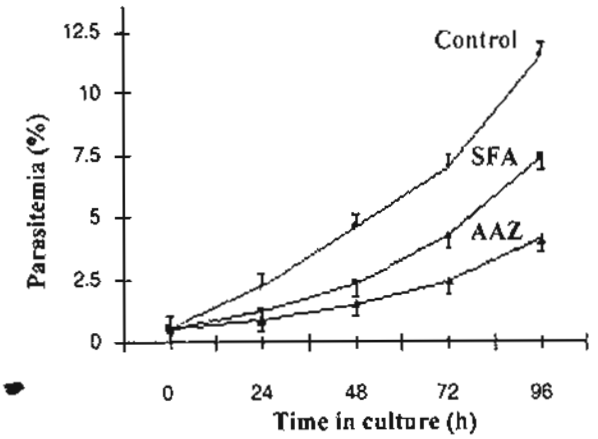

FIGURE 4 Antimalarial activity of the PfCA1 inhibitors, acetazolamide (AAZ) and sulfanilamide (SFA). *P. falciparum* growth was started with 0.5% parasitemia (mixed stages) at 2.5% red cell suspension and monitored every 24 h for up to 96 h at 37°C. The growth of *P. falciparum* in the absence of inhibitors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

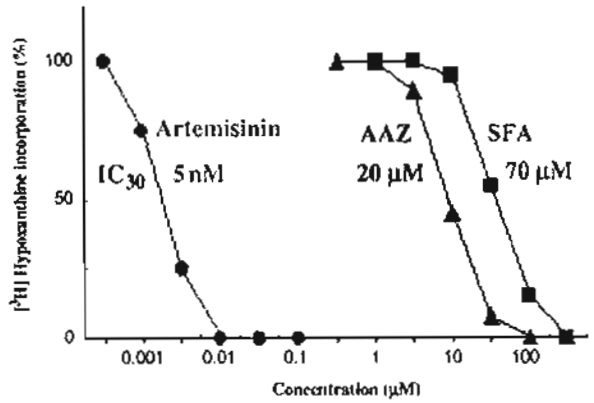

FIGURE 6 Inhibition of [^{3}H]hypoxanthine incorporation by *in vitro* culture of *P. falciparum* at various concentrations of drugs. Growth of *P. falciparum* was started with 0.25% parasitemia (mixed stages) at 0.5% red cell suspension. (●) antimalarial artemisinin, a Chinese traditional drug; (▲)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

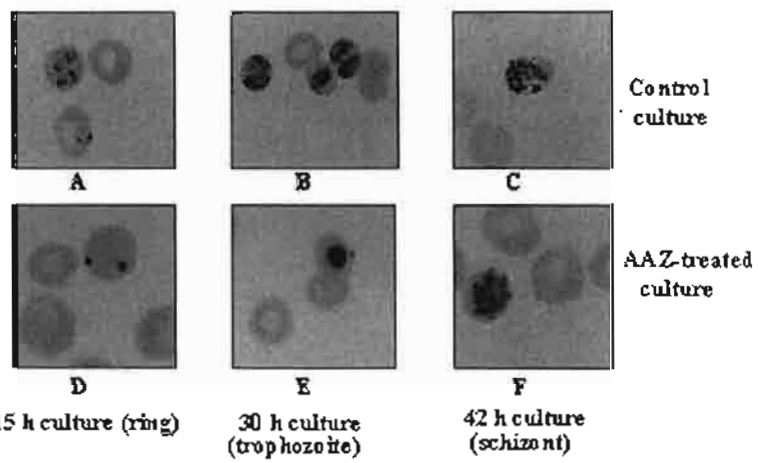

FIGURE 5 Effect of acetazolamide (AAZ) on *P. falciparum* morphology during an intraerythrocytic cycle (ring, trophozoite and schizont stages). The morphological changes were examined in the absence (panels A, B and C; control culture) or in the presence of 100 μM AAZ (panels D, E and F; AAZ-treated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

Our thanks are to P. Prapunwattana, S. Kudan, A. Bhumirattana, R. Kanchanarithisak, P. Learngaramgul and N. Wuttipraditkul for initial study of the *P. falciparum* and *P. berghei* enzyme characterizations. This work was supported by the Thailand Research Fund (to S.R., S.K. and J.K.).

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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

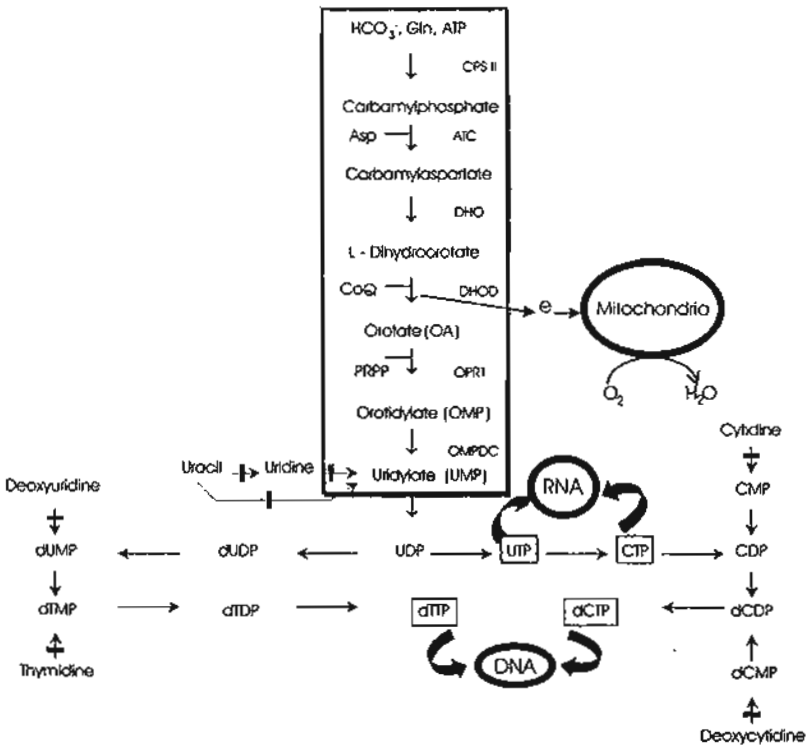

Fig 1- Proposed pyrimidine synthetic pathway in the human malaria parasite *P. falciparum* (Krungkrai, 2000). The first six enzymes of the *de novo* pathway are shown in the box. An uracil pyrimidine salvage pathway, inter-converting uracil, uridine and UMP, is shown by a broken line. The arrow with 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

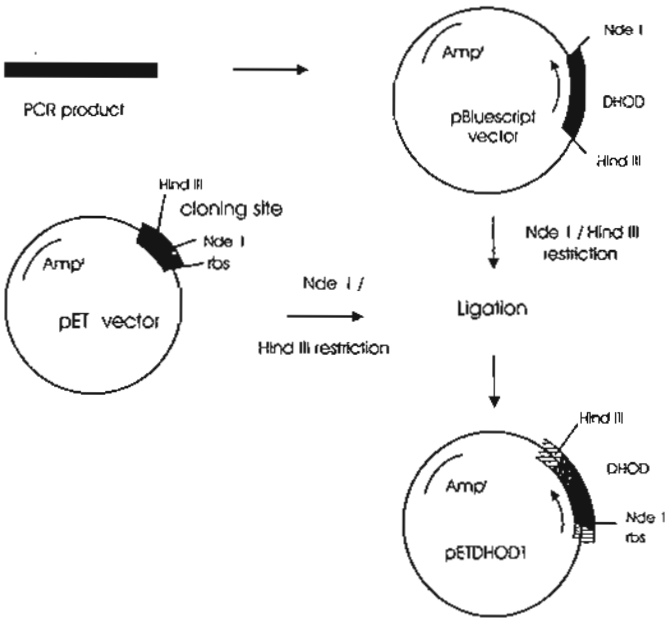

Fig 2- Molecular cloning and expression of *P. falciparum* DHOD homologue1 (*pfDHOD1*) in *E. coli* using pET vector.

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

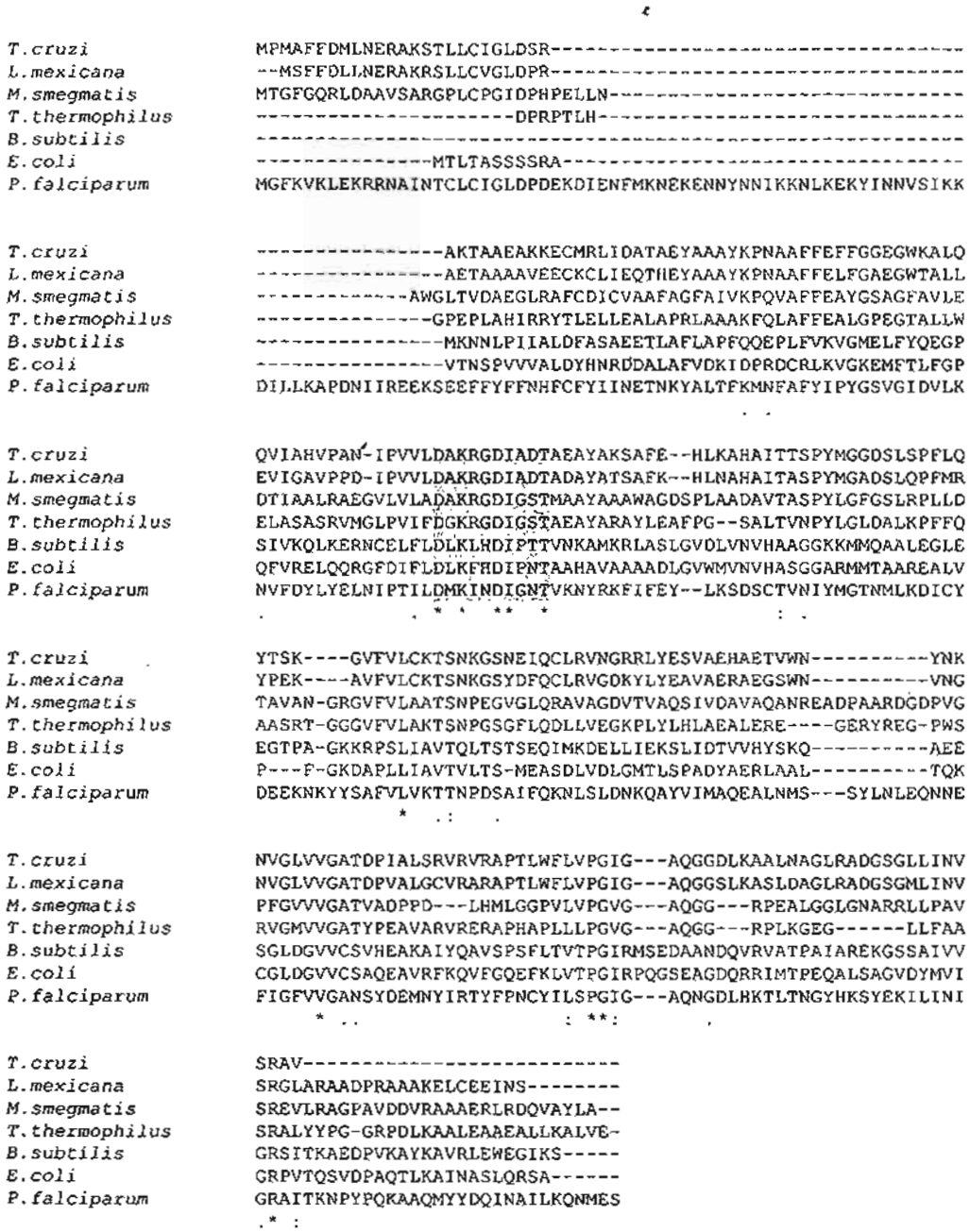

Fig 3- Comparison of deduced amino acid sequence for *P. falciparum* OMPDC and OMPDCs sequences from other species of protozoa, eubacteria and archaebacteria.

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

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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---------
                ***.  *::::. **.**  *: :  :
```

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

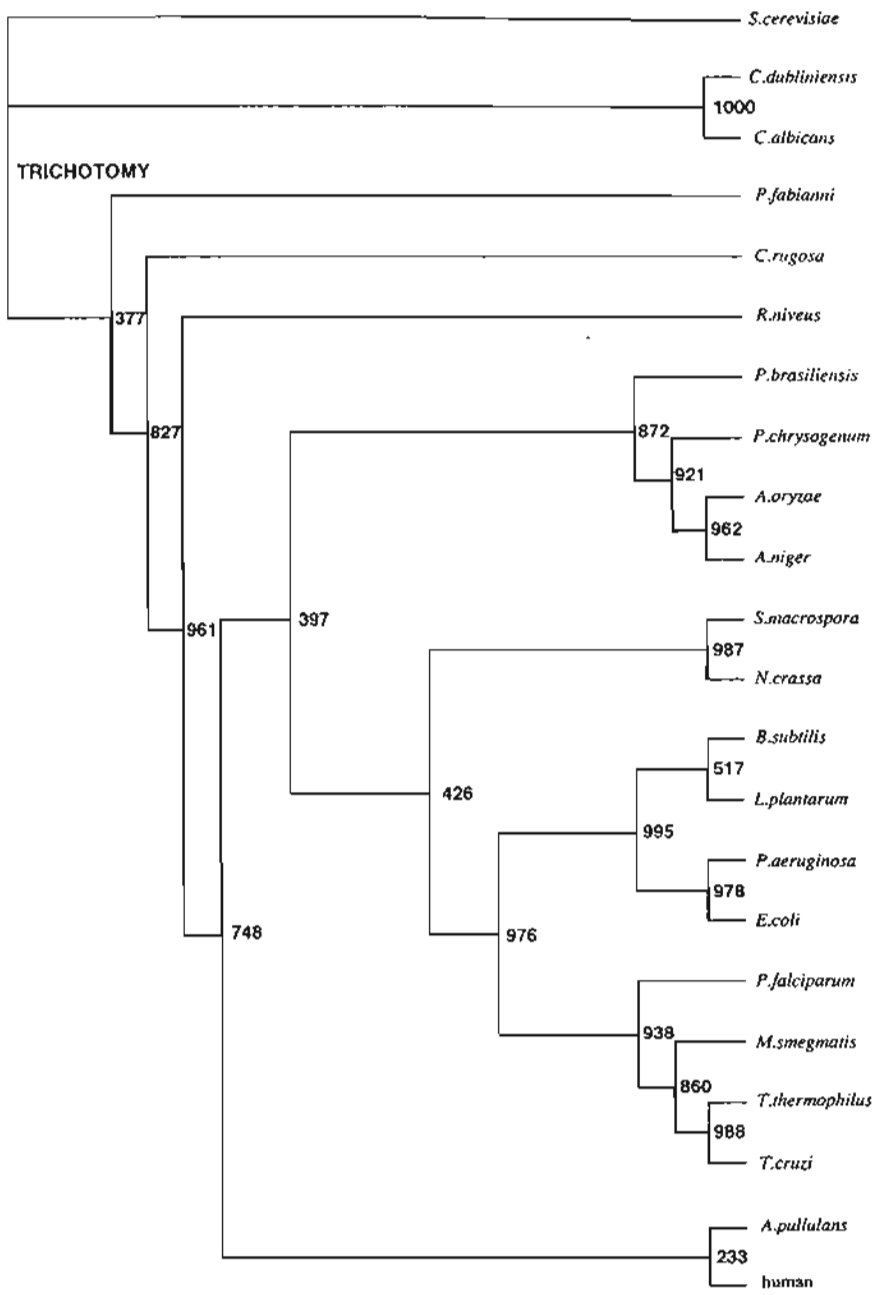

Fig 5- Phylogenetic analyses of the selected OMPDC sequences from various organisms using neighbor-joining methods.

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.

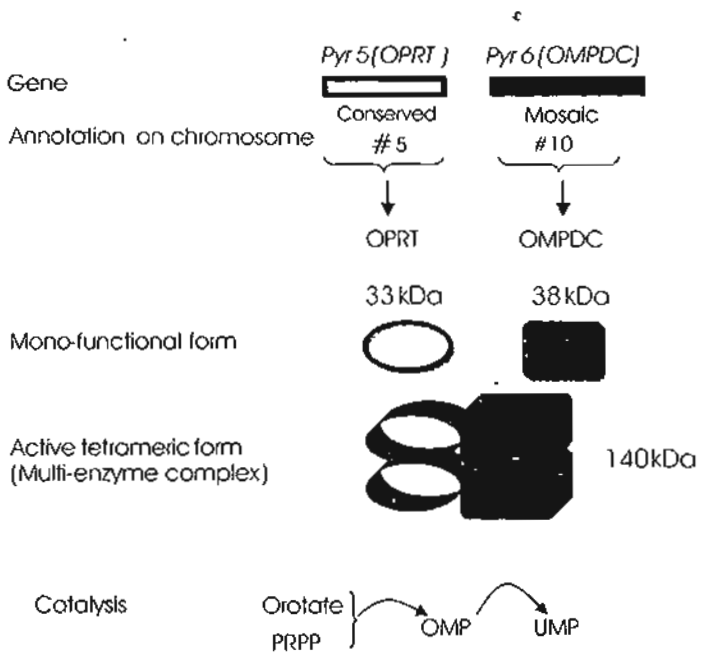

Fig 6- Organizations of genes and enzymes of *P. falciparum* OPRT and OMPDC. PRPP is 5-phosphoribosyl-1-pyrophosphate.

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.

ACKNOWLEDGEMENTS

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6th INTERNATIONAL CONFERENCE ON THE CARBONIC ANHYDRASES

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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.

This work was supported by the Thailand Research Fund.

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

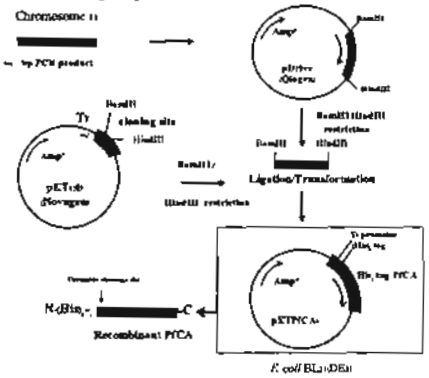
Homology search of the parasite PfCA was performed with the BLAST program of the U.S. National Center for Biotechnology Information server.[1] Using α-CA sequences from other organisms, significant homology for PfCA was found within a sequence on chromosome 11 in a malaria genome database.

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).

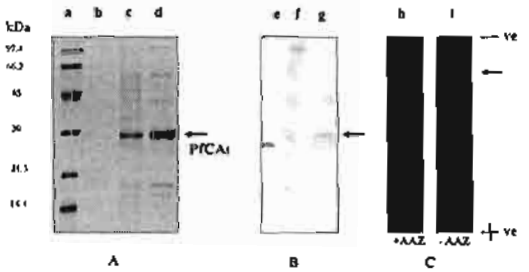


Figure 2. Analysis of recombinant *P. falciparum* carbonic anhydrase from IPTG-induced *E. coli* harboring PfCA construct pET15b plasmid.

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.

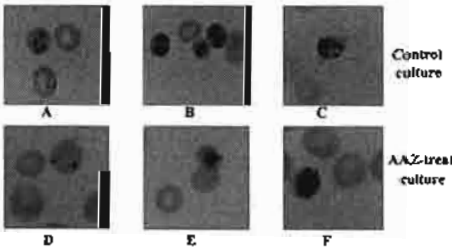


Figure 3. Effect of acetazolamide (AAZ) on *P. falciparum* morphology during an intraerythrocytic cycle (ring, trophozoite and schizont stages).

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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