิต

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

ทุนส่งเสริมนักวิจัยรุ่นใหม่

กิตติกรรมประกาศ

โครงการวิจัยเรื่องการโคลนและการแสดงออกของยีนคาร์โบนิค แอนไฮเดรสของเชื้อพลาสโมเดียม ฟัลซิปารัม ได้ดำเนินการมาตลอดเวลา 2 ปี ภายใต้การดูแลของ ศ.ดร. จิระพันธ์ กรึงไกร ในฐานะนักวิจัยพี่เลี้ยง ดิฉันขอขอบพระคุณ ศ.ดร. จิระพันธ์ กรึงไกร เป็นอย่างสูงที่ได้ให้คำแนะนำที่มีประโยชน์ ให้การสนับสนุนในทุกๆ ด้าน รวมทั้งเอาใจใส่ดูแลในระหว่างการทำวิจัยอย่างดียิ่ง จึงทำให้งานวิจัยในครั้งนี้สำเร็จลุล่วงไปได้ด้วยดี

ดิฉันขอขอบพระคุณอาจารย์สุดารัตน์ กรึงไกร ที่ได้ให้คำแนะนำพร้อมทั้งสละเวลาสอนเทคนิคต่างๆ ในการทำงานวิจัยในครั้งนี้

ดิฉันขอขอบคุณภาควิชาชีวเคมี คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัยที่อำนวยความสะดวกในระหว่างการใช้ห้องปฏิบัติการวิจัย

ดิฉันขอขอบคุณมหาวิทยาลัยรังสิตที่เปิดโอกาสและให้การสนับสนุนการทำงานวิจัยครั้งนี้

ท้ายสุดนี้ดิฉันขอขอบพระคุณ สำนักงานกองทุนสนับสนุนการวิจัย ที่ได้ให้การสนับสนุนเงินทุนวิจัยในครั้งนี้ ทำให้งานวิจัยสำเร็จลุล่วงไปด้วยดี ดิฉันหวังเป็นอย่างยิ่งว่าจะได้มีโอกาสได้รับการสนับสนุนจากทุกท่านที่กล่าวมาในโอกาสต่อไป

บทคัดย่อ

รหัสโครงการ	หมายเลข TRG 4580036
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ระยะเวลาโครงการ	2 ปี

วัตถุประสงค์ของงานวิจัยครั้งนี้เพื่อสืบหา ทำการโคลนยีนพร้อมทั้งศึกษาการแสดงออกของยีน carbonic anhydrase (CA) ของเชื้อ *Plasmodium falciparum* (PfCA) ใน *Escherichia coli* จากการสืบค้นหายีนคาร์โบนิค แอนไฮเดรส (CA) ของเชื้อ *P. falciparum* ในฐานข้อมูลมาเลเรีย โดยใช้โปรแกรม BLAST พบว่าลำดับเบสของยีน CA ที่พบในเชื้อ *P. falciparum* อยู่บนโครโมโซมที่ 11 มีความเหมือนกันกับ CA ใน *P. yoelii* และใน Human และจัดอยู่ในกลุ่มอัลฟ่า โดยมีจำนวนกรดอะมิโน 235 ตัว เมื่อทำการโคลนยีนและหาลำดับเบสของยีน CA รวมทั้งศึกษาการแสดงออกของยีน CA ของเชื้อ *P. falciparum* (PfCA) ใน *E. coli* พบว่าเอนไซม์ที่สกัดได้จาก recombinant PfCA อยู่ในรูปที่ละลายน้ำได้มีน้ำหนักโมเลกุลประมาณ 29 ± 1 กิโลดัลตัน สามารถเร่งปฏิกิริยาได้และถูกยับยั้งได้โดย acetazolamide และ sulfanilamide เอนไซม์นี้มีคุณสมบัติทางด้านจลนศาสตร์เหมือนกับเอนไซม์ที่สกัดจากเชื้อ *P. falciparum* หรือ native enzyme นอกจากนี้ยังพบว่า acetazolamide มีผลยับยั้งการเจริญเติบโตของเชื้อ *P. falciparum* ที่เลี้ยงในจานทดลอง (*in vitro*) ผลจากงานวิจัยในครั้งนี้สามารถใช้เป็นแนวทางในการค้นหายาชนิดต่างๆ ที่มีคุณสมบัติยับยั้งการทำงานของเอนไซม์ CA เพื่อใช้เป็นประโยชน์ในการพัฒนายาเป้าหมายในการรักษาโรคมาเลเรียต่อไป

Keywords : Malaria, *Plasmodium falciparum*, Drug target, Carbonic anhydrase, Acetazolamide

Abstract

Project code : TRG 4580036

Project Title : Molecular cloning and expression of *Plasmodium falciparum* carbonic anhydrase

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Project Period : 2 years

The objective of this study is to clone and functional express *Plasmodium falciparum* carbonic anhydrase (CA) in *Escherichia coli*. The search of the malarial genome database yielded an open reading frame (ORF) on chromosome 11 similar to the ∝-CAs from various organisms, including human. The primary amino acid sequence of the PfCA gene has ~60% identity with a rodent parasite enzyme, namely *P. yoelii* (PyCA). The single ORF encoded 235 amino acid protein for PfCA. The PfCA gene was cloned, sequenced and expressed in *E. coli*. The purified recombinant PfCA enzyme was catalytically active. The recombinant protein was analyzed by SDS-PAGE and has a molecular mass of 29 ± 1 kDa, close to the molecular mass of deduced amino acid sequence of PfCA and the native CA purified from the malarial culture. It was sensitive to acetazolamide and sulfanilamide inhibition. Kinetic properties of the recombinant PfCA revealed the authenticity to the wild type enzyme purified from *P. falciparum in vitro* culture. Furthermore, the PfCA inhibitors acetazolamide and sulfanilamide showed good antimalarial effect on the *in vitro* growth of *P. falciparum*. Our molecular tools developed for the recombinant enzyme expression will be useful for developing potential antimalarials directed at *P. falciparum* carbonic anhydrase.

Keywords : Malaria, *Plasmodium falciparum*, Drug target, Carbonic anhydrase, Acetazolamide

เนื้อหางานวิจัย

บทนำ

การกลับมาระบาดอีกครั้งของโรคมาเลเรียในรอบ 10 ปีที่ผ่านมา เกิดจากการดื้อยาอย่างรวดเร็วของเชื้อมาเลเรียโดยเฉพาะสายพันธุ์พลาสโมเดียม ฟัลซิปารัม (*Plasmodium falciparum*) ซึ่งเป็นสายพันธุ์ที่ก่อให้เกิดความรุนแรงต่อมนุษย์มากที่สุด ในแต่ละปีจะมีผู้เสียชีวิตจากโรคนี้ประมาณ 1-2 ล้านคน โดยเฉพาะเด็กๆ ในทวีปแอฟริกา และเนื่องจากในปัจจุบันยังไม่มีวัคซีนที่ใช้ในการป้องกันโรคมาเลเรียที่มีประสิทธิภาพเพียงพอ จึงมีความจำเป็นและเร่งด่วนในการค้นหาและพัฒนาตัวยาใหม่ๆ เพื่อใช้เป็นยาเป้าหมายในการรักษาโรคมาเลเรียให้ได้ผลอย่างเต็มที่ โดยเฉพาะยาที่ออกฤทธิ์โดยตรงต่อกระบวนการเมตาโบลิสมของเชื้อมาเลเรีย และเมื่อเร็วๆ นี้จากการศึกษาของทีมงานวิจัย (Krungkrai et al. 2001) ได้ค้นพบเอนไซม์ที่สำคัญอีกตัวหนึ่งในเชื้อ *P. falciparum* ได้แก่ เอนไซม์คาร์โบนิค แอนไฮเดรส (carbonic anhydrase) โดยศึกษาพบว่าเอนไซม์ตัวนี้มีบทบาทและความสำคัญเป็นอย่างมากต่อการเจริญเติบโตของเชื้อ *P. falciparum* และมีคุณสมบัติดังต่อไปนี้

1. ทำหน้าที่เกี่ยวข้องกับการดำรงชีพของ *P. falciparum*
2. มีคุณลักษณะทางชีวเคมีที่มีลักษณะจำเพาะและแตกต่างจากเอนไซม์ที่พบในมนุษย์
3. ตัวยับยั้ง (inhibitor) ของเอนไซม์มีผลในการขัดขวางการเจริญเติบโตของ *P. falciparum* เมื่อศึกษาในจานทดลอง (*in vitro*)

ดังนั้นงานวิจัยครั้งนี้ได้มุ่งเป้าไปที่การสืบหาและศึกษายีนของ carbonic anhydrase (CA) ใน *P. falciparum* เพื่อจะเป็นแนวทางใหม่และเป็นอีกทางเลือกหนึ่งในการค้นหาและพัฒนายาเป้าหมายสำหรับใช้รักษาโรคมาเลเรียให้ได้ผลดีและมีประสิทธิภาพมากที่สุด

วิธีการทดลอง

การวิจัยครั้งนี้มุ่งศึกษาไปที่ยีนของ carbonic anhydrase (CA) ของเชื้อ *P. falciparum* (PfCA) และเนื่องจากการแยกและสกัดเอนไซม์ CA ที่ได้จากการเพาะเลี้ยงเชื้อมาเลเรียในจานทดลอง (*in vitro*) มีข้อจำกัดเพราะได้ปริมาณน้อยมาก จึงนำเอาวิธีพันธุวิศวกรรม (genetic engineering) มาใช้ในการโคลนยีน และให้ยีนแสดงออกใน *E. coli* เพื่อให้ได้เอนไซม์ในปริมาณที่เพียงพอเพื่อนำไปศึกษาและค้นหาตัวยับยั้งของเอนไซม์เพื่อใช้เป็นยาเป้าหมายในการรักษาโรคมาเลเรียต่อไป ซึ่งมีขั้นตอนในการดำเนินงานดังต่อไปนี้

1. สืบหายีน carbonic anhydrase ของเชื้อ *P. falciparum* (PfCA) ในฐานข้อมูลของมาเลเรีย (malaria genome data bank) โดยใช้โปรแกรม BLAST (Altschul et al. 1990) และเปรียบเทียบความเหมือน (homology) ของลำดับเบสบริเวณที่ถูกอนุรักษ์ (conserved region) ของยีน CA ในสิ่งมีชีวิตชนิดอื่น เช่น ในแบคทีเรีย มนุษย์ เป็นต้น และนำไปใช้ในการออกแบบ primer เพื่อใช้ในการเพิ่มจำนวนยีนในจีโนม (genomic DNA) เพื่อให้ได้ยีน CA ที่มีความยาวมากที่สุดโดยวิธี PCR (polymerase chain reaction)

2. นำยีน CA ที่ได้จากการทำ PCR จากข้อ 1(full-length PfCA) ไปหาลำดับเบส และนำไปโคลนในพลาสมิดที่มีลำดับของฮิสทิดีนติดอยู่ (His-tagged sequence) เมื่อทราบลำดับเบสของ PfCA รวมทั้งลำดับของกรดอะมิโนแล้วนำไปเปรียบเทียบความเหมือน (homology) กับ CA ที่ได้จากสิ่งมีชีวิตชนิดอื่นๆ ซึ่งได้ทำการศึกษาลงรายละเอียดถึงในระดับโครงสร้างของผลึก (crystal structure) ไปแล้ว (Supuran and Scozzafava 2000, 2001)

3. ศึกษาหาสภาวะที่เหมาะสมเพื่อให้ได้เอนไซม์ที่บริสุทธิ์ในปริมาณ 10-30 มิลลิกรัม เช่น ดูเวลาที่เหมาะสมในการเหนี่ยวนำของ IPTG (time for IPTG induction) หาปริมาณที่เหมาะสมของIPTG ในการเหนี่ยวนำ หรือศึกษาหาอุณหภูมิที่เพาะเลี้ยงเชื้อที่เหมาะสมในระหว่างการเหนี่ยวนำ เป็นต้น

4. นำโปรตีนที่ได้จากข้อ 3 ไปทำให้บริสุทธิ์ขึ้นโดยวิธีโครมาโตกราฟฟีโดยใช้ Ni^{2+}-NTA-agarose affinity chromatography แล้วนำไปแยกต่อโดยใช้เทคนิค FPLC (Fast-Protein Liquid Chromatography) ใช้คอลัมน์ gel filtration เรียกเอนไซม์ที่สกัดจากวิธีนี้ว่า recombinant PfCA และนำไปศึกษาการทำงานของเอนไซม์(enzyme activity) โดยวิธีสเปคโตรโฟโตเมตรี (Krungkrai et al. 2001) โดยใช้ esterase assay รวมถึงการศึกษาลักษณะทางชีวเคมีทั่วไป เช่น ศึกษาทางจลนศาสตร์เพื่อหาค่า K_m K_i เป็นต้น และเปรียบเทียบค่าที่ได้กับเอนไซม์ที่สกัดจากการเพาะเลี้ยงในจานทดลอง (*in vitro*) และศึกษาการทำงานของเอนไซม์แบบ staining on non-denaturating-PAGE gel

5. เพาะเลี้ยงเชื้อ *P. falciparum* ในจานทดลอง (*in vitro*) โดยอาศัยวิธีของ Trager และ Jensen (Trager and Jensen 1976) หลังจากนั้นทำการสกัดและทำให้เอนไซม์บริสุทธิ์เพื่อนำไปศึกษาคุณสมบัติของเอนไซม์ในเชื้อมาเลเรียทั้ง 3 ไอโซไซม์ และศึกษาการแบ่งชนิดของ CA ออกเป็น α, β, γ (α-CA, β-CA,- γ-CA family) ให้เข้าใจละเอียดมากขึ้น

6. ศึกษารูปแบบ (model) และโครงสร้างของเอนไซม์ CA ในรูปที่อิสระ (free) และรูปที่จับกันเป็นคอมเพล็ก (complexed) กับตัวยับยั้งซัลโฟนาไมด์ (sulfonamide-based CA inhibitor) พร้อมทั้งทดสอบกับซัลโฟนาไมด์ที่มีอยู่ในห้องปฏิบัติการของ Associate Professor Dr. Claudiu T. Supuran, University of Florence, Florence, Italy. โดยใช้ structure-activity relationship (SAR)

ผลการทดลอง (โปรดดูรายละเอียดใน manuscript ที่แนบมาด้วย)

1. จากการสืบค้นหายีนคาร์โบนิค แอนไฮเดรส (CA) ของเชื้อ *Plasmodium falciparum* ในฐานข้อมูลมาเลเรีย โดยใช้โปรแกรม BLAST พบ open reading frame อยู่บนโครโมโซมที่ 11 โดยมีจำนวนกรดอะมิโน 235 ตัว และลำดับเบสของยีน CA ที่พบในเชื้อ *P. Falciparum* มีความเหมือนกันกับ CA ใน *P. yoelii* ประมาณ 60 % เมื่อศึกษาถึงบริเวณ active site พบว่าจัดอยู่ในกลุ่มอัลฟา
2. จากการโคลนยีนและหาลำดับเบสของยีน CA รวมทั้งศึกษาการแสดงออกของยีน CA ของเชื้อ *P. falciparum* (PfCA) ใน *E. coli* พบว่าเอนไซม์ที่สกัดได้อยู่ในรูปที่ละลายน้ำได้ และสามารถเร่งปฏิกิริยาได้และมีน้ำหนักโมเลกุลประมาณ 2.9 $\pm$ 1 กิโลดัลตัน
3. นำเอนไซม์ที่สกัดได้จาก recombinant PfCA มาทดสอบกับกับตัวยับยั้งในกลุ่ม acetazolamide (AAZ) และ sulfanilamide (SFA) พบว่าตัวยับยั้งเหล่านี้มีผลยับยั้งการทำงานของเอนไซม์ carbonic anhydrase ได้ และเมื่อเปรียบเทียบค่าทางชีวเคมีต่าง ๆ เช่น K_m, k_{cat}, K_I ของตัวยับยั้งทั้งสองที่มีต่อเอนไซม์ CA ที่เป็น recombinant CA และ native CA พบว่าให้ผลคล้ายกัน นอกจากนั้น AAZ ยังมีผลยับยั้งการเจริญเติบโตของเชื้อมาเลเรีย *P. Falciparum* เมื่อศึกษาในจานทดลอง (*in vitro*)

บทวิจารณ์ (โปรดดูรายละเอียดใน manuscript ที่แนบมาด้วย)

การศึกษาวิจัยครั้งนี้พบว่ายีน CA ของเชื้อ *P. Falciparum* หรือ *P. falciparum* (PfCA) อยู่บนโครโมโซมที่ 11 มีลำดับกรดอะมิโน 235 ตัวและมีน้ำหนักโมเลกุลประมาณ 2.9 $\pm$ 1 กิโลดัลตัน เมื่อสกัดเอา recombinant PfCA มาศึกษาพบว่าสามารถเร่งปฏิกิริยาได้และมีคุณสมบัติทางชีวเคมีเหมือนกับเอนไซม์ CA ที่สกัดจากเชื้อ *P. Falciparum* หรือ native enzyme แต่มีคุณสมบัติแตกต่างจาก CA ใน human

ตัวยับยั้งในกลุ่ม acetazolamide (AAZ) และ sulfanilamide (SFA) มีผลยับยั้งการทำงานของเอนไซม์ recombinant PfCA และ acetazolamide (AAZ) มีผลยับยั้งการเจริญเติบโตของเชื้อมาเลเรีย *P .Falciparum* เมื่อศึกษาในจานทดลอง (*in vitro*)

เอนไซม์ CA ที่ได้จากการศึกษาในครั้งนี้สามารถใช้เป็นแนวทางในการค้นหายาตัวอื่นๆ (drug screening test) และศึกษาถึงโครงสร้างสามมิติของเอนไซม์ เพื่อให้เข้าใจถึงกลไกการการทำงานและการยับยั้งการทำงานของเอนไซม์ CA อย่างลึกซึ้งเพื่อใช้ในการออกแบบยาเพื่อยับยั้งเชื้อมาเลเรียต่อไปในอนาคต

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การนำผลงานวิจัยไปใช้ประโยชน์

1. เชื่อมโยงทางวิชาการกับ Andrea Scozzafava และ Claudiu Supuran , University of Florence, Italy. ในด้านการศึกษาการยับยั้งการทำงานของเอนไซม์ CA กับตัวยับยั้งชนิดต่างๆ
2. มอบหมายให้ น.ส. ชุติมา โชติประสิทธิ์ นักศึกษาระดับปริญญาตรีคณะวิทยาศาสตร์ สาขาวิทยาศาสตร์ชีวการแพทย์ทำงานวิจัยเรื่อง "Enhanced expression of recombinant PfCA in *E. Coli* by co-transforming cell with RIG plasmid.

ผลงานอื่นๆ

1. เสนอผลงาน ในการประชุม **6th International conference on the carbonic** anhydrase ในระหว่างวันที่ 20-25 มิถุนายน 2546 ที่ Smolenice castle , Slovakia. **Sutarnthip Ruengprapavut**, Sudaratana R Krungkrai and Jerapan Krungkrai. Human malarial parasite carbonic anhydrase : molecular cloning, functional expression and characterization.

2. เสนอผลงานในรูปโปสเตอร์ เรื่อง "Molecular cloning and expression of *Plasmodium falciparum* carbonic anhydrase" ในวันที่ 9-11 มกราคม 2547 ที่โรงแรมเฟลิกซ์ จ. กาญจนบุรี

3. เป็นวิทยากรเรื่อง "Molecular cloning of *P. Falciparum* carbonic anhydrase" ในการประชุมวิชาการของภาควิชาวิทยาศาสตร์การแพทย์ คณะวิทยาศาสตร์ มหาวิทยาลัยรังสิต ในเดือนมีนาคม 2546

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Special Issue

Plasmodium falciparum Carbonic Anhydrase is a Possible Target for Malaria Chemotherapy

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Plasmodium falciparum **is responsible for the majority of life-threatening cases of human malaria. The global emergence of drug-resistant malarial parasites necessitates identification and characterization of novel drug targets. Carbonic anhydrase (CA) is present at high levels in human red cells and in *P. falciparum*. Existence of at least three isozymes of the α class was demonstrated in *P. falciparum* and a rodent malarial parasite *Plasmodium berghei*. The major isozyme CA1 was purified and partially characterized from *P. falciparum* (PfCA1). A search of the malarial genome database yielded an open reading frame similar to the α-CAs from various organisms, including human. The primary amino acid sequence of the PfCA1 has 60% identity with a rodent parasite *Plasmodium yoelii* enzyme (PyCA). The single open reading frames encoded 235 and 252 amino acid proteins for PfCA1 and PyCA, respectively. The highly conserved active site residues were also found among organisms having α-CAs. The *PfCA1* gene was cloned, sequenced and expressed in *Escherichia coli*. The purified recombinant PfCA1 enzyme was catalytically active. It was sensitive to acetazolamide and sulfanilamide inhibition. Kinetic properties of the recombinant PfCA1 revealed the authenticity to the wild type enzyme purified from *P. falciparum in vitro* culture. Furthermore, the PfCA1 inhibitors acetazolamide and sulfanilamide showed good antimalarial effect on the *in vitro* growth of *P. falciparum*. Our molecular tools developed for the recombinant enzyme expression will be useful for developing potential antimalarials directed at *P. falciparum* carbonic anhydrase.**

Keywords: Malaria; *Plasmodium falciparum*; *Plasmodium berghei*; Drug target; Carbonic anhydrase; Acetazolamide

INTRODUCTION

Malaria is a disease caused by protozoan parasites of the genus *Plasmodium*. The disease afflicts approximately 5 hundred million and kills up to 2.5 million annually, mainly children in African countries.[1] Four species infect humans but *P. falciparum* is responsible for the majority of deaths. The limitation and toxicity of antimalarial drugs currently used, and the spread of drug-resistant malarial parasites accompanied by a world-wide resurgence of malaria, requires the development of new drugs for management of the disease. In intraerythrocytic stage of development, the parasites require purines and pyrimidines for DNA and RNA synthesis during their exponential growth and replication. The parasites, known as purine auxotroph, salvage the preformed purines from the human host, but they have to synthesize pyrimidines *de novo* from HCO_3^-, adenosine 5′-triphosphate, glutamine and aspartate.[2–4] These properties on both purine and pyrimidine biosynthesis represent key differences between the parasite and human host.

Carbonic anhydrase (CA; EC 4.2.1.1) is a zinc-containing enzyme catalyzing the reversible hydration of CO_2[$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$]. The first CA was purified from bovine red cells in 1933,[5] followed by the identification of several isozymes widely distributed in mammals, plants and bacteria.[6–8] Recent advances regarding the crystal structure and biochemistry of CAs from various

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organisms revealed that they evolved independently and have been categorized into 4 classes: α, β, γ and δ.[9] The first three classes have no significant sequence identity and structural differences, their active sites function with a single zinc ion essential for catalysis. Recently, we have demonstrated the existence of three active CA isozymes in the human malarial parasite *P. falciparum*.[10] The major isozyme, *P. falciparum* CA1 (PfCA1), has been purified and its characteristics have been partially studied due to the paucity of parasitic materials from *in vitro* culture with human red cells.

Based on our previous observation, this prompted us to identify the *PfCA1* gene in the malarial genome database, and then clone and heterologously express it in *Escherichia coli*. This is the first CA cloned and functionally expressed from a protozoan parasite. The recombinant enzyme is catalytically active for both *p*-nitrophenylacetate and α-naphthylacetate as substrates, and was strongly inhibited by acetazolamide (2-acetylamino-1,3,4-thiazole-5-sulfonamide, AAZ) and sulfanilamide (4-aminobenzenesulfonamide, SFA). Physical and kinetic properties indicate authenticity of the recombinant PfCA1 to the wild type enzyme purified from *P. falciparum*. The PfCA1 also differs from the human CA II. The PfCA1 inhibitors AAZ and SFA show good antimalarial effect on the *in vitro* growth of *P. falciparum*. In addition to *P. falciparum* CAs, we report the existence of four isozymes of CA and their sensitivity to AAZ inhibition in a rodent parasite *P. berghei*.

MATERIALS AND METHODS

Materials and Chemicals

Restriction enzymes, biochemicals and chemicals were purchased from Promega, Sigma, Fluka and Amersham Biosciences. These were of the highest quality commercially available and were used without further purification. Nickel(II)-nitrilotriacetic acid (Ni^{2+}-NTA)-agarose affinity gel was obtained from Qiagen. Molecular mass markers for SDS-PAGE and gel filtration chromatography were purchased from Amersham Biosciences and BioRad.

Cultivation of *P. berghei* and *P. falciparum*

P. berghei was cultivated in Swiss albino mice with 50–60% parasitemia before collecting the blood. The *P. berghei*-infected blood, mainly at the trophozoite stage, was passed through CF-11 cellulose columns to remove all white blood cells and platelets. *P. falciparum* (T_9 isolate) was cultivated by a modification of the candle jar method of Trager and Jensen,[11] using a 5% hematocrit of human red cell type O suspended in RPMI 1640 medium supplemented with 25 mM Hepes, 32 mM $NaHCO_3$ and 10% fresh human serum type O. The cultures, started at low parasitemia (~1–2%), were changed with the medium twice daily until the cultures had ~30% parasitemia and then harvested for enzyme and nucleic acid preparations. The parasites were isolated from the infected red cells by incubating in 0.15% saponin in the RPMI medium for 20 min at 37°C. The host cell-free parasites were obtained after centrifugation at 8,000 × g for 10 min and washed at least 4 times with phosphate buffered saline (5 mM phosphate buffer/145 mM NaCl/pH 8.0) (PBS) and 1 mM phenylmethylsulfonyl fluoride (PMSF), and then lysed according to the reported procedure.[4]

Purification of Enzyme on Fast Protein Liquid Chromatographic System

The parasite supernatant obtaining after centrifugation of the parasite lysate at 27,000 × g for 60 min was dialyzed with 20 mM Na_2HPO_4 (pH 6.0) containing 1 mM PMSF, and concentrated. It was then loaded onto a Pharmacia Mono S cation-exchange fast protein liquid chromatographic (FPLC) column, which had been equilibrated with the phosphate buffer. The column was washed with the same buffer and then eluted with a linear gradient of phosphate buffer from pH 6.0–8.0 at a flow rate of 1 ml per min. The eluates were collected into 30 fractions, each 1.0 ml-fraction was assayed for CA activity and protein concentration. All *P. berghei* CA activities were eluted at fractions 3–8, pooled and prepared for activity staining on nondenaturing-PAGE. For the *P. falciparum* CA, the major isozyme CA1 was further purified to near homogeneity using two more sequential columns on Mono Q anion-exchange and Superose 12 gel filtration FPLC as described previously.[10]

Enzyme Assay and Kinetic Studies

The esterase activity of CA was measured by using the modified method of Armstrong *et al.*[12] The enzyme activity was determined by following the change in absorbance at 348 nm of *p*-nitrophenylacetate to 4-nitrophenoxide ion (extinction coefficient = 18.1 $M^{-1}\,cm^{-1}$) over a period of 3 min at 37°C using a Shimadzu 1601 spectrophotometer equipped with a temperature-controlled unit. The enzymatic reaction, in a total volume of 1.0 ml, contained 10 mM Tris–HCl buffer, pH 8.0, 0.25 mM *p*-nitrophenylacetate and 10–100 μl enzyme preparations. This measurement was then repeated in the presence of the inhibitor AAZ at a concentration of 0.1 mM, to obtain the net CA activity. One unit of enzyme activity was expressed as 1 μmol of *p*-nitrophenylacetate hydrolyzed per min at 37° C. Kinetic constants, K_m and k_{cat}, were determined by fitting data to the Michaelis-Menten equation using non-linear regression with an Elsevier

Biosoft Enzfitter program. Inhibitor constants (K_i) were determined from Dixon's plots as described.[13]

Identification and Characterization of *P. falciparum* Carbonic Anhydrase Homolog

Homology search of the parasite PfCA1 was performed with the BLAST program of the U.S. National Center for Biotechnology Information server.[14] Using α-CA sequences from other organisms, significant homology for PfCA1 was found within a sequence on chromosome 11 in a malaria genome database. A single continuous *P. falciparum* open reading frame (ORF) encoding CA homolog was further characterized by the TBLASTN program.[14] In addition, an ORF encoding CA homolog was identified in *P. yoelii* genome database (PyCA). Pair-wise and multiple sequence alignments of PfCA1 and PyCA with other organisms were performed using the CLUSTALW program.[15]

Cloning and Sequencing of *P. falciparum* Carbonic Anhydrase

Genomic DNA was isolated from *P. falciparum* by DNAzol™ reagent (Invitrogen). PCR was used to amplify DNA encoding PfCA1. The forward primer was 5′TCTGGATCCATGAAAGATTTAAAGGAGAGAGAA3′ and the reverse primer was 5′CCCAAGCTTTTATTTATTACCTGAGCCGACGTG3′, which introduce BamH I and Hind III restriction sites respectively (shown in bold).

The PCR cycling parameters include denaturation at 95°C (1 min), annealing at 55°C (1 min) and extension at 68°C (3 min). After 30 cycles, a single band of the predicted size was visualized on an 0.8% agarose gel. The PCR products from gDNA were purified from the gel by using gel extraction kit (Qiagen). PCR products were ligated into a cloning vector pDrive (Qiagen). The PfCA1 sequence was determined in both directions by the dideoxy chain-termination method using an automated Applied Biosystems Procise sequencer. The construct plasmid was subcloned into expression vectors. Attempts were done with at least three expression vectors having different promoters, i.e., pQE30 (Qiagen), pTOPO (Invitrogen), pET 15b (Novagen). The *PfCA1* was expressed only with the pET15b expression vector. This approach will produce the recombinant protein fused to N-terminal His_6-thrombin cleavage site and the expressed recombinant protein can be detected by monoclonal antibody directed against His_6-tag.

Recombinant Protein Expression and Purification of *P. falciparum* Carbonic Anhydrase from *E. coli*

The competent *E. coli* BL21 (DE3) cells were transformed with the pET15b having the fused His_6-PfCA1. The cells were grown in LB medium (37°C) to an $OD_{600\ nm}$ of 0.5, and induced with 1 mM isopropyl α-D-thiogalactopyranoside (IPTG), harvested by centrifugation (8,000 × g) after IPTG induction for 3 h at 37°C, washed three times with ice-cold PBS, and stored as cell paste by freezing in −80°C until used.

All protein purification steps were performed at 4°C or on ice. Frozen cell pellets were suspended in four cell paste volume of buffer A (50 mM NaH_2PO_4, pH 8.0/300 mM NaCl/10 mM imidazole). A protease inhibitor cocktail was added to the cell suspension. The mixture was sonicated with an ultrasonic homogenizer. The *E. coli* lysate was then centrifuged for 30 min at 18,000 × g.

The supernatant (8 ml) was loaded onto a 2 ml bed volume of Ni^{2+}-NTA-agarose affinity gel equilibrated with buffer A. The column was washed with 20 ml of buffer B (50 mM NaH_2PO_4, pH 8.0/300 mM NaCl/20 mM imidazole), and then eluted with 6 ml of buffer C (50 mM NaH_2PO_4, pH 8.0/300 mM NaCl/250 mM imidazole). The eluted protein from the Ni^{2+}-NTA-agarose column was dialyzed extensively against PBS, prior to thrombin protease treatment (10 unit/mg protein, overnight at 22°C) for His_6-tag removal. The recombinant protein, after concentration using centricon 10 devices, was assayed for CA activity staining on nondenaturing-PAGE gel. The purified enzyme was determined for kinetic properties and inhibitory effect by AAZ and SFA inhibitors using the esterase assay as described.[12]

In Vitro Antimalarial Test

Growth of *P. falciparum* during drug-screening tests was measured by using incorporation of [^{3}H] hypoxanthine into parasite DNA and RNA and asynchronized culture with starting parasitemia of 0.5% as described.[16] Aliquots of stock solution of drugs were placed in 96-well tissue culture plates, to final concentrations of 0.001–1000 μM in sterile water after the addition of *P. falciparum* infected red cell suspension (0.5%) in RPMI 1640 culture medium. The plates were incubated in candle jars at 37°C for 24 h. [^{3}H] hypoxanthine (0.5 μCi; 1 Ci/mol) in 25 μl of the culture medium was then added to each well. The incorporation of [^{3}H]hypoxanthine in each well was examined after 48 h of drug-treated culture and the radioactivity was measured by liquid scintillation counting. The drug-free control of *P. falciparum*-infected red cells incubated under the same condition had radioactivity of 18,000 ± 1,000 cpm. The control red cells without harboring parasites incubated as described had 400 ± 50 cpm. All compounds were run in triplicate at each concentration. The 50% inhibitory concentration (IC_{50}) was defined as the concentration of the compound causing 50% inhibition of the [^{3}H]

hypoxanthine incorporation, compared with the drug-free control of the parasite culture. In parallel studies, antimalarial activity against *P. falciparum* in *in vitro* growth was quantified by measuring % parasitemia in a 96-hour culture in the presence of the drugs at various concentrations.[16] All compounds were tested in triplicate at each concentration used. The morphological changes of *P. falciparum* were also observed in the culture treated with 100 μM AAZ in one intraerythrocytic cycle (~44–48 h) starting with the synchronized ring stage.

Other Methods

Human CA II was purified from normal red blood cells, which had been cultivated as well as *P. falciparum*-infected red cell using the same procedure as described for the malarial CA. Mouse red cells were prepared from the normal Swiss albino mice. The number of cells was determined by a hematocytometer. Parasitemia and parasite morphological characteristics were performed on methanol-fixed and Giemsa-stained blood film of the malarial culture and then counted by using a Nikon microscope, equipped with a camera unit.

Protein concentrations were determined by the method of Bradford[17] using bovine serum albumin as standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a Bio-Rad minislab gel apparatus with a 5% acrylamide stacking gel and 10% acrylamide running gel in the discontinuous buffer system of Laemmli.[18] Nondenaturing-PAGE was performed by using the modified Laemmli's method in the absence of SDS and reducing agents of all reagents used, and 5% acrylamide running gel was applied. The enzyme activity was also detected on the nondenaturing-PAGE gel after electrophoresis in the presence of substrate α-naphthylacetate using the method described by Tashian.[19] The inhibitor AAZ (at 1 mM) was also included during enzyme activity staining as a control. The proteins on the SDS-PAGE gels were stained with Coomassie Blue R dye and visualized by a Kodak 1D image analysis software.

Western blot analysis was performed to confirm the authenticity of the His_6-tagged recombinant protein by using the method as described.[20] The detection system was QIA express™ kit (Qiagen) containing monoclonal antibody directed against His_6-tag with horseradish peroxidase-conjugate.

RESULTS AND DISCUSSION

The enzyme CA has previously been characterized from many species of animals, plants, yeast, bacteria, including human protozoa.[6–8,10] The crystal structures of the enzyme CA isolated from many sources have been identified.[9] The enzyme is the target for sulfonamide drugs, such as AAZ and methazolamide, for the treatment of human glaucoma.[8,21–22] In the present study, it is shown that in addition to the human parasite *P. falciparum*, the rodent parasite *P. berghei* propagated in the mouse system has its own CA activity and exhibits at least four isozymes, which are sensitive to the inhibitor AAZ. The *PfCA1* gene encoding *P. falciparum* CA1 was identified in the malarial genome database, and then cloned and functionally expressed in *E. coli*. The recombinant enzyme is catalytically active and sensitive to AAZ and SFA inhibition. It has authenticity to the wild type native enzyme purified from *P. falciparum*, and is also different from the human CA II. The PfCA1 inhibitors AAZ and SFA show good antimalarial effects on the *in vitro* growth of *P. falciparum* as observed by both [^{3}H] hypoxanthine incorporation and morphological abnormality, suggesting the therapeutic potential of the malarial parasite enzyme.

Existence of Carbonic Anhydrase Activity in Rodent Malarial Parasite *P. berghei*

To demonstrate the existence of CA activity in *P. berghei*, the isolated parasites freed from mouse red cells were used for CA assay by the esterase method, including the specific inhibitor AAZ, and compared to the CA activities in normal and *P. berghei*-infected red cells. The results are shown in Table I. There was a 4–5-fold increase in total activity of the enzyme in the infected red cells, compared to the uninfected and normal red cells. It was found that specific activity (mU/mg protein) of the enzyme in isolated parasites was ~9–10 times more than that in the normal red cells. When subjecting the parasite supernatant to the cation-exchange Mono S FPLC column, most CA activities were associated in the fractions 3–8 eluting at pH 6.2–6.6. These fractions were pooled, concentrated and then analyzed on nondenaturing-PAGE, followed by CA activity staining on the gels (Figure 1). There were four-activity bands, typing

TABLE I Carbonic anhydrase activity in normal and *P. berghei*-infected mouse red cells and in isolated parasites

	Activity[a]	
Cell	mU/10^9 cells	mU/mg protein
Normal mouse red cell	250 ± 20[b]	0.04 ± 0.005
P. berghei-infected red cell[c]	1,100 ± 130	0.63 ± 0.07
Isolated parasites	960 ± 90	0.35 ± 0.03

[a] mU of enzyme activity is expressed as nmol per min at 37°C. [b] The values are mean ± S.D., taken from three separate experiments of parasite and enzyme preparations. [c] The red cells were infected with ~60–70% parasitemia, harboring mainly the trophozoite stage.

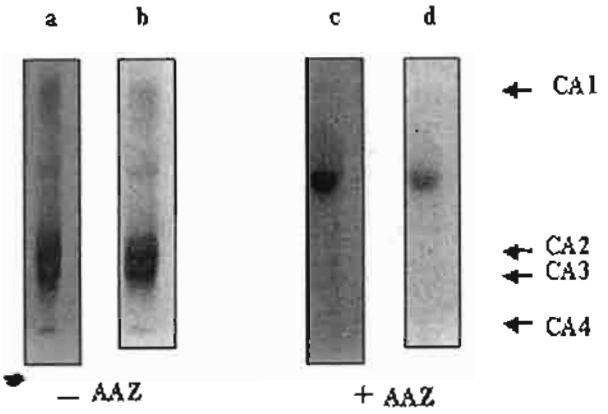

FIGURE 1 Enzyme activity staining of *P. berghei* carbonic anhydrase on nondenaturing-PAGE gels using α-naphthylacetate as substrate in the absence (a,b) and presence (c,d) of 1 mM acetazolamide (AAZ). The enzyme was concentrated from the pooled fractions 3–8 eluting at pH 6.2–6.6 of the cation-exchange Mono S FPLC column. Four-activity bands were observed to be sensitive to AAZ inhibition, and typing as CA1, CA2, CA3, and CA4 based on their mobility of the gels.

as isozymes CA1, CA2, CA3, and CA4, in the order of their mobility on the gel from cathode to anode, as described previously for the *P. falciparum* enzyme.[10] The isozymes CA2 and CA3 were major forms. In the presence of 1 mM AAZ, all four malarial CA activities were completely inhibited. Our results indicate the existence of at least four CA isozymes in *P. berghei*. This is consistent to our previous study on the occurrence of three CA isozymes in *P. falciparum*,[10] however, the majority of CA isozymes and their sensitivity to AAZ inhibition are markedly different between the human and the rodent malarial parasites. Purification of the major isozymes in *P. berghei* remains to be studied.

Cloning and Expression of *P. falciparum* Carbonic Anhydrase in *E. coli*

Recently, the nucleotide sequence of the *P. falciparum* genome, having 14 chromosomes with 23 Mb, has been completed.[23] It is now possible to identify the sequences that encode CA isozymes in this parasite. By using the bioinformatics approaches, TBLASTN searching of the malarial genome database was performed with the protein CA sequences obtained from other organisms. The search of the malarial genome database yielded an open reading frame (ORF) on chromosome 11 similar to the α-CAs from various organisms, including human. The primary amino acid sequence of the PfCA1 gene has ~60% identity with a rodent parasite enzyme, namely *P. yoelii* (PyCA). The single ORFs encoded 235 and 252 amino acid proteins for PfCA1 and PyCA, respectively. Low homology (~35–51%) of the PfCA1 and PyCA were found when compared to the insect *Drosophila melanogaster* and human CA I, II sequences. Nevertheless, the highly conserved active site residues responsible for binding of substrate and catalysis were also found among organisms having α-CAs (Figure 2). The consensus signature sequence remained variable.

The full-length *PfCA1* gene was cloned using PCR and genomic DNA extracted from *P. falciparum*, and the nucleotide of the cloned gene was sequenced to confirm it authenticity. The construct plasmid having

```
D.melanogaster      MSHHWGYTEENGPAHWAKEYPQASGHRQSPVDITPSSAKKGSELNVAPLKWKYVPEHTKS
H.sapiens           MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDP--SLKPLSVSYDQATSLR
P.falciparum        MKDLKERELKNISDVYLNLFDD-DNYAWNNYNKPWMKG------DFFYYYEYFIKKIVIN
P.yoelii            MHTLKERELKNLSDFYLNAFYDNDEYSWNNFNRPWFKG------DIFYYYENLINKIIIN
                    *         .* .  : : :   .    .  :    ..       ..

D.melanogaster      LVNPGYCWRVDVN-GADSELTGGPLGDQIFKLEQFHCHWGCTDSKGSEHTVDGV--SYSG
H.sapiens           ILNNGHAFNVEFDDSQDKAVLKGGPLDGTYRLIQFHFHWGSLDGQGSEHTVDKK--KYAA
P.falciparum        RQNNIFQIKAARDGIIPFGVLFTTEQPAMFYADQIHFHA------PSEHTFQGSGNRREI
P.yoelii            RQNNMFKIKASNNEIIPFGVLFTTDEPTIFYSHHINFHS------PSEHTFEGSGNRRHI
                      *   .  ..  :     :          :   ::: *        ****.:

D.melanogaster      ELHLVHWNTTKYKSFGEAAAAPDGLAVLGVFLKAGNHHAELDKVTSLLQFVLHKGDRVTL
H.sapiens           ELHLVHWN-TKYGDFGKAVQQPDGLAVLGIFLKVGSAKPGLQKVVDVLDSIKTKGKSADF
P.falciparum        EMQIFHSTNYFYDIQDDKSKYKKKYGLHIYNNLKKNSKETSKKDSSRYHSYLMSFLMNSL
P.yoelii            EMQIYHSTNEIYDYDENK-----WNGVFEKKNYKKKNNETN-----IQHSYILTFLMNSL
                    *::: * .   *    .        .:        . :          .    .     :

D.melanogaster      PQGCDPGQLLPDVHTYWTYEGSLTTPPCSESVIWIVFKTPIEVSDDQLNAMRNLNAYDVK
H.sapiens           TN-FDPRGLLPESLDYWTYPGSLTTPPLLECVTWIVLKEPISVSSEQVLKFRKLNFNGEG
P.falciparum        SNEQLQNKYNKKKR-------------------IKKMKNQYEVISITFTSAEINASTINA
P.yoelii            SNPHLGQQYTKNKKRNKRSKSLYNSIRLDENGKNTKRENQYQVISITFSSAEIDKSTINN
                    .:        .                        :   .* .   .    .

D.melanogaster      EECPCNEFNGKVINNFRPPLPLGKRELREIGGH-
H.sapiens           EPE------ELMVDNWRPAQPLKNRQIKASFK--
P.falciparum        FKKLPSEKFLRTIINVSSAVHVGSGNK-------
P.yoelii            FKKLPSEKFLKTILEASQNVPVGSGEKNIFIYFS
                          : :       : . :
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FIGURE 2 Amino acid sequences of *P. falciparum* and *P. yoelii* CAs, deduced from the open reading frame of *PfCA1* and *PyCA* genes. The predicted amino acid sequences of PfCA1 (235 residues) and PyCA (252 residues) are shown aligned with *D. melanogaster* (270 residues) and human CA II isozyme (260 residues). The amino acids responsible for binding and catalysis are identical among the four sequences and shown by boldface letters. The identical amino acids and conservative replacements are shown by star and dot symbols, respectively.

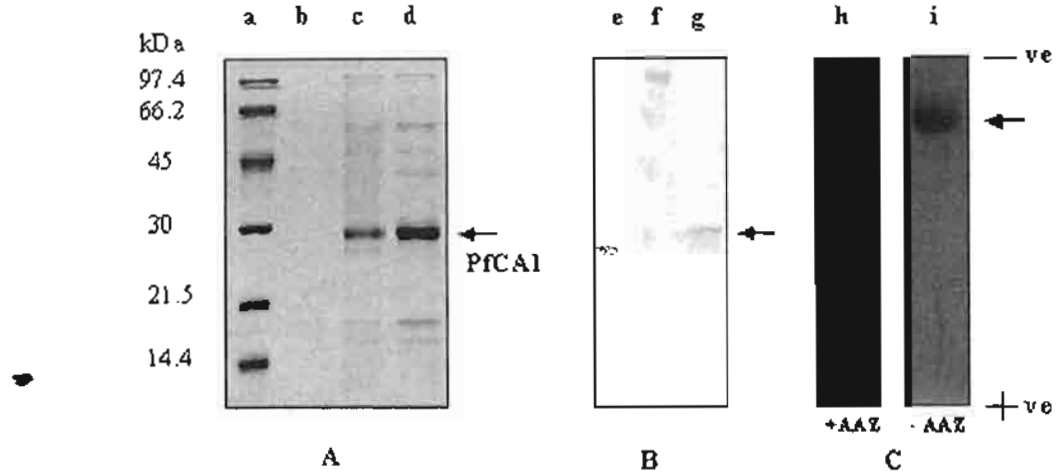

FIGURE 3 Analysis of recombinant *P. falciparum* carbonic anhydrase from IPTG-induced *E. coli* harboring PfCA1 construct pET15b plasmid. Panel A shows a SDS-PAGE analysis of purified PfCA1 from Ni^{2+}-NTA chromatography (>90% purity). The gel was stained with Coomassie Blue R; lane a, known proteins with molecular masses indicated in kDa; lanes b and c, 7.5 and 15 μg of the purified enzyme respectively; arrow indicates position of the recombinant enzyme. Panel B shows Western blot analysis of purified PfCA1 with monoclonal antibody directed against His_6 with HRP conjugate; lane d, 2 μg of control recombinant His_6-tagged dihydrofolate reductase (molecular mass of 26 kDa) obtained from Qiagen; lane f, pre-stained marker proteins; lane g, 2 μg of purified PfCA1. Panel C shows activity staining on denaturing-PAGE gels using α-naphthylacetate as substrate in the presence (lane h) or absence (lane i) of 1 mM acetazolamide (AAZ). The concentrated recombinant PfCA1 enzyme, after His_6-tag removal by thrombin, was used.

PfCA1 gene was then expressed in *E. coli*. The recombinant PfCA1 protein expression was obtained by a cloning and expression strategy using a His_6-tagged fusion protein at N-terminus, containing a thrombin cleavage site. It was shown that the *PfCA1* was functionally expressed in *E. coli* as soluble protein. The recombinant protein in the IPTG-induced *E. coli* supernatant was purified using Ni^{2+}-NTA agarose-affinity chromatographic column, and analyzed by SDS-PAGE (Figure 3A). The purity of the enzyme preparation was >90% after analysis by a Kodak 1D image software. It has a molecular mass of 29 ± 1 kDa, close to the molecular mass of the deduced amino acid sequence of PfCA1 (Figure 2) and the native CA1 purified from the malarial culture.[10] The authentic recombinant protein having N-terminal His_6-tag was confirmed using the Western blot analysis and monoclonal antibody directed against His_6-tag (Figure 3B). Furthermore, the recombinant PfCA1 was shown to express its activity after His_6-tag removal on the nondenaturing-PAGE gels, and was completely inhibited by 1 mM AAZ (Figure 3C). The purified recombinant PfCA1 was then studied kinetically for comparison with the wild type native *P. falciparum* CA1.

Kinetic Characterization of Recombinant *P. falciparum* Carbonic Anhydrase

Characterization of the purified recombinant PfCA1 was performed and compared with the purified human red cell CA II and the native CA1 purified from *P. falciparum* culture as described previously.[10] The kinetic parameters including K_m, k_{cat}, K_i of two specific inhibitors, AAZ and SFA, were found to be similar between the native and recombinant enzymes (Table II). AAZ and SFA were found to be typical competitive inhibitors for the malarial enzyme as determined by Dixon's plots (data not shown). The kinetic and inhibitory constants were different between the human and the parasite enzymes. These results suggest that the recombinant PfCA1 enzyme shows most properties similar to the native enzyme. The protein obtained here will be used for drug-screening tests and crystal structure analysis for a purpose of mechanism-based drug design in the near future.

TABLE II Comparison of kinetic parameters and inhibitory constants of human red cell CA II, native and recombinant *P. falciparum* carbonic anhydrases

Source	K_m[a] (mM)	k_{cat} (min^{-1})	K_i^{AAZ} (nM)	K_i^{SFA} (μM)
Human CA II	10.1 ± 0.8[b]	74.1 ± 5.7	99 ± 6	145 ± 2
Native PfCA1	3.7 ± 0.2	10.4 ± 1.2	247 ± 14	56 ± 4
Recombinant PfCA1	5.6 ± 0.3	8.2 ± 1.6	315 ± 26	84 ± 10

[a]This assay is based on esterase assay using *p*-nitrophenylacetate as substrate. [b]The values are mean ± S.D., taken from 3–4 separate experiments of the enzyme preparations.

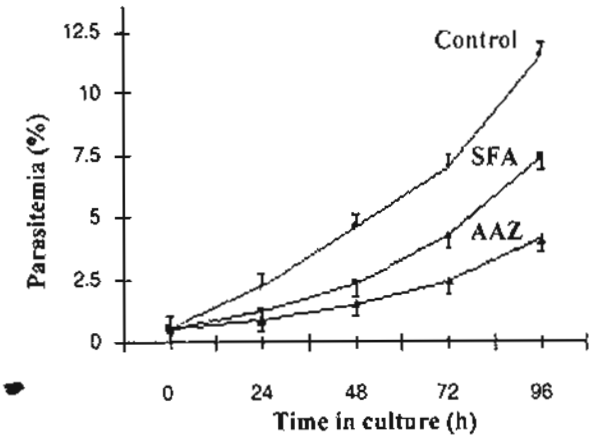

FIGURE 4 Antimalarial activity of the PfCA1 inhibitors, acetazolamide (AAZ) and sulfanilamide (SFA). *P. falciparum* growth was started with 0.5% parasitemia (mixed stages) at 2.5% red cell suspension and monitored every 24 h for up to 96 h at 37°C. The growth of *P. falciparum* in the absence of inhibitors is shown (●). 100 μM AAZ (▲) or 100 μM SFA (■) was present during the 96-h growth.

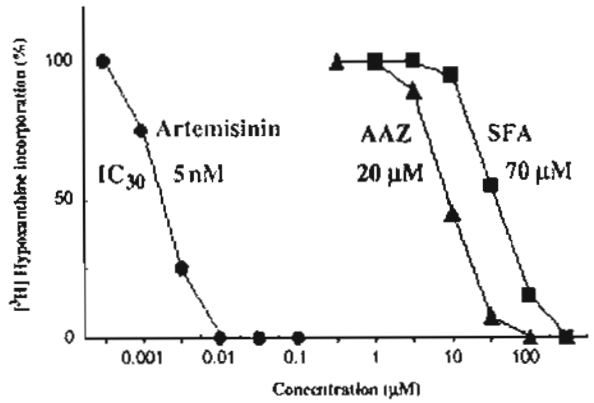

FIGURE 6 Inhibition of [^{3}H]hypoxanthine incorporation by *in vitro* culture of *P. falciparum* at various concentrations of drugs. Growth of *P. falciparum* was started with 0.25% parasitemia (mixed stages) at 0.5% red cell suspension. (●) antimalarial artemisinin, a Chinese traditional drug; (▲) acetazolamide, AAZ; (■) sulfanilamide (SFA).

Antimalarial Properties of *P. falciparum* CA Inhibitors

We hypothesized that inhibition of *P. falciparum* growth in the erythrocytic stage requires inhibition of both human and *P.falciparum* CAs, since both human host cell and parasite contain relatively high CA activities.[10] The antimalarial properties of CA inhibitors were tested against *in vitro* growth of *P. falciparum* by lowering the % red cell suspension from 10% to 2.5%. Both AAZ and SFA drugs (100 μM) tested at 2.5% red cell suspension showed a strong antimalarial effect on *P. falciparum* growth with higher than 50% inhibition (Figure 4), whereas at 10% red cell suspension they showed little activity (data not shown). Interestingly, AAZ at 100 μM shows its antimalarial property by interfering with the intracellular development of *P. falciparum* in a stage-dependent manner (Figure 5). The morphological abnormality, as shown by clumping of nucleus and cytosol, of the AAZ-treated parasites in the human host red cells were markedly enhanced at the latter stages of development, i.e., trophozoite and schizont (Figure 5, D–F). The control culture shows healthy parasites during an intraerythrocytic development (Figure 5, A–C). By using [^{3}H] hypoxanthine incorporation for monitoring growth of *P. falciparum* in *in vitro* culture, which were started with mixed stages at 0.5% red cell suspension, the IC_{50} values in the mixed stages of parasite development in the human red cell for AAZ and SFA were determined to be 20 μM and 70 μM, respectively (Figure 6). This condition was also used for the control antimalarial drug artemisinin which had IC_{50}

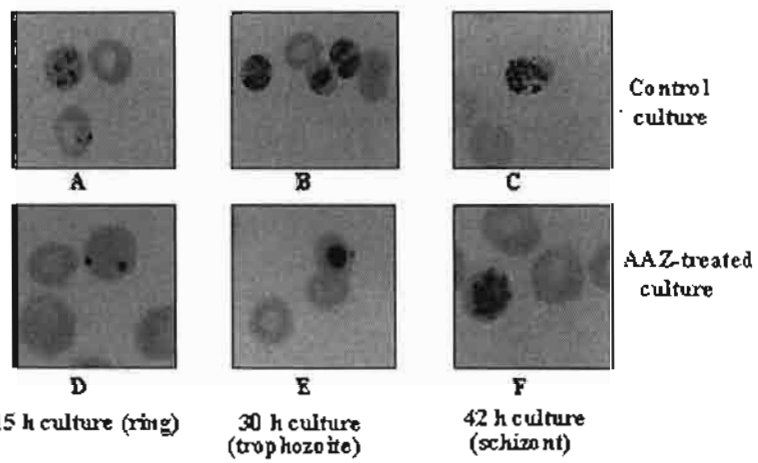

FIGURE 5 Effect of acetazolamide (AAZ) on *P. falciparum* morphology during an intraerythrocytic cycle (ring, trophozoite and schizont stages). The morphological changes were examined in the absence (panels A, B and C; control culture) or in the presence of 100 μM AAZ (panels D, E and F; AAZ-treated culture) at various times of *P. falciparum* culture starting with ring stage parasite.

of 5 nM. The IC_{50} values for AAZ and SFA reported here are ~10-fold less than the values reported under different conditions for drug testing, i.e., 5% red cell suspension.[10]

Based on these results, which are consistent with the role of carbonic anhydrase in the malarial parasite[10] and the inhibition of *P. falciparum* enzyme by the sulfonamide-based drugs, it is appropriate to target this enzyme for the development of new antimalarial drugs.

Acknowledgements

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MOLECULAR BIOLOGY AND BIOCHEMISTRY OF MALARIAL PARASITE PYRIMIDINE BIOSYNTHETIC PATHWAY

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Abstract. Metabolic pathways in the malarial parasite are markedly different from the host, *eg*, hemoglobin, fatty acids, folate and nucleic acids. Understanding of metabolic function will illuminate new chemotherapeutic targets for drug development, including the identification of target(s) for drugs in current use. The parasite-contained pyrimidine biosynthetic pathway is essential for growth and development in the human host. *Plasmodium falciparum* carbonic anhydrase, producing HCO_3^- as a pyrimidine precursor, was identified as α- type and the encoded gene was cloned and sequenced. The first six enzymes, catalyzing the conversion of HCO_3^-, ATP, L-aspartate and L-glutamine to uridine 5′-monophosphate (UMP), were partially characterized. The genes encoding these enzymes were identified in order, from the first to the sixth step, as *CPSII* (carbamyl phosphate synthase II), *ATC* (aspartate transcarbamylase), *DHO* (dihydroorotase), *DHOD* (dihydroorotate dehydrogenase, DHOD), *OPRT* (orotate phosphoribosyltransferase, OPRT), and *OMPDC* (orotidine 5′-monophosphate decarboxylase, OMPDC). Unlike its analogous parasitic protozoan, *Trypanosoma*, the organization of the malarial genes was not an operon-like cluster. The *CPSII, DHO* and *OPRT* genes were conserved to bacterial counterparts, whereas the *ATC, DHOD* and *OMPDC* were mosaic variations. The data support the mosaic pyrimidine pathway in the malarial parasite. The human host had five enzymes out of the six associated into two different multifunctional proteins, in that a single gene *CPSII-ATC-DHO* encoded the first three enzymes, and another gene *OPRT-OMPDC* encoded the last two enzymes. In the malarial parasite, the CPSII and ATC were not characterized. The DHO was partially characterized in *Plasmodium berghei*. The DHOD was well characterized in both *P. falciparum* and *P. berghei*. It was functionally expressed in *Escherichia coli*. The physical and kinetic properties of the recombinant pfDHOD were similar to the native enzyme. The OPRT and OMPDC were also partially characterized. These lines of evidence indicate that the malarial pyrimidine enzymes are mono-functional forms. In addition, the enzymatic activities inter-converting uracil, uridine and UMP of the pyrimidine salvage pathway, were demonstrated, and the gene encoding uridine phosphorylase was cloned. Our results suggest that the pyrimidine enzymes are possible new drug targets.

INTRODUCTION

Malaria afflicts approximately 2.5 million people deaths annually, making it a major cause of human morbidity and mortality worldwide. Four malarial species infect humans, the most deadly being *Plasmodium falciparum*. In the fight against this disease, there is an urgent need to develop new antimalarials and an effective vaccine because of widespread resistance to current chemotherapeutic agents (Nchinda, 1998; Ridley, 2002). At present, the complete nucleotide sequences of the 23-megabase nuclear genome of *P. falciparum* consists of 14 chromosomes, encoding about 5,300 genes, and is the most (A+T)-rich genome sequenced to date (Gardner *et al*, 2002). In the post-genomic era, metabolism of the malarial parasite has been mapped based on the current knowledge of parasite biochemistry and on pathways known to occur in other eukaryotes (Gardner *et al*, 2002). Some metabolic pathways in the parasite are unique and found to be markedly different from the mammalian host, *eg*, hemoglobin catabolism, fatty acid synthesis, folate biosynthesis and metabolism of nucleic acids (Ridley, 2002). Understanding of metabolic functions should illuminate new chemotherapeutic targets for drug development, including the identification of target(s) for drugs in current use. Recently, it has been proposed that the pyrimidine metabolic pathway may be a target for the design of new antimalarial drugs (Krungkrai *et al*, 1992; Krungkrai, 1993a; McRobert and McConkey, 2002; Ridley, 2002).

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The erythrocytic malarial parasites require purines and pyrimidines for DNA/RNA synthesis and other metabolic pathways during exponential multiplication in the human host. They use preformed purines from the host and must synthesize pyrimidines *de novo* (Gero and O'Sullivan, 1990). The parasites lack thymidine kinase, which is responsible for salvaging the preformed thymidine from the host (Reyes *et al*, 1982). Several lines of evidence suggest that there are some key differences between malarial parasites and the human host in the pyrimidine pathway. The first six enzymes of the pathway (Fig 1), catalyzing the conversion of HCO_3^-, ATP, L-glutamine and L-aspartate to uridine 5′-monophosphate (UMP), are demonstrated in both *P. falciparum* and a rodent parasite *P. berghei* (Reyes *et al*, 1982; Rathod and Reyes, 1983; Gero and O'Sullivan, 1990; Krungkrai *et al*, 1990; 1991; 1992; Krungkrai, 1995). Some genes encoding the six enzymes are partially sequenced, in order, from the first to the sixth step; these are *CPSII* (carbamyl phosphate synthase II, CPSII) (Flores *et al*, 1997), *ATC* (aspartate transcarbamylase, ATC), *DHO* (dihydroorotase, DHO), *DHOD* (dihydroorotate dehydrogenase, DHOD) (LeBlanc and Wilson, 1993), *OPRT* (orotate phosphoribosyltransferase, OPRT), and *OMPDC* (orotidine 5′-monophosphate decarboxylase, OMPDC)(van Lin *et al*, 2001). The human host has five enzymes out of the six, associated into two different multifunctional proteins, in that a single gene *CPSII-ATC-DHO* encoded the first three enzymes and another gene *OPRT-OMPDC* encoded the last two enzymes (Jones, 1980).

In this report, the six genes encoding the pyrimidine *de novo* pathway are identified on various chromosomes of the *P. falciparum* genome. Multiple alignments and phylogenetic analyses of these genes suggest the mosiac evolution of the pyrimidine pathway in *P. falciparum*. The *pfDHOD*, *pfOPRT* and *pfOMPDC* genes are cloned and sequenced. The *pfDHOD* is expressed in *E. coli*. The physical and kinetic properties of the recombinant enzyme are similar to the native enzyme. In addition, *P. falciparum* carbonic anhydrase (CA), catalyzing the inter-conversion of CO_2 and the pyrimidine precursor

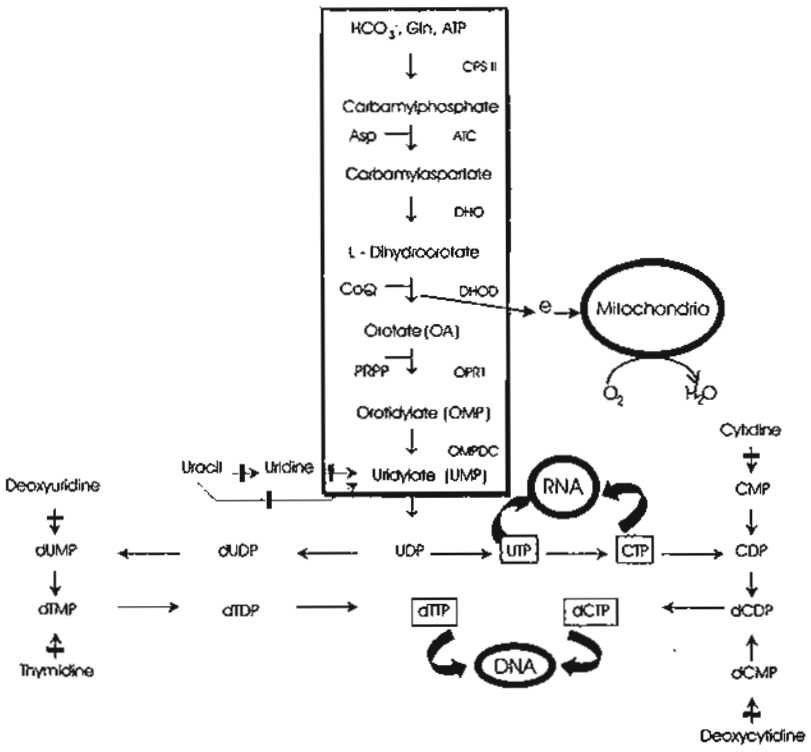

Fig 1- Proposed pyrimidine synthetic pathway in the human malaria parasite *P. falciparum* (Krungkrai, 2000). The first six enzymes of the *de novo* pathway are shown in the box. An uracil pyrimidine salvage pathway, inter-converting uracil, uridine and UMP, is shown by a broken line. The arrow with crossing bars indicates no enzymatic activities in the parasites.

HCO_3^-, is identified and the *pfCA* gene is cloned, sequenced and expressed in *E. coli*. Furthermore, the enzyme activities inter-converting uracil, uridine and UMP of the pyrimidine salvage pathway (uracil phosphoribosyltransferase, UPRT; uridine phosphorylase, UP; uridine kinase, UK) are demonstrated in *P. falciparum* and *P. berghei* and the *pfUP* is cloned and sequenced.

MATERIALS AND METHODS

Chemicals and malarial parasite materials

Oligonucleotides were custom-synthesized and purified by the Bioservice Unit of the National Center for Biotechnology and Genetic Engineering of Thailand. Restriction endonucleases, *Pfu* DNA polymerase, all supplies and reagent kits for molecular biology work were obtained from Promega Corp, Life Technologies, Inc, Invitrogen Inc, and Qiagen Inc. All other chemicals, materials, and reagents used in this work were of the highest grade commercially available and purchased from Aldrich and Sigma Co. *P. falciparum* (a multidrug-resistant T9 isolate from Thailand) was cultivated by a minor modification of the candle jar method of Trager and Jensen (1976), using 5% human red cells type O suspended in RPMI 1640 medium supplemented with 25 mM Hepes, 32 mM $NaHCO_3$ and 10% fresh human serum type O. The cultures with ~15-20% parasitemia, mainly of trophozoites, were then harvested for DNA preparation, enzymatic determination and antimalarial activity testing on pyrimidine analogs. *P. berghei* was cultivated in Swiss albino mice. Cell-free extracts of the parasites were prepared as described previously (Krungkrai *et al*, 1990).

Nucleic acids preparation

The total genomic DNA from the parasites, freed from the host red cells as previously described, were isolated using a lysis buffer (100 mM Tris-HCl, pH 8.3, 5 mM EDTA, 1% SDS) and then digested with proteinase K, followed by phenol-chloroform extraction as previously described (Sambrook *et al*, 1989).

Identification of pyrimidine genes on *P. falciparum* genome

The six pyrimidine genes (*pfCPSII*, *pfATC*, *pfDHO*, *pfDHOD*, *pfOPRT*, *pfOMPDC*), including *pfCA* and *pfUP*, were identified by BLAST searching of the Institute of Genome Research (TIGR), malaria databases with sequences from various prokaryotes and eukaryotes using the BLAST program default search parameters (Altschul *et al*, 1997). Sequencing of the *P. falciparum* chromosome was accomplished as part of the International Malaria Genome Project and was supported by Burroughs Wellcome, the National Institute of Allergy and Infectious Diseases, National Institute of Health, and the US Department of Defense.

Pair-wise amino acid sequence and multiple sequence alignments of pyrimidine enzymes from *P. falciparum* with other organisms were performed using CLUSTALW (Thompson *et al*, 1994). All other sequence data used in this study were collected from the EMBL, GenBank, DDBJ and SWISSPROT databases. Determinations of hydrophobicity and secondary structure (α-helix, β-pleated sheet) of the malarial enzymes were done using Hitachi DNASIS version 2.6 software. Phylogenetic analyses to produce the gene tree were performed by the neighbor-joining (NJ) method (Saitou and Nei, 1987) using a distance matrix estimated by the maximum likelihood method (Kishino *et al*, 1990). The reliability was assessed by the bootstrap method with 1,000 pseudo-replications.

Cloning and sequencing of *P. falciparum* pyrimidine genes

Polymerase chain reactions (PCRs) were employed to isolate *pfDHOD*, *pfOPRT*, *pfOMPDC*, *pfCA* and *pfUP* genomic clones from *P. falciparum* DNA. The open reading frames (ORFs) of these 5 genes were amplified from the genomic DNA with *Pfu* DNA polymerase (Promega). The PCR amplification conditions were optimized as follows: initial denaturation for 3 minutes at 95°C, followed by 30 cycles of annealing at 55 °C for 1 minute, extension at 68 °C for 3 minutes, and denaturation at 95 °C for 1 minute, and final cycle 1 minute at 55 °C, 10 minutes incubation at 68 °C. The expected PCR fragments amplified from each pair of primers designed for the above genes were ligated into a pBluescript vector (Stratagene) and the recombinant DNA was transformed into the *E. coli* XL1-Blue. To confirm the authenticity of the cloned genes, the nucleotide sequence of each gene was determined by the dideoxy chain-termination method using an automated Applied Biosystems Procise sequencer.

Recombinant expression of *P. falciparum* dihydroorotate dehydrogenase

In order to express the *pfDHOD* in the *E. coli* system using a pET expression vector, primers were designed using the *pfDHOD* ORF as follows: sense primer, (5′GAGGATCC**CATATG**ATCTCTAAATTG AAACC 3′) containing a *Bam*HI site (underlined) and a *Nde*I site (boldface) and antisense primer, (5′

GAAAGCTTGCGGCCGCTTAACTTTTGCTATG 3') containing a *Hind*III site (underlined) and a *Not*I site (boldface). The PCR amplification conditions were used as described earlier. The 1.7-kb PCR amplified fragment was cloned into the pBluescript vector. The verified clone of the gene corresponding to the *pfDHOD* ORF was ligated into the pET vector. The construct plasmid with *pfDHOD*, namely pETDHOD1 (Fig 2), was then transformed into *E. coli* strain BL21(DE3) (Novagen). The cells were grown in LB medium containing 25 µg/ml chloramphenicol and 40 µg/ml ampicillin to optical density at 600 nm of 0.4, induced with 1mM IPTG and harvested three hours after induction at 37 °C by centrifugation, and washed three times with phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride. The cell paste was suspended in a buffer and disrupted by rapid freezing in liquid nitrogen and thawing at 37°C at least 3 times, and then by an ultrasonic disrupter (Bandelin Inc) with 5-s pulse for at least 10 cycles on ice. To the cell lysate, 0.15% TritonX-100 was added, and DHOD activity was then assayed immediately using the methods described later. The supernatant, obtained after centrifugation, was subjected to purification by the procedure that had been used for the native enzyme from *P. falciparum* (Krungkrai *et al*, 1991; Krungkrai, 1995).

Enzymatic assays

DHOD activity was assayed using 2,6-dichlorophenolindophenol (DCIP) as a terminal electron acceptor, L-dihydroorotate and CoQ_0 as co-substrates. The enzyme reaction was monitored by the loss of DCIP absorbance at 610 nm (extinction coefficient 21,500 $M^{-1} cm^{-1}$) (Krungkrai *et al*, 1991). OPRT and OMPDC activities were assayed using high-performance liquid chromatographic (HPLC) methods to detect both substrate and product (*ie*, orotate, OMP, UMP) simultaneously (Krungkrai *et al*, 2001b). UPRT, UP and UK activities were determined using the HPLC methods (Krungkrai *et al*, 2001a). CA was assayed based on acetazolamide-inhibited esterase activity (Krungkrai *et al*, 2001b).

Miscellaneous methods

Kinetic constants, K_m and k_{cat}, were determined by fitting data to the Michaelis-Menten equation using non-linear regression of an Elsevier Biosoft enzfitter program. Inhibitor constants (K_i) were determined from Dixon's plots (Segel, 1975). I_{50} was defined as the concentration of compound having 50% inhibitory effect against the purified enzyme. Antimalarial activity on the growth of *P. falciparum in vitro* was quantified by measuring % parasitemia in a 96-hour culture in the presence of the tested compounds at various

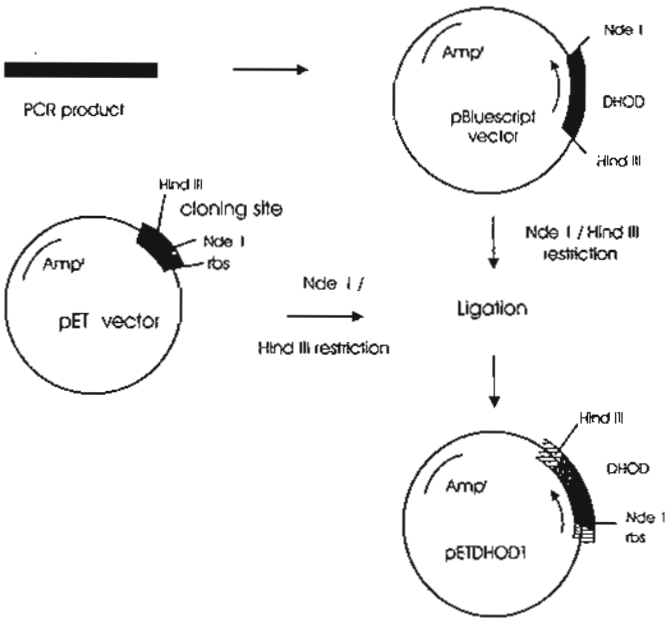

Fig 2- Molecular cloning and expression of *P. falciparum* DHOD homologue1 (*pfDHOD1*) in *E. coli* using pET vector.

concentrations (Krungkrai *et al*, 1992). All compounds were tested in triplicate at each concentration used. The 50% inhibitory concentration (IC_{50}) was defined as the concentration of the compound causing 50% inhibition of parasite growth in a 96-hour culture, compared with the compound-free control of the parasite culture.

RESULTS AND DISCUSSION

Identification of pyrimidine genes on the *P. falciparum* genome

Recently, sequencing of the *P. falciparum* genome has been completed (Gardner *et al*, 2002). It is now possible to identify the sequences that encode the pyrimidine enzymes in this parasite. Using the bioinformatics approach, TBLASTN searching of the TIGR malaria genome databases was performed with the protein sequences from bacteria (*eg*, *Escherichia coli*), yeast (*eg*, *Saccharomyces cerevisae*), other parasites (*eg*, *Trypanosoma*, *Leishmania*, *Caenorhabditis elegans*, *Ascaris suum*) and mammalian enzymes (*eg*, mouse, human) as query sequences. The ORFs of the first six enzymes of the pyrimidine *de novo* pathway *pfCPSII* (*PYR1*, chromosome 13), *pfATC* (*PYR2*, chromosome 13), *pfDHO* (*PYR3*, chromosome 14), *pfDHOD* (*PYR4*, chromosomes 7 & 9), *pfOPRT* (*PYR5*, chromosomes 5 & 7), *pfOMPDC* (*PYR6*, chromosome 10), including *pfCA* (chromosome 11) and *pfUP* (chromosomes 5&7), were identified and located on various chromosomes, as indicated by the numbers in parentheses. It was found that the *pfDHOD* (*PYR4*), *pfOPRT* (*PYR5*) and the *pfUP* genes had two homologues mapping on different chromosomes of the *P. falciparum* genome. The functions of these homologues remain to be studied. It is then concluded that the molecular organization of the malarial pyrimidine genes is separate from each other and is not an operon-like cluster. This differs from its analogous parasitic protozoa, *Trypanosoma* and *Leishmania*, in which the *PYR1-PYR6* genes (as an operon-like cluster) constitute a polycistronic transcript unit on a 25 kb segment of the 800 kb chromosomal DNA (Gao *et al*, 1999). The malarial pyrimidine genes are also different from human, in that the single gene *PYR1-PYR2-PYR3* (chromosome 2p22-21) encodes the multifunctional CAD protein catalyzing the first three enzymes' activities and the other gene *PYR5-PYR6* (chromosome 3q13) produces the bifunctional UMP synthase activity (Jones, 1980; Gao *et al*, 1999).

The identified ORFs of the *PYR1- PYR6* genes of *P. falciparum* were deduced to amino acid sequences of the pyrimidine enzymes. Using multiple sequence alignments and phylogenetic analyses of these sequences, the malarial *CPSII*, *DHO* and *OPRT* were conserved to bacterial counterparts. The malarial *ATC*, *DHOD* and *OMPDC* were mosaic variations that were homologous to both bacterial and eukaryotic counterparts, including human (Fig 3 for the OMPDC as a representative gene). An analysis with the ATC sequence of *Toxoplasma gondii*, a parasitic protozoan, revealed only 30% identity to the *pfATC* gene. The pfDHO sequence is close to most bacterial sequences, yeast *S. cerevisae* and plant *Arabidopsis thalina*, indicating that *P. falciparum* may carry the monofunctional DHO whose gene might have been acquired by the horizontal transfer from proteobacteria, *ie*, *E. coli*, *Neisseria gonorrhoeae*. The pfDHOD was ~ 48-51%, similar to the human and *E. coli* DHODs. The pfOPRT had 60% and 28% sequence similarity to *E. coli* and human OPRTs, respectively. The sequences between *P. falciparum* and *T. cruzi* OPMDCs were 50% similar, whereas for the malarial and human enzymes it was 37% (Fig 3). In addition, the OMPDC were identified in other *Plasmodium* species (Fig 4), *eg*, *P. knowlesi* (a monkey parasite), *P. berghei* and *P. yoelii* (rodent parasites). These four malarial OMPDCs were highly similar. Fig 5 shows an example of phylogenetic analysis of the pyrimidine enzymes, where the *P. falciparum* OMPDC is placed in the monophyletic subtree containing *Mycobacterium smegmatis*, *Thermus thermophilus* and *T. cruzi*, and is also close to other bacterial OMPDCs, *ie*, *E. coli* and *Bacillus subtilis*. The OMPDC sequences of many eukaryotes examined, except the trypanosome and malaria parasites, are relatively monophyletic. The results on the malarial OMPDC sequence are consistent with the observation of Gao *et al* (1999) and Nara *et al* (2000) on the trypanosomatid parasites. This suggests that the malarial parasite or its ancestor may have acquired an eubacterial OMPDC (*ie*, *Mycobacterium*) and elaborated a new gene product, OMPDC, which is the longest sequence (323 amino acids) to date. The origin of this *pfOMPDC* remains to be determined. Our results in a human malarial parasite also support the evolutionary implications of the mosaic pyrimidine biosynthetic pathway in many eukaryotes. Horizontal gene transfer(s) and endo-symbiosis may be responsible for establishing this mosaic pathway (Nara *et al*, 2000).

In addition, when the *pfCA* gene was used to TBLASTN search other malaria genome databases, the rodent parasite *P. yoelii CA* gene was also identified with >70% sequence identity. Highly conserved signature sequences were also found among human, malaria and bacteria CAs. The presence of the

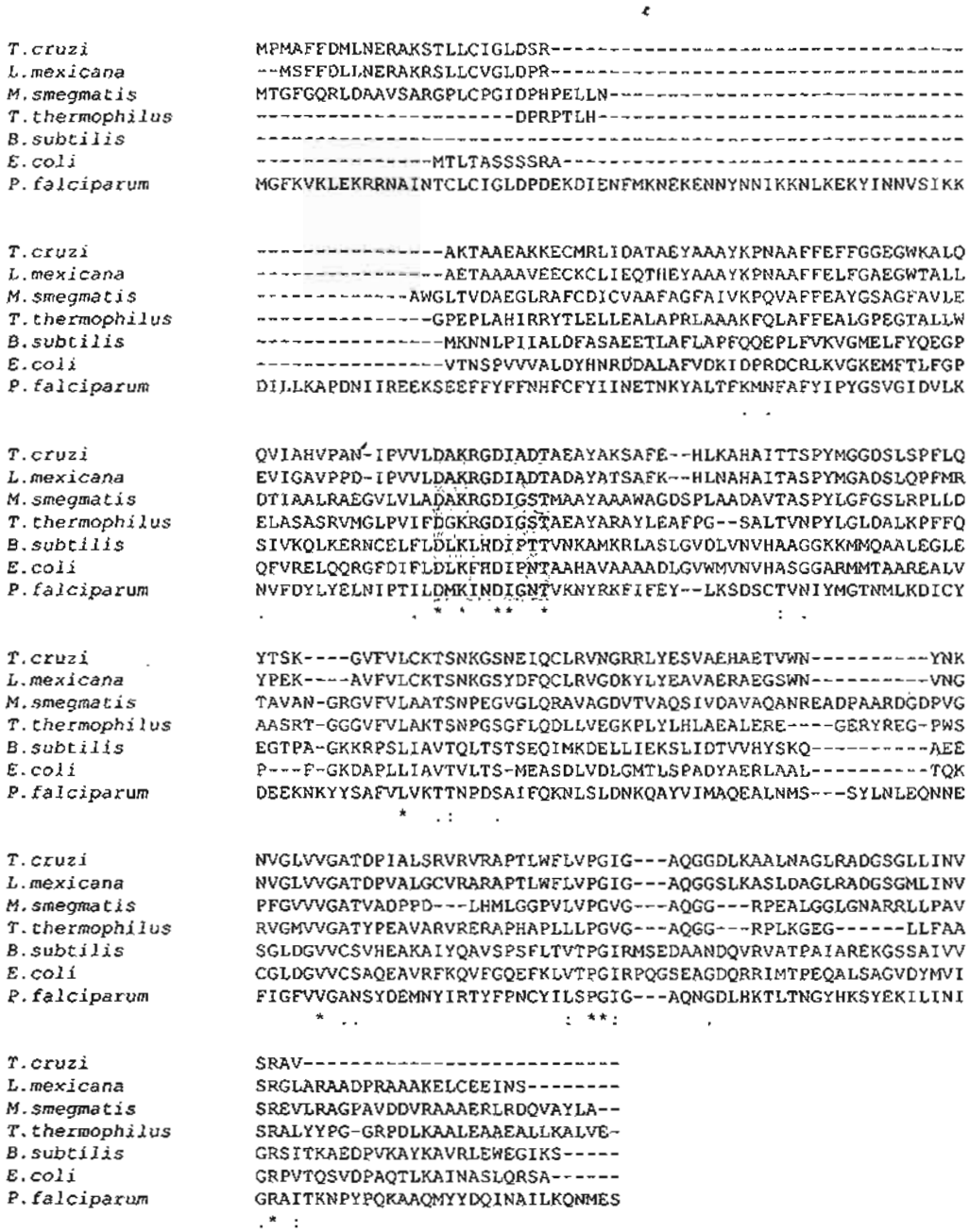

Fig 3- Comparison of deduced amino acid sequence for *P. falciparum* OMPDC and OMPDCs sequences from other species of protozoa, eubacteria and archaebacteria.

HxHxxxE motif in both *pfCA* and *pyCA* suggests the α- type of carbonic anhydrase in the malarial parasites.

Biochemical characterization and recombinant expression of pyrimidine genes

In the malarial parasite, the first three enzymes catalyzing the conversion of HCO_3^-, ATP, L-glutamine and L-aspartate to dihydroorotate (CPSII, ATC and DHO) were partially characterized in *P. berghei* (Krungkrai *et al*, 1990; 1992). These three enzymatic activities were separated by analytical gel filtration chromatography. Our preliminary results for the three

```
P.berghei       MHFKTKLKNRRNEVNTCLCIGLDPDEDDIKNFMRNEEKNGYKNVKNNMNSNNNRIEN--V
P.yoelii        ------------------------------------------------------------
P.falciparum    MGFKVKLEKRRNAINTCLCIGLDPDEKDIENFMKNEKENNYNNIKKN---LKEKYIN--N
P.knowlesi      MNLKTKLQKRRDEVNTCLCIGLDPDEADIKSFMQSEKQNGYQSIKKNLSNHGSSSQGGLF

P.berghei       IKIGKEILLTDEENIENLSEEDKFFYFFNHFCFYIINNTKEYALIYKMNFAFYIPYGSVG
P.yoelii        ------------------------------------------NFAFYIPYGSVG
P.falciparum    VSIKKDILLKAPDNIIREEKSEEFFYFFNHFCFYIINETNKYALTFKMNFAFYIPYGSVG
P.knowlesi      TPQVGGTMLLAENPPKEVQEKDEFFYFFNHFCFYIINETKQYALSYKMNFAFYLPYGSLG
                                                          *****:****:*

P.berghei       INALKNVFDYLNSMNIPTMLDMKINDIGNTVKNYRKFIFEYLKSDSCTINVYMGTSMLKD
P.yoelii        INALKNVFDYLNSMNIPTMLDMKINDIGNTVKNYRKFIFEYLKSDSCTINVYMGTNMLKD
P.falciparum    IDVLKNVFDYLYELNIPTILDMKINDIGNTVKNYRKFIFEYLKSDSCTVNIYMGTNMLKD
P.knowlesi      VDVLKNVFDYLHYLDVPTILDIKMNDIGNTVKHYSKFIFHYLRSDSCTANIYMGTHMLRD
                ::.******** :::**:**:*:********:* ****.**:***** *:**** **:*

P.berghei       ICFDYEKNKYYSAYVLIKTTNKDSFIFQNELSINDKQAYIVMAEETQKMATDLKIDQNNE
P.yoelii        ICFDYEKNKYYSAYVLIKTTNKDSFIFQNELSINDKQAYIVMADETQKMATELKIEQNNE
P.falciparum    ICYDEEKNKYYSAFVLVKTTNPDSAIFQKNLSLDNKQAYVIMAQEALNMSSYLNLEQNNE
P.knowlesi      ICLDEECKRHYSTFVLIKTTNPDSHIFQNRLSLDGKEAYVVIADEAQKMAKQLHLEENGE
                ** * * :::**::**:**** ** ***:.**::.*:**:::*:*: :*:. *::::*.*

P.berghei       FIGFVVGSNAFEEMKIIRNKFPECYILSPGIGAQNGDLYKTLKNGYNKDYEKLLINVGRA
P.yoelii        FIGFVVGSNAFEEMKIIRNKFPDSYILSPGIGAQNGDLYKTLKNGYNKDYEKLLINVGRA
P.falciparum    FIGFVVGANSYDEMNYIRTYFPNCYILSPGIGAQNGDLHKTLTNGYHKSYEKILINIGRA
P.knowlesi      FVGFVVGANCYDEMKKIRELFPDCYILAPGVGAQKGDLRKTLCNGYSKNYERLLINVGRA
                *:*****:*.::**: **  **:.***:**:***:*** *** *** *.**::***:***

P.berghei       ITKSPNPKKSSESYYNQIIQIFKDIENGGNIEQVYL
P.yoelii        ITKSPDPKKSSESYYNQIIQIFKDIENGDNIEQV--
P.falciparum    ITKNPYPQKAAQMYYDQINAILKQNMES--------
P.knowlesi      ITKSGNPQQAAREYYHQIKEILAELRE---------
                ***.  *::::. **.**  *: :  :
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Fig 4- Multiple sequence alignments of four Plasmodia species identified in the genome databases of rodent, monkey and human parasites.

enzymes in *P. falciparum* are consistent with the results obtained for *P. berghei*. These results suggest that the malarial CPSII, ATC and DHO enzymes carry on separate proteins as mono-functional forms, differing from the human enzymes. This is strongly supported by the evidence that the three gene (*PYR1, PYR2, PYR3*) organization in the *P. falciparum* genome are not clustered.

The fourth enzyme DHOD, catalyzing the conversion of L-dihydroorotate (L-DHO) to orotate (OA), was well characterized in both *P. berghei* (Krungkrai *et al*, 1991) and *P. falciparum* (Krungkrai, 1995). It is localized in the mitochondria organelle and is linked to the organelle electron transport system, similar to the human enzyme (Krungkrai, 2000). It was shown earlier that the *P. falciparum* genome contained two homologues of DHOD on chromosome 7 and 9. Based on multiple sequence alignments with other DHODs, the DHOD homologue 1 (*pfDHOD1*) on chromosome 7 represents the mitochondrial-associated enzyme in which it exhibits a long N-terminal part having the typical feature of a mitochondrial targeting (Hartl and Neupert, 1990; Neupert, 1997). The DHOD homologue 2 (*pfDHOD2*) on chromosome 9 represents the cytosolic form, in that it has been previously characterized in *P. falciparum* and *P. berghei* (Krungkrai, 1993b). In this study, the full-length ORF of the pfDHOD1 was cloned into an expression vector pET and functionally expressed in *E. coli* (Fig 2). Typically, the amount of purified recombinant pfDHOD1 (molecular mass ~55,000 Da) obtained from 1 liter of bacterial culture was ~0.7-1.0 mg active protein, with a turnover number of 16 s^{-1}. The recombinant pfDHOD1 contained flavin mononucleotide as the prosthetic group. It had an optimal pH 8.0 and required both substrates: L-dihydroorotate (L-DHO) and coenzyme Q (CoQ) for maximal catalysis. The kinetic properties of the recombinant enzyme were compared with native

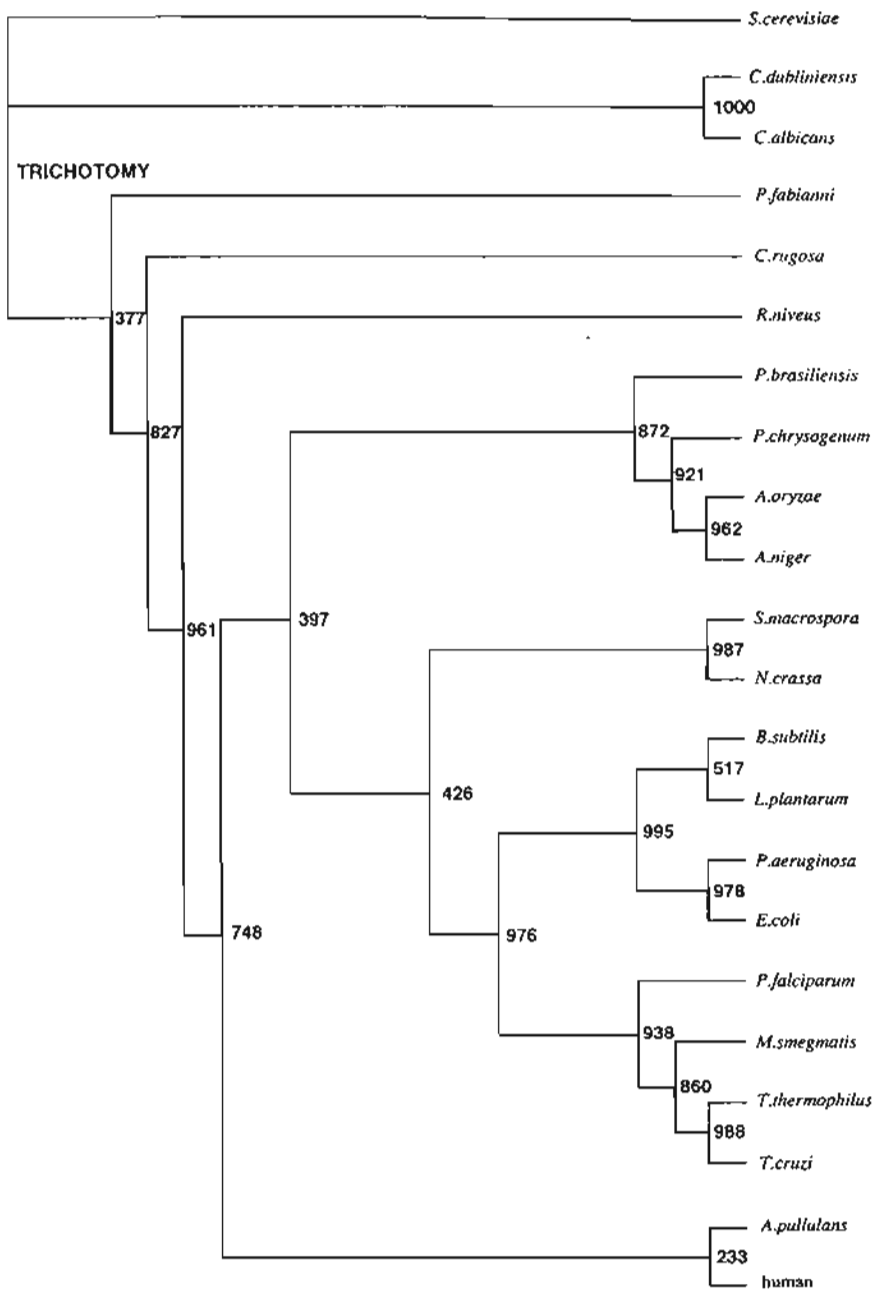

Fig 5- Phylogenetic analyses of the selected OMPDC sequences from various organisms using neighbor-joining methods.

malarial enzymes from *P. falciparum* (Krungkrai, 1995) and *P. berghei* (Krungkrai *et al*, 1991; 1992) and with recombinant human enzyme (Copeland *et al*, 1995; Knecht and Loffler, 2000; Knecht *et al*, 2000) (Table 1). It was shown that the antimalarial CoQ analogue atovaquone (Fry and Pudney, 1992) inhibited both human and malarial enzymes but the I_{50} values were >1,000-fold different. The drug was tested on growth and proliferation, the IC_{50} values were also markedly different (>5,000-fold) between human and parasite (Table 1). Based on these lines of evidence, it is therefore concluded that the recombinant enzyme

shows some physical and kinetic properties somewhat similar to those of the native enzymes, and it may be a possible target for the current chemotherapeutic drug, *ie*, atovaquone.

The fifth enzyme OPRT and the sixth enzyme OMPDC, catalyzing the conversion of orotate (OA) to OMP and OMP to UMP, respectively, have been partially characterized in *P. falciparum* by Rathod and Reyes (1983). Their results suggest the two enzyme activities are active in mono-functional forms. This is supported by the evidence of the genes *PYR5* and *PYR6* mapping on chromosome 5 and 10, respectively, in the *P. falciparum* genome. We have cloned and sequenced both *pfOPRT* and *pfOMPDC* using PCR methods. The single ORFs (containing 1 exon) of both genes encoded proteins with 281 (molecular mass ~ 33,000 Da) and 323 (molecular mass ~ 38,000 Da) amino acid residues, respectivley. We have purified both enzymes from *P. falciparum* and found them to be a multi-enzyme complex with a molecular mass of 140,000 Da, containing two OPRT and two OMPDC mono-functional forms (Fig 6). This represents the first study of a unique multi-enzyme complex of OPRT and OMPDC in the parasite, whereas *Trypanosoma*, *Leishmania* and human enzymes existing ~52,000 Da single bifunctional polypeptide chain encoded by the single gene of fused *PYR5* and *PYR6*, that occurs during evolution (Jones, 1980; Gao *et al*, 1999). More recently, the *P. falciparum OMPDC* gene has been expressed in *E. coli* with a relatively low turnover number (Cinquin *et al*, 2001; Menz *et al*, 2002).

Carbonic anhydrase (CA), catalyzing the inter-conversion of CO_2 and the pyrimidine precursor HCO_3^-, has been biochemically identified and partially characterized in *P. falciparum* (Krungkrai *et al*, 2001b). In addition, *P. berghei* contained CA activities. Both CA activities were found to be sensitive to acetazolamide, a specific inhibitor of α- type CA family. However, this remains to be further investigated, *eg*, recombinant expression and molecular modeling studies.

Demonstration of a pyrimidine salvage pathway in malarial parasites

The use of sensitive assays of radiometric (Reyes *et al*, 1982) and HPLC methods (Krungkrai *et al*, 1989), provides evidence that *P. falciparum* and *P. berghei* lack the enzyme activity of thymidine kinase in the salvage of preformed thymidine from the host to form thymidine 5´-monophosphate (TMP), suggesting that there is no thymidine pyrimidine salvage pathway operating in the parasites. However, UMP may not be only produced by synthesis *de novo* but also from preformed uracil via salvage pathways (Fig 1). This is achieved either in one step by UPRT, or by the sequential action of UP and UK. In this study, the three enzyme activities were assayed in the cell-free extracts of *P. falciparum* and *P. berghei* using the developed HPLC methods. As shown in Table 2, both parasites contained the three enzymes, in order from high to low specific activities: UPRT, UK and UP. The human red cell enzymes were not detected, the mouse red cells contained detectable activities of UK and UP

Table 1
Comparison of kinetics and inhibitory properties between the malarial parasites and human dihydroorotate dehydrogenase enzymes[a].

Enzyme sources	$K_m^{L\text{-}DHO}$ (μM)	$K_m^{CoQ_0}$ (μM)	$K_m^{CoQ_n}$[b] (μM)	K_i^{OA} (μM)	I_{50}[c] (μM)	IC_{50}[d] (nM)
Recombinant *P. falciparum*	12.5	66.6	20.5	33.3	0.01	4.9
Native *P. falciparum*[e]	14.4	58.4	22.5	18.2	N.D.[f]	N.D.
Native *P. berghei*[e]	7.9	28.0	21.6	30.5	N.D.	N.D.
Recombinant human[e]	9.4	13.7	9.9	N.D.	15	27,400

[a] Values are averages, taken from 2-4 separate experiments with the enzyme preparations.
[b] CoQ_n (n=8 for the parasite, n =10 for human enzyme).
[c] I_{50} is a concentration of atovaquone showing 50% inhibition of enzyme activity.
[d] IC_{50} is a concentration of atovaquone having 50% inhibitory effect on the growth and viability of parasite and human cells.
[e] Data are taken for *P. falciparum* (Krungkrai, 1995), *P. berghei* (Krungkrai *et al*, 1991; 1992), and human enzymes (Knecht and Loffler, 2000; Knecht *et al*, 2000).
[f] N.D., value not determined.

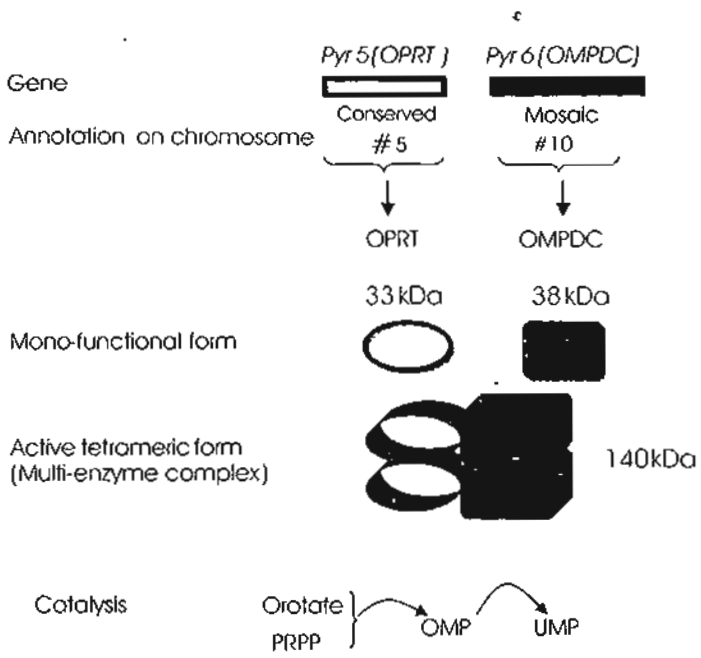

Fig 6- Organizations of genes and enzymes of *P. falciparum* OPRT and OMPDC. PRPP is 5-phosphoribosyl-1-pyrophosphate.

Table 2
Enzymatic activities inter-converting uracil, uridine and UMP of a pyrimidine salvage pathway (uracil phosphoribosyltransferase, UPRT; uridine kinase, UK; uridine phosphorylase, UP) in the malarial parasites, human and mouse red cells.

Sources	Enzyme-specific activity (nmol/min/mg protein)[a]		
	UPRT	UK	UP
P. falciparum	0.325±0.044	0.221±0.010	0.076±0.005
P. berghei	0.266±0.072	0.179±0.016	0.092±0.010
Human red cell	N.F.[b]	N.F.	N.F.
Mouse red cell	N.F.	<0.015	<0.015

[a] Values are mean ± SD of 4-7 separate experiments of the enzyme preparations from cell-free extract.
[b] N.F., enzyme activity not found.

at a lower level than those of the rodent parasite. The *pfUP* homologues encoding uridine phosphorylase activities were identified as mentioned earlier. One of this homologue on chromosome 5 (*pfUP1*) was cloned and sequenced using PCR methods. The ORF of the pfUP1 was 68% and 37% sequence similarity to *E. coli* and human enzymes. The pfUP1 sequence was also close to other bacterial UPs. Our results indicate that a uracil pyrimidine salvage pathway is present in the malarial parasites. This is consistent with the observation of the salvage pathway in other protozoa, *ie*, *T. brucei* (Hammond and Gutteridge, 1982) and *T. gondii* (Schumacher *et al*, 1998).

Concluding remarks and future prospects

In this report, our observations on both biochemical and molecular approaches suggest that: 1) the *P. falciparum* genes of the first six pyrimidine enzymes are genetically and physically unlinked and mosaically evolved; 2) the malarial pyrimidine enzymes are mono-functional forms; 3) the uracil pyrimidine salvage pathways do exist in the parasite from exploration of

both gene and enzymatic activities; 4) the gene and enzyme carbonic anhydrase providing the pyrimidine precursor bicarbonate ion are demonstrated; 5) pyrimidine enzymes are new targets for antimalarial development. A validation of the fourth enzyme dihydroorotate dehydrogenase of the malarial pyrimidine pathway as the drug target has been recently reported by the growth inhibition of *P. falciparum* using RNA interference that encodes a segment of the *pfDHOD* gene (McRobert and McConkey, 2002). We intend to make large amounts of *P. falciparum* pyrimidine enzymes by cloning, expression and purification of potential drug targets, to rule out the technical difficulties in obtaining large quantities of pure enzyme from parasites grown in erythrocytic culture. This will enable complete characterization of interactions with inhibitors and determination of three-dimensional structures.

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Human malarial parasite carbonic anhydrase: molecular cloning, functional expression and characterization

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Plasmodium falciparum is responsible for the majority of life-threatening cases of human malaria. The global emergence of drug-resistant malarial parasites necessitates identification and characterization of novel drug targets. Carbonic anhydrase (CA) is present at high levels in human erythrocytes and in *P. falciparum*. Existence of three isozymes of the ∝-CA has been demonstrated in the malarial parasites (Krungkrai SR, Suraveratum N, Rochanakij S and Krungkrai J. (2001) *Inter. J. Parasitol.* **31**, 661-668). The major isozyme CA I has been purified and characterized from the human parasite, but the two minor isozymes CA II and CA III has not been characterized. The malarial CA I was the most sensitive to acetazolamide inhibition. A search of the malarial genome database yielded an open reading frame (ORF) similar to the ∝-CAs from various organisms, including bacteria, mosquito and human. The primary amino acid sequence of the PfCA1 gene has 70% identity with the rodent malarial parasite (*P. yoelli*) enzyme (PyCA). The single ORFs encoded 235 and 252 amino acid proteins for PfCA1 and PyCA, respectively. The highly conserved signature sequences were also found among human, malaria and bacteria ∝-CAs. The PfCA1 was cloned, sequenced, expressed in a heterologous system of *E. coli*, purified and characterized. The purified recombinant PfCA1 was catalytically active for both p-nitrophenyl acetate and ∝-naphthyl acetate. Its activity was sensitive to acetazolamide inhibition. Kinetic properties of the recombinant PfCA1 revealed the authenticity to the wild type enzyme isolated from *P. falciparum*. This is the first CA cloned and expressed from protozoan parasites. The CA from *P. falciparum* also differed from the human enzyme CA II. Furthermore, the PfCA inhibitor acetazolamide showed some antimalarial effect on the *in vitro* growth of *P. falciparum*. Our molecular tools developed for the recombinant enzyme expression will be useful for developing potential antimalarials directed at *P. falciparum* CA.

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Molecular cloning and expression of *Plasmodium falciparum* carbonic anhydrase

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Objective

To clone and functional express *Plasmodium falciparum* carbonic anhydrase (CA) in *Escherichia coli*

Materials and Methods

Gene identification and characterization of *P. falciparum* carbonic anhydrase homolog

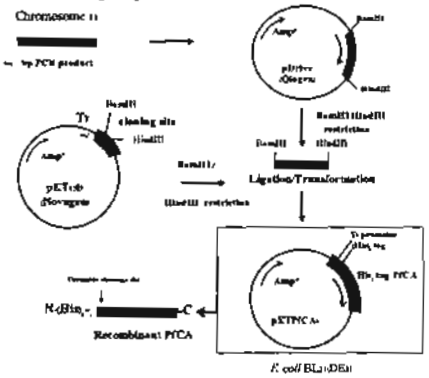
Homology search of the parasite PfCA was performed with the BLAST program of the U.S. National Center for Biotechnology Information server.[1] Using α-CA sequences from other organisms, significant homology for PfCA was found within a sequence on chromosome 11 in a malaria genome database.

Cloning and sequencing of *P. falciparum* carbonic anhydrase

Genomic DNA was isolated from *P. falciparum* by DNAzol™ reagent (Invitrogen). PCR was used to amplify DNA encoding PfCA.

Recombinant protein expression of *P. falciparum* carbonic anhydrase

The competent *E. coli* BL21 (DE3) cells were transformed with the pET15b having the fused His6-PfCA. The basics of creating recombinant *P. falciparum* carbonic anhydrase is summarized in the following diagram.



Purification of *P. falciparum* carbonic anhydrase from *E. coli*

Enzyme purification was performed with Ni^{2+}-NTA-agarose affinity chromatography. The recombinant protein was assayed for CA activity staining on nondenaturing-PAGE gel and determined for kinetic properties using esterase assay as described.[2]

In vitro antimalarial test

The morphological changes of *P. falciparum* was observed in the culture treated with 100 μM acetazolamide (AAZ) in one intraerythrocytic cycle (~44-48 h) starting with synchronized ring stage.

Results and discussion

Cloning and Expression of *P. falciparum* carbonic anhydrase in *E. coli*

The search of the malarial genome database yielded an open reading frame (ORF) on chromosome 11 similar to the α-CAs from various organisms, including human. The primary amino acid sequence of the PfCA gene has ~60% identity with a rodent parasite enzyme, namely *P. yoelii* (PyCA). The single ORF encoded 235 amino acid protein for PfCA. Low homology (~35-51%) of the PfCA and PyCA were found when compared to the insect *Drosophila melanogaster* and human CA I, II sequences. Nevertheless, the highly conserved active site residues responsible for binding of substrate and catalysis were also found among organisms having α-CAs (**Figure 1**)[3]. The recombinant protein was analyzed by SDS-PAGE and has a molecular mass of 29 ± 1 kDa, close to the molecular mass of deduced amino acid sequence of PfCA (**Figure 2A**) and the native CA purified from the malarial culture.[4] The authentic recombinant protein having N-terminally His6-tag was confirmed using the Western blot analysis and monoclonal antibody directed against His6-tag (**Figure 2B**). Furthermore, the recombinant PfCA is shown to express its activity after His6-tag removal on the nondenaturing-PAGE gels, and completely inhibited by 1 mM AAZ (**Figure 2C**).

Kinetic characterization of recombinant *P. falciparum* CA

The kinetic parameters including K_m, k_{cat}, K_i of two specific inhibitors, acetazolamide (AAZ) and sulfanilamide (SFA), were found to be similar between the native and recombinant enzymes (**Table I**). The kinetic and inhibitory constants were different between the human and the parasite enzymes. These results suggest the recombinant PfCA enzyme shows most properties similar to the native enzyme.

Antimalarial properties of *P. falciparum* CA inhibitors

AAZ at 100 μM shows its antimalarial property by interfering with the intracellular development of *P. falciparum* in the stage-dependent manner (**Figure 3**). The morphological abnormality, as shown by clumping of nucleus and cytosol, of the AAZ-treated parasites in the human host red cells were markedly enhanced at the latter stages of development, i.e., trophozoite and schizont (**Figure 3, D-F**). The control culture shows healthy parasites during an intraerythrocytic development (**Figure 3, A-C**).

Figure 1. Amino acid sequences of *P. falciparum* and *P. yoelii* CAs, deduced from the open reading frame of *PfCA* and *PyCA* genes. The predicted amino acid sequences of PfCA (235 residues) and PyCA (252 residues) are shown aligned with *D. melanogaster* (270 residues) and human CA II isozyme (260 residues).

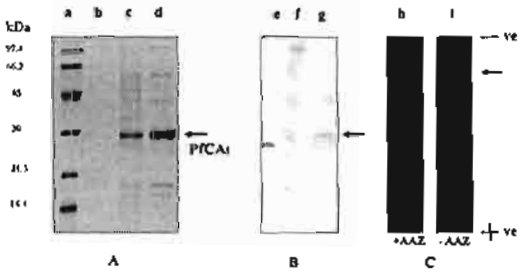


Figure 2. Analysis of recombinant *P. falciparum* carbonic anhydrase from IPTG-induced *E. coli* harboring PfCA construct pET15b plasmid.

Table I Comparison of kinetic parameters and inhibitory constants of human red cell CA II, native and recombinant *P. falciparum* carbonic anhydrases.

Source	K_m[a] (mM)	k_{cat} (min^{-1})	K_i^{AAZ} (nM)	K_i^{SFA} (μM)
Human CA II	10.1 ± 0.8 b	74.1 ± 5.7	99 ± 6	145 ± 2
Native PfCA1	3.7 ± 0.2	10.4 ± 1.2	247 ± 14	56 ± 4
Recombinant PfCA	5.6 ± 0.3	8.2 ± 1.6	315 ± 26	64 ± 10

[a]This assay is based on esterase assay using p-nitrophenylacetate as substrate

[b]The values are mean ± S.D., taken from 3-4 separate experiments of the enzyme preparations.

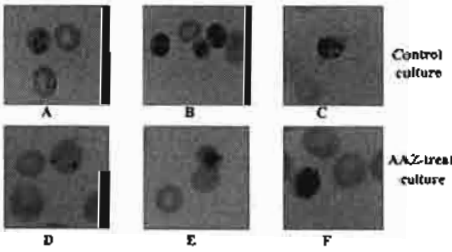


Figure 3. Effect of acetazolamide (AAZ) on *P. falciparum* morphology during an intraerythrocytic cycle (ring, trophozoite and schizont stages).

Conclusions

The *PfCA* gene encoding *P. falciparum* CA is identified in the malarial genome database, and then cloned and functionally expressed in *E. coli*. The recombinant enzyme is catalytically active and sensitive to AAZ and SFA inhibition. It has authenticity to the wild type native enzyme purified from *P. falciparum*, and is also different from the human CA II. The PfCA inhibitor AAZ shows good antimalarial effect on the *in vitro* growth of *P. falciparum* as observed by morphological abnormality, suggesting the therapeutic potential of the malarial parasite enzyme.

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