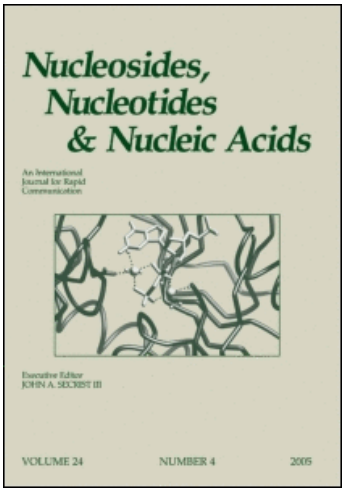

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Cloning and Expression of Malarial Pyrimidine Enzymes

R. I. Christopherson[a]; O. Cinquin[a]; M. Shojaei[a]; D. Kuehn[b]; R. I. Menz[b]
[a] School of Molecular and Microbial Biosciences, University of Sydney, Sydney, Australia [b] School of Biological Sciences, Flinders University of South Australia, Adelaide, Australia

Online publication date: 27 October 2004

To cite this Article Christopherson, R. I. , Cinquin, O. , Shojaei, M. , Kuehn, D. and Menz, R. I.(2004) 'Cloning and Expression of Malarial Pyrimidine Enzymes', Nucleosides, Nucleotides and Nucleic Acids, 23: 8, 1459 — 1465
To link to this Article: DOI: 10.1081/NCN-200027678
URL: http://dx.doi.org/10.1081/NCN-200027678

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS
Vol. 23, Nos. 8 & 9, pp. 1459–1465, 2004

Cloning and Expression of Malarial Pyrimidine Enzymes

R. I. Christopherson,[1,*] O. Cinquin,[1] M. Shojaei,[1] D. Kuehn,[2] and R. I. Menz[2]

[1]School of Molecular and Microbial Biosciences, University of Sydney, Sydney, Australia
[2]School of Biological Sciences, Flinders University of South Australia, Adelaide, Australia

ABSTRACT

We have cloned genes encoding three enzymes of the de novo pyrimidine pathway using genomic DNA from *Plasmodium falciparum and* sequence information from the Malarial Genome Project. Genes encoding dihydroorotase (reaction 3), orotate phosphoribosyltransferase (reaction 5), and OMP decarboxylase (reaction 6) have been cloned into the plasmid pET 3a or 3d with a thrombin cleavable 9xHis tag at the C-terminus and the enzymes were expressed in *Escherichia coli*. To overcome the toxicity of malarial OMP decarboxylase when expressed in *E. coli,* and the unusual codon usage of the malarial gene, a hybrid plasmid, pMICO, was constructed which expresses low levels of T7 lysozyme to inhibit T7 RNA polymerase used for recombinant expression, and extra copies of rare tRNAs. Catalytically-active OMP decarboxylase has been purified in tens of milligrams by chromatography on Ni-NTA. The gene encoding orotate phosphoribosyltransferase includes an extension of 66 amino acids from the N-terminus when compared with sequences for this enzyme from other organisms. We have found that other pyrimidine enzymes also contain unusual protein inserts. Milligram quantities of pure recombinant malarial enzymes

*Correspondence: R. I. Christopherson, School of Molecular and Microbial Biosciences, University of Sydney, Sydney NSW 2006, Australia.

DOI: 10.1081/NCN-200027678
1525-7770 (Print); 1532-2335 (Online)

from the pyrimidine pathway will provide targets for development of novel antimalarial drugs.

Key Words: Pyrimidines; Malaria; Inserts; Genes; De novo pathway and cloning.

INTRODUCTION

Drug resistance has become a major problem in malaria-endemic areas. New drugs with novel mechanisms of action are required. Malaria is a major world health problem with 300 million people infected and 2–3 million deaths annually. There are 4 species

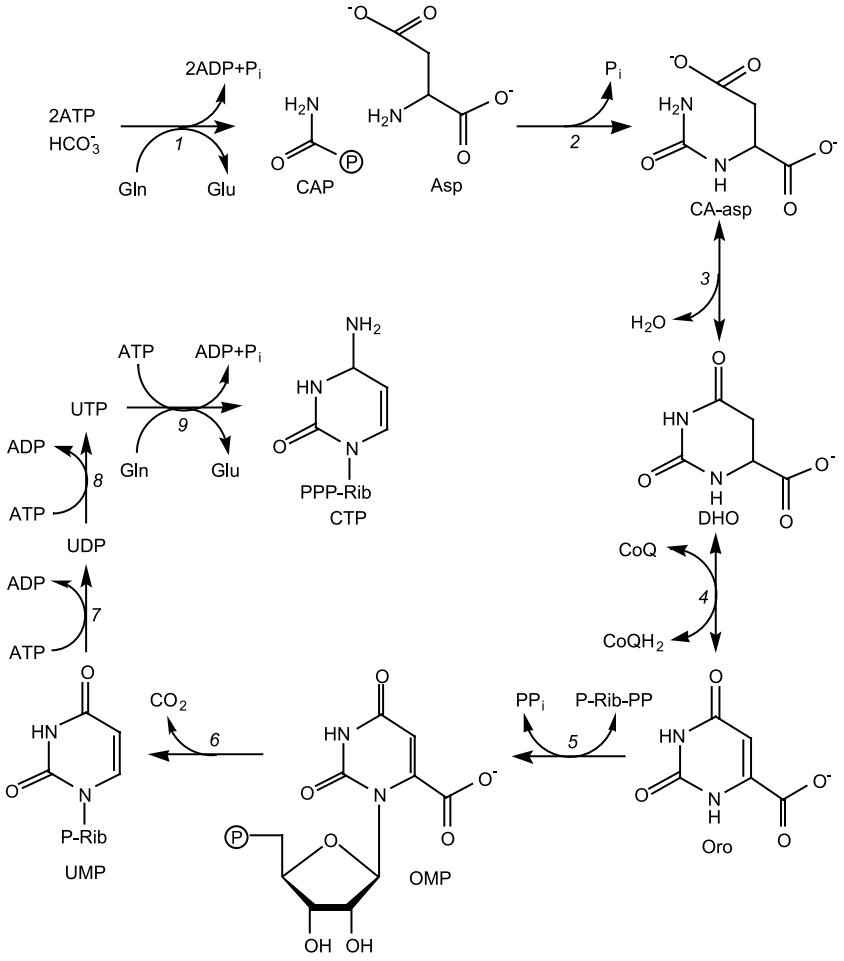

Figure 1. The de novo pathway for biosynthesis of pyrimidine nucleotides. The enzymes are: 1, carbamyl phosphate synthetase (CPSase); 2, aspartate transcarbamylase (ATCase); 3, dihydroorotase (DHOase); 4, dihydroorotate dehydrogenase (DHODHase); 5, orotate phosphoribosyltransferase (OPRTase); 6, OMP decarboxylase (ODCase); 7, UMP kinase; 8, nucleoside diphosphokinase; 9, CTP synthase (CTPSase).

of malaria which infect man and *Plasmodium falciparum* is responsible for the most fatalities. The parasite has 14 chromosomes constituting approximately 25 Mb of DNA. The AT content of the DNA is very high, approximately 80%, which makes expression of recombinant malarial proteins in other organisms, such as *E. coli,* quite difficult. Malarial genes often lack introns which means that genes can be cloned by PCR directly from genomic DNA. Sequencing of the genome in the Malarial Genome Project started in 1996 with contributions from the Sanger Centre, The Institute for Genomic Research (TIGR) and Stanford University; and was completed in October 2002.[1] The annotated sequence of the malarial genome is available at http://plasmodb.org.

The malarial parasite can only synthesise pyrimidine nucleotides via the de novo pathway[2] (Fig. 1) whereas the human patient has an alternative salvage pathway for pyrimidine nucleotides. Inhibitors of de novo pyrimidine biosynthesis may therefore have selective toxicity against the parasite. The suitability of the de novo pyrimidine pathway as an antimalarial target is demonstrated by the successful drug, atovaquone,[3] which inhibits the malarial electron transport chain at Complex III and indirectly inhibits dihydroorotate dehydrogenase (reaction 4). We have designed and synthesised several dihydropyrimidine analogues which are potent inhibitors of DHOase. Two good examples are 2-oxo-1,2,3,6-tetrahydro-pyrimidine-4-6-dicarboxylate (HDDP, Ki = 0.74 μM) and 6-L-thiodihydroorotate (TDHO, Ki = 0.85 μM).[4] We showed that exposure of *P. falciparum* in erythrocytic culture to TDHO or atovaquone induces accumulation of N-carbamyl-L-aspartate (CA-asp), and CA-asp and L-dihydroorotate (DHO), respectively,[2] with depletion of dTTP but not dCTP[5] resulting in inhibition of DNA synthesis and antimalarial activity. Combination chemotherapy with several potent inhibitors of the pyrimidine pathway would overcome *metabolic resistance*[6] which results from accumulation of the substrate for an inhibited enzyme to levels sufficient to out-compete the inhibitor. HDDP and TDHO are effective inhibitors of DHOase, but more potent inhibitors will be required for clinical antimalarial activity. The cloning and expression of malarial enzymes described here is a first step toward obtaining three-dimensional structures, and designing and synthesizing a second generation of more potent inhibitors.

MATERIALS AND METHODS

Cloning

Primers were designed based upon malarial genomic DNA sequences flanking genes of interest and incorporating restriction sites and a protease cleavable His tag sequence. Pyrimidine genes were transcribed by PCR from *P. falciparum* 3D7 genomic DNA with the extension step at 65°C rather than 72°C due to the high AT content. The PCR product was ligated into a pET expression vector. *E. coli* strain PMC103 was used as the primary transformant host as it has reduced recombination mechanisms.[7]

Over-Expression and Purification

E. coli BL-21 (DE3) cells were transformed with the expression plasmid and the hybrid plasmid, pMICO, containing both pLysS expressing T7 lysozyme which inhibits RNA polymerase, and the RIG plasmid expressing rare tRNAs for Arg, Ile and Gly.[8]

Table 1. Summary of malarial enzymes.

	DHOase	OPRTase (215)	OPRTase (281)	ODCase
Chromosome no.	14	5	5	11
Length	1074 bp	645 bp	846 bp	969 bp
%AT	70.4 %	77.9 %	77.2%	75.6 %
Introns	None	None	None	None
Protein size	358 aa	215 aa	281 aa	323 aa
	41.7 kDa	25.1 kDa	33.0 kDa	37.8 kDa
E. coli identity	29.0%	31.4%	30.4%	19.5 %
Human identity	20.1 %	31.1 %	30.4%	14.7 %
% rare codons	8.4 %	7.8 %	7.5%	7.1 %

The *E. coli* and human identity figures are for amino acid sequences; %rare codons is the percentage of AGA/AGG, Arg, ATA, Ile and CTA Leu codons relative to the total number of codons.

Bacteria were induced with 0.5 mM IPTG, the temperature was reduced to 25°C, and cells were grown for a further 4–16 h. Cells were then lysed and the soluble fraction was applied to a Ni-NTA chromatography column, and the His tagged protein was purified using step-wise elution with imidazole buffers. Enzymes were further purified as required by ion-exchange chromatography on a Poros HQ cartridge using a Biocad Model 700E. Radioassays for DHOase, OPRTase and ODCase have been described elsewhere.[4]

RESULTS AND DISCUSSION

Open Reading Frames (ORFs) homologous to the nucleotide sequences of genes encoding DHOase, OPRTase and ODCase from other species have been identified in sequences from the Malarial Genome Project (Table 1).

These ORFs were then cloned into the expression vector, pETMCSIII, which encodes an N-terminal hexa-histidine tag, using a polymerase chain reaction (PCR) strategy and standard techniques. The tag enables purification of the recombinant protein product by nickel-affinity chromatography on a Ni-NTA column. A Factor Xa cleavage site was engineered to allow the tag to be removed after purification of the

Table 2. Summary of plasmid constructs and protein expression.

Construct	Tag	Protease	Soluble	Purified	Active
pETMCSIII-DHO	6 × His N-term	Factor Xa	Partial	Yes	Yes
pET3a-DHO3	9 × His C-term	Thrombin	20%	No	n/a
pETMCSIII-OPRT215	6 × His N-term	Factor Xa	Yes	Yes	No
pET3a-OPRT281	9 × His C-term	Thrombin	No	No	n/a
pETMCSIII-ODC	6 × His N-term	Factor Xa	Yes	Yes	Yes
pET3d-ODC3	9 × His C-term	Thrombin	Yes	Yes	Yes

recombinant enzyme. The three malarial genes are very AT rich (70–78%) and utilise many codons rare in *E. coli.* Therefore, malarial enzymes have been expressed in *E. coli* strain BL21 (DE3) containing the pMICO plasmid.[8] Recombinant, N-terminal tagged malarial DHOase was expressed in an active, soluble form using the CodonPlus^{TM}-RIL system. However the yields were low, the protein had low affinity for the nickel column and the His tag could not be removed by Factor Xa treatment. Therefore, a new DHOase expression construct was produced with a 9 × His C-terminal tag, the new construct produces large quantities of recombinant DHOase however, the majority of this protein is insoluble (Table 2).

Initial results suggested that malarial ODCase was toxic to *E. coli* due to low level expression prior to induction with IPTG, probably due to leakage of the inducible promoter that controls T7 RNA polymerase. A hybrid plasmid, pMICO, was constructed containing the functionalities of both pLysS expressing T7 lysozyme which inhibits RNA polymerase, and the RIG plasmid expressing rare tRNAs[8] *E. coli* strain BL21 (DE3), transformed with the pETMCSIII expression vector and pMICO, gave high level expression of malarial ODCase after induction with IPTG (Table 2). As with the recombinant DHOase the 6 × His N-terminal tagged ODCase had low affinity for the Ni-NTA column, and a new 9 × His C-terminal tagged construct was generated. The new construct has allowed tens of milligrams of essentially pure, active recombinant ODCase to be prepared by a single purification on a nickel-NTA column, followed by cleavage with protease and repurification on nickel-NTA.[9] The turn-over number for recombinant ODCase of 5 UMP s^{-1} is similar to those reported for other species.[9] Crystallization trials have now commenced with pure malarial ODCase.

For OPRTase we initially identified and cloned a protein of 215 amino acids which contained all the conserved residues of OPRTases from other species. Preliminary experiments produced low levels of soluble protein which was inactive. Subsequent completion of the malarial genome sequence and it's annotation using the DNA sequence analysis algorithms, Genefinder and FullPhat, located two putative OPRTase

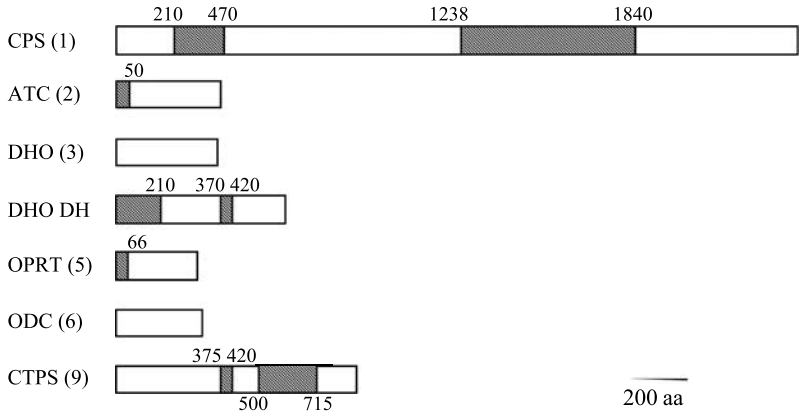

Figure 2. Graphical representation of the amino-acid sequences of pyrimidine biosynthetic enzymes from *P. falciparum*. The relative positions and sizes of the unique inserts not found in other organisms are shaded.

genes in the genome on chromosomes 5 and 7 with identical predicted amino acid sequences which were 281 amino acids in length, OPRTase(281). Alignment of the sequences of the malarial OPRTase(281) gene with those of other species shows a 66-amino acid extension at the N-terminus unique to the malarial enzyme (Fig. 2). The function of this extension is currently unknown. We have cloned the full length OPRTase(281) gene but like the OPRTase(215) it expresses poorly. Interestingly, the OPRTase(281) is less soluble than OPRTase(215). Further work is required to establish if the 66 amino acid extension is required for function of the enzyme or whether it is involved in localization of the enzyme.

When the amino acid sequences of the enzymes for de novo pyrimidine biosynthesis are compared to those of analogous enzymes from other organisms, it becomes apparent that several of these enzymes contain *Plasmodium* unique amino acid sequence inserts of approximately 50 to 600 amino acids (Fig. 2). The functions of these unique amino acid sequence inserts are unknown. Blast searching sequence databases reveals that several of the inserts do not have any significant homology to other known proteins (E value <0.001). The unique protein inserts found in the pyrimidine biosynthetic enzyme, CTP synthetase, show homology to CTP synthetase sequences from other *Plasmodium* species, these particular inserts are not restricted to *P. falciparum*. The CTP synthetase inserts also have homology to short sequences found in a variety of *P. falciparum* and *Dictyostelium discoideum* proteins. These homologous proteins are not from a single family of proteins but display a diverse range of functions, whilst many are hypothetical proteins predicted from genome analysis. This diverse range of functions coupled with homologues only being found in two species of lower eukaryotes suggests that these unique inserts may have a universal function in *Plasmodium* or other lower eukaryotes not restricted to the de novo pyrimidine pathway. This hypothesis is further strengthened by reports of other unique protein inserts being found in a variety of *Plasmodium* enzymes.[10] These inserts are highly likely to play an important role in the biology of *Plasmodium*. They are not found in other organisms and may be found in a variety of malarial enzymes. The inserts may provide contact points for formation of multi-enzyme complexes for pyrimidine nucleotide biosynthesis, they may localize the enzymes to a particular site in the parasite, or they may be involved in regulation of enzymic activities. Their roles are fundamental to understanding the enzymology and cell biology of the parasite and may provide potential targets for development of novel antimalarial drugs. Milligram quantities of pure recombinant malarial enzymes from the pyrimidine pathway will provide targets for development of novel antimalarial drugs.

REFERENCES

1. Gardner, M.J.; Hall, N.; Fung, E.; White, O.; Berriman, M.; Hyman, R.W.; Carlton, J.M.; Pain, A.; Nelson, K.E.; Bowman, S.; Paulsen, I.T.; James, K.; Eisen, J.A.; Rutherford, K.; Salzberg, S.L.; Craig, A.; Kyes, S.; Chan, M.S.; Nene, V.; Shallom, S.J.; Suh, B.; Peterson, J.; Angiuoli, S.; Pertea, M.; Allen, J.; Selengut, J.; Haft, D.; Mather, M.W.; Vaidya, A.B.; Martin, D.M.; Fairlamb, A.H.; Fraunholz, M.J.; Roos, D.S.; Ralph, S.A.; McFadden, G.I.; Cummings, L.M.; Subramanian, G.M.; Mungall, C.; Venter, J.C.; Carucci, D.J.; Hoffman, S.L.;

Newbold, C.; Davis, R.W.; Fraser, C.M.; Barrell, B. Genome sequence of the human malaria parasite Plasmodium falciparum. Nature **2002**, *419*, 498–511.
2. Seymour, K.K.; Lyons, S.D.; Phillips, L.; Christopherson, R.I. Cytotoxic effects of inhibitors of de novo pyrimidine biosynthesis upon *Plasmodium falciparum*. Biochemistry **1994**, *33*, 5268–5274.
3. Looareesuwan, S.; Viravan, C.; Webster, H.K.; Kyle, D.E.; Hutchinson, D.B.; Canfield, C.J. Clinical studies of atovaquone, alone or in combination with other antimalarial drugs, for treatment of acute uncomplicated malaria in Thailand. Am. J. Trop. Med. Hyg. **1996**, *54*, 62–66.
4. Christopherson, R.I.; Schmalzl, K.J.; Szabados, E.; Goodridge, R.G.; Harsanyi, M.C.; Sant, M.E.; Algar, E.M.; Anderson, J.E.; Armstrong, A.; Sharma, S.C.; Bubb, W.A.; Lyons, S.D. Mercaptan and dicarboxylate inhibitors of hamster dihydroorotase. Biochemistry **1989**, *28*, 463–470.
5. Seymour, K.K.; Yeo, A.E.T.; Rieckmann, K.H.; Christopherson, R.I. dCTP levels are maintained in Plasmodium falciparum subjected to pyrimidine deficiency or excess. Ann. Trop. Med. Parasitol. **1997**, *91*, 603–609.
6. Christopherson, R.I.; Duggleby, R.G. Metabolic resistance: the protection of enzymes against drugs which are tight-binding inhibitors by the accumulation of substrate. Eur. J. Biochem. **1983**, *134*, 331–335.
7. Doherty, J.P.; Lindeman, R.; Trent, R.J.; Graham, M.W.; Woodcock, D.M. Escherichia coli host strains SURE and SRB fail to preserve a palindrome cloned in lambda phage: improved alternate host strains. Gene **1993**, *124*, 29–35.
8. Cinquin, O.; Christopherson, R.I.; Menz, R.I. A hybrid plasmid for expression of toxic malarial proteins in Escherichia coli. Mol. Biochem. Parasitol. **2001**, *117*, 245–247.
9. Menz, R.I.; Cinquin, O.; Christopherson, R.I. Identification, cloning and functional expression of the gene encoding OMP decarboxylase from *Plasmodium falciparum*. Ann. Trop. Med. Parasitol. **2002**, *96*, 469–476.
10. Rozmajzl, P.J.; Kimura, M.; Woodrow, C.J.; Krishna, S.; Meade, J.C. Characterization of P-type ATPase 3 in Plasmodium falciparum. Mol. Biochem. Parasitol. **2001**, *116*, 117–126.