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Phylogenetic Analysis and Protein Modeling of *Plasmodium falciparum* Aspartate Transcarbamoylase (ATCase)

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ABSTRACT

Unlike most mammalian cells, *Plasmodium* sp., are unable to utilize preformed pyrimidine bases and nucleosides hence they are reliant solely on *de novo* pathway. Aspartate transcarbamoylase (ATCase, EC 2.1.3.2) catalyzes the first committed step in *de novo* pyrimidine biosynthesis pathway, is a potential target for novel anti-parasitic including antimalarial drugs. *P. falciparum* ATCase has not been studied extensively. To reveal whether it has a regulatory subunit or no and how its evolution, phylogenetic analysis and protein modeling of ATCase *P. falciparum* were studied. The structural model can be used to identify the possible differences between active sites of mammalian and *Plasmodium* enzyme. This is important in a relation with antimalarial drug development. Analogous sequences from *P. falciparum* were searched by 'tBLASTn search' carried out using the web based tools provided by National Center for Biotechnology Information (NCBI). After alignment of ATCase residues sequences, phylogram was constructed by means of MEGA 2.1 software. The results show that the residues sequences of *P. falciparum* ATCase in the phylogenetic tree constructed clearly positioned *P. falciparum* as Class C or "A" consistent with Wild and Wales structural organization. In addition the models for the three dimensional protein structures of the catalytic domain of human and *P. falciparum* ATCase were also generated. As far as our concerned, our study was the first to reveal of *P. falciparum* ATCase classification based on its ATCase amino acid sequences. However, the structure of *P. falciparum* ATCase needs to be determined experimentally to confirm this and to assist the rational design of antimalarial drugs.

Key words: ATCase, *Plasmodium falciparum*, pyrimidine, biosynthesis, phylogenetic, protein modeling, N-(phosphonoacetyl)-L-aspartate (PALA)

INTRODUCTION

The synthesis of pyrimidine nucleotides in pathogenic as well as cancer cells is one of the essential targets for novel anti-viral or anti-parasitic (Ridley, 2002), as well as anti-cell proliferative drugs (Galmarini *et al*., 2003; Striepen *et al*., 2004). Aspartate transcarbamylase (ATCase) is one of the targets, since ATCase is an enzyme that plays an important role in the first committed step in series of reactions that leads to synthesis of pyrimidine nucleotides (Purcarea *et al*., 2003; Labedan *et al*., 2004).

The functional organisation of the enzyme activities is significantly different in prokaryotes, protists, fungi, plants and animals (Nara *et al*., 2000; Roos, 2005; Ginger, 2006). Prokaryotic ATCases have been classified into three classes (A, B and C) based on their molecular weights, subunit architectures and regulatory responses while the eukaryotes ATCases studied fall into either class "A" or the multifunctional polypeptides such as CA and CAD (Wild and Wales, 1990). Santiago and West (2003, 2008) showed that the ATCase can be used for the analysis of taxonomic diversity within the genus *Pseudomonas*. *E. coli* ATCase has both catalytic and regulatory chains which are encoded by *pyrB* and *pyrI* genes, respectively reviewed by Helmstaedt *et al*. (2001). ATCase classification in some parasitic protozoa, such as plasmodium is still unclear (Nara *et al*., 2000).

In the present study, *Plasmodium falciparum* ATCase was chosen as a model since *Plasmodium* have been the serious parasitic protozoa that causes of malaria diseases and *P. falciparum* is the species that causes most human deaths especially in Asia and Africa (Ursos and Roepe, 2002; Osamor, 2010). As mentioned above, ATCase classification in *Plasmodium* is still unclear whether the ATCase is composed of catalytic and regulatory polypeptides or if these functions are found on the same polypeptide or if the catalytic chain is unregulated. However, the sequence of a polypeptide with homology to the catalytic subunit has been reported in *Plasmodium falciparum* and submitted to the EMBL/GenBank/DDBJ databases (Hillier, 2001) with Primary Accession Number O15804. For these reasons, study on *P. falciparum* ATCase needs to be carried out.

In this study, it was hypothesised that ATCase genes with similar sequences will display similar types of regulation. In order to assign the *Plasmodium* enzyme to a particular class and thereby predict if the *Plasmodium* enzyme may have a regulatory subunit or not, phylogenetic analysis of the ATCase amino acid sequence from a variety of species was conducted.

Furthermore, it is becoming apparent that *Plasmodium falciparum* increasingly resistant to antimalarial drugs, particularly chloroquine which then in turn result in increased morbidity and mortality especially among children (Umar *et al*., 2007, 2008). It is necessary to develop alternative antimalarial drugs pursued with a clear mechanism of *Plasmodium* inhibition without disrupting the host's essential metabolic pathway (Ridley, 2002).

In order to elucidate whether the protein structure of human ATCase has similarity to that of *Plasmodium falciparum*, a model for the three dimensional protein structures of the catalytic domain of human and *P. falciparum* ATCase were generated. It was anticipated that these structural models may be able to explain differences observed in the kinetic analysis of the mammalian and *Plasmodium* enzymes in the future. This should be important in a relation with antimalarial drug development.

MATERIALS AND METHODS

The bioinformatics operations: The bioinformatics operations were carried out mainly using the "BioManager" interface provided by ANGGIS (http://www.angis.org.au), (ANGIS, 2002). Searching for analogous sequences from *P. falciparum* by 'tBLASTn search' was carried out using the web based tools provided by NCBI (http://www.ncbi.nlm.nih.gov/projects/Malaria).

Data bank retrieval and multiple sequence alignments: Genes of *E. coli* which encoded the catalytic (*pyrB*) and regulatory (*pyrI*) subunits of ATCase were used as queries to retrieve all ATCase sequence present in the SwissProt and SpTrEMBL data bases (as of November 2010). Multiple protein sequence alignments were generated using Clustasl W (accurate) program (Thompson *et al*., 1994).

Phylogenetic tree construction: Phylogenetic tree constructed by MEGA 2.1 software (GENETYX-WIN 5.1) after alignment of ATCase sequences (Tamura *et al.*, 2007).

Protein modeling: Models for three dimensional structures of catalytic domain of human and *P. falciparum* ATCase were generated either using the automated protein modeling package Swiss-Model (Peitsch, 1996) directly or obtaining the model from the Swiss-Model repository (http://swissmodel.expasy.org/repository/). In all cases the models were based on the *E. coli* structure (PDB id 1d09).

Model structures and the *E. coli* structure complexed with the bisubstrate analog N-phosphonacetyl aspartate, PALA (Krause *et al.* 1987), were aligned using the 'lsqkab' program which is part of CCP4 suite of programs, Collaborative Computational Project (Winn *et al.*, 2002; Krissinel *et al.*, 2004).

Residues, identified as crucial for the *E.coli* ATCase catalysis according to Helmstaedt *et al.* (2001) and Macol *et al.* (2001) i.e., Ser-52, Thr-53, Arg-54, Thr-55, Arg-105, His-134, Gln-137 and Arg-167 were used for alignment. The active sites of the aligned structures were analysed and diagrams were constructed using Phyton-enhanced molecular graphics program, PyMOL software, DeLano Scientific, California, USA (DeLano, 2002).

RESULTS

The catalytic domain: Text searches were used to find the gene sequences from a variety of organisms, for the ATCase catalytic subunit or domain (*pyrB*) as presented in material and methods. The results show that 124 mono-functional ATCase genes were retrieved. In addition the sequence for the trifunctional CAD protein complex was identified for a further 93 eukaryotic organisms. The sequence corresponding to the *pyrB* gene from *Plasmodium falciparum* was located by text searching with the ATCase EC number (2.1.3.2), this resulted in a single hit: SwissProt+SpTrEMBLO15804.

The regulatory domain: A similar strategy to that used to find the catalytic gene sequences was used to find genes encoding the regulatory subunit (*pyrI*). The regulatory gene sequences for 39 organisms were found. Text searching failed to identify any *P. falciparum* genes that are annotated as ATCase regulatory subunits (*pyrI*).

Classification of ATCase: Selected ATCase sequences which were representative of a diverse array of organisms were manually classified based on their size and structural organization according to the classification system of Wild and Wales (1990). The results of this classification are presented in Table 1. It appears here that the ATCase of *P. falciparum* seems rather difficult to put into which classes among the Wild and Wales's system. For these reasons it was performed alignment of amino acids and then proceeded with the constructing of a dendogram (Fig. 1).

Alignment and phylogenetic tree: Figure 1 shows the alignment of the amino acid sequences of the ATCase catalytic genes (*pyrB*) for all the representative organisms used in the manual classification presented in Table 1. A phylogenetic tree from the aligned sequences was derived and the result is presented in Fig. 2. With this construction the position of *P. falciparum* ATCase became more clear that it was among eukaryote class "A" and archea class C.

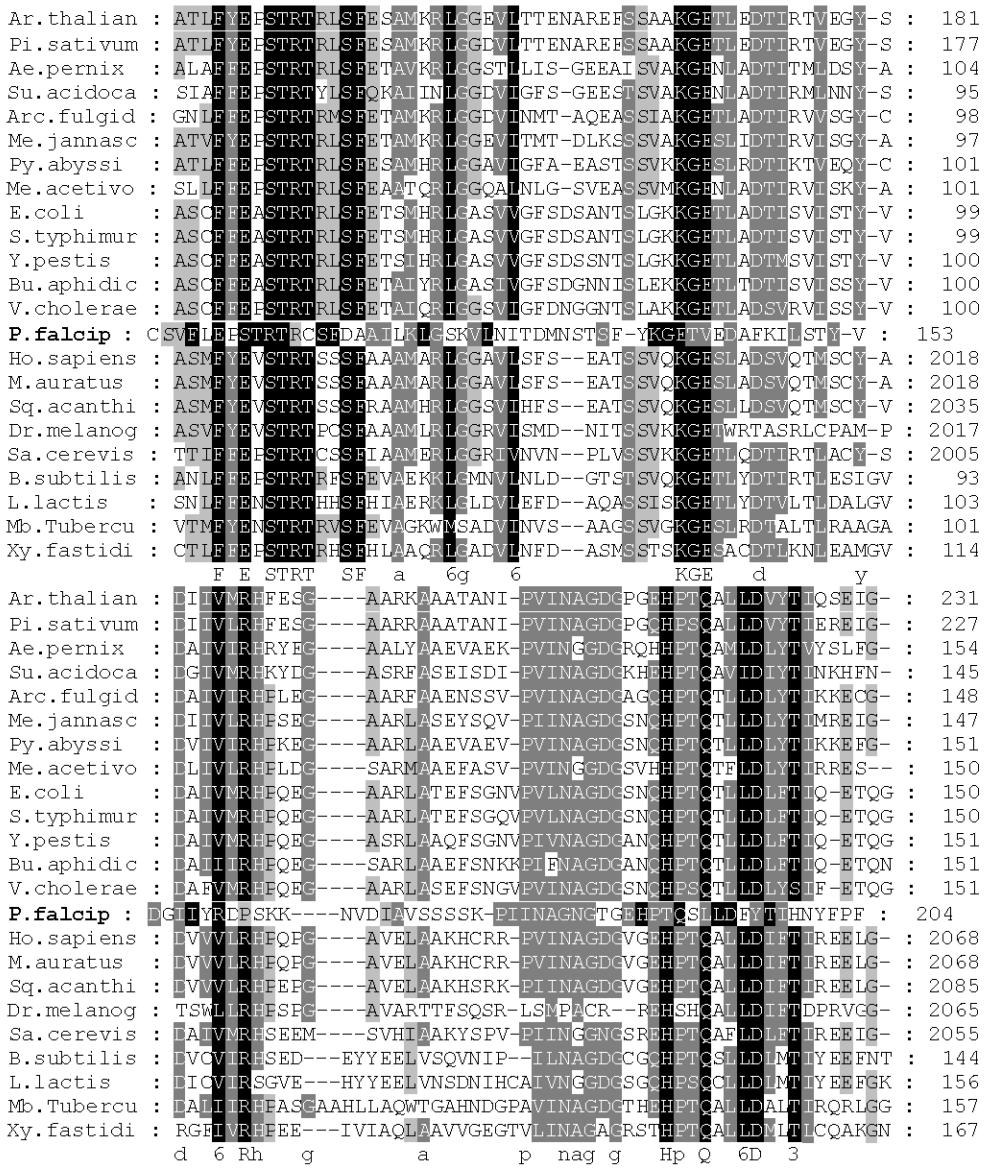

Fig. 1: Representative of multiple alignment of aspartate transcarbamoylase catalytic domains of organisms presented in Table 1. Completely conserved resuidues and patially conserved residues are shaded black and grey, respectively

Protein modeling: The models for the three dimensional protein structures of the catalytic domain of human and *P. falciparum* ATCase were generated based on the sequence alignment of *P. falciparum*, the Human and *E. coli* (Fig. 3). Figure 3 shows the alignment revealed only 20% identity of *P. falciparum* ATCase to *E. coli* and human ATCase. Four amino acids, serine, threonine, arginine and threonine (S-107, T-108, R-109 and T-110) were used in PALA binding as presented in three dimensional structures (Fig. 4).

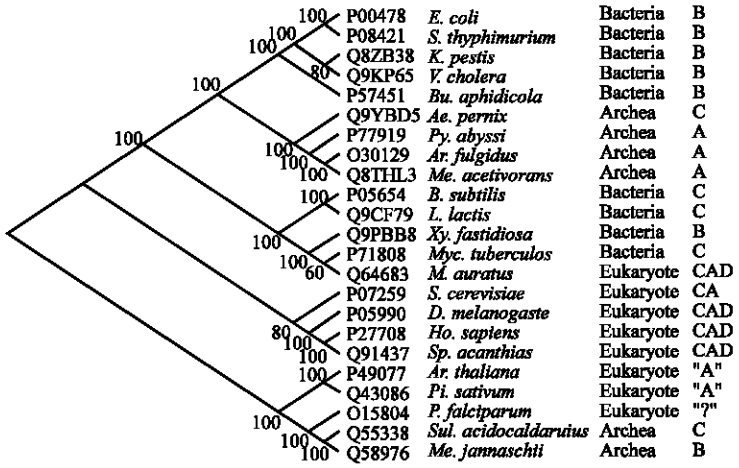

Fig. 2: The phylogenetic tree of asparatate transcarbamoylase sequences. The dendogram was constructed using the program Protpars as described in text

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                52         62         72         82         92
consensus       -      K  I I D     L    L T        LK N     L   KV  S
P_falcipar      .---KN.SV.N.D.VDDEE.LAI.Y.SKQFEKI..N.EDSKY.EN..FC.
PYRB_ECOLI      ANPLYQ.HI.S.N.LSRDD.NLV.A.AAK----..A.PQPEL.KH..IA.
H_sapiens       .----GQHILSVQQFTKDQMSHLFNVAHTLRMMVQKERSLDI.KG..MA.

                102        112        122        132        142
consensus        F E STRTR SF AA   LG  VL   D  STS - KGET  D     S
P_falcipar      V.L.P....C..D..ILK..SK..NIT.MN...F.Y....VE.AFKIL.
PYRB_ECOLI      C.F.A....L..ETSMHR..AS.VGFS.SAN..LGK....LA.TISVI.
H_sapiens       M.Y.V....SS..A..MAR..GA..SFSEAT.SVQ.-...SLA.SVQTM.

                152        162        172        182        192
consensus       TYVD I  R P    V  A   S-  P INAG G GEHPTQ LLD  TI
P_falcipar      ....G.IY.D.SKKN.DI.VSS..SK.I....N.T......S...FY..H
PYRB_ECOLI      ....A.VM.H.QEGAARL.TEF.GNV.VL...D.SNQ....T...LF..Q
H_sapiens       C.A.VVVL.H.QPGA.EL.AKHC.RR.V....D.V......A...IF..R

                202        212        222        232        242
consensus             LD      L IA VGDLK GRTVHSL  LL  Y VS-F  FV
P_falcipar      NYFPFI..RNINKK.N..F.....N.......SK..SR.N....N..SCK
PYRB_ECOLI      -ETQGR..N-----.HV.M.....Y.......TQA.AKFDGNR.Y.IAPD
H_sapiens       -EELGTVNG-----MT.TM.....H.......AC..TQ.R...LRY.APP

                252        262        272        282        292
consensus       SL  P  I          K    S           EE    V I YMTR QK
P_falcipar      ..NI.KD.VNTITYNLK.NNFY.DDSIKYFDNL..GLED.H.I....I..
PYRB_ECOLI      A.AM.QY.LDMLD---E.GIAW.---LH--SSI..VMAE.D.L....V..
H_sapiens       ..RM.PTVRAFVASR-----------------------------------

                247        257        267        277        287
consensus       ER  D  EY   K  F L    L N     K LHPLPRV EI   VD  P
P_falcipar      ..FT.VD..NQY.NA.I.SNKT.E.TRDDT.I.......N..KVE..SN.
PYRB_ECOLI      ..L-.PS..ANV.AQ.V.RASD.H.AKANM.V.......D..ATD..KT.
H_sapiens       --------------------------------------------------

                297        307        317
consensus          YF QA NG   R ALL L       --
P_falcipar      KSV..T..E..LYV.M...Y.I-----..
PYRB_ECOLI      HAW..Q..G..IFA.Q...A.VLNRDLVL
H_sapiens       -------------------------..
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Fig. 3: Alignment of *P. Falciparum, E. Coli* and Human ATCase catalytic residues. Homologous residues are represented by does. Residues proposed to be involved in PALA binding are shaded grey (STRT, Ser-107, Thr-108, Arg-108 and Thr-110). The positions of the resuidues are numbered after *P. Falciparum* ATCase sequence

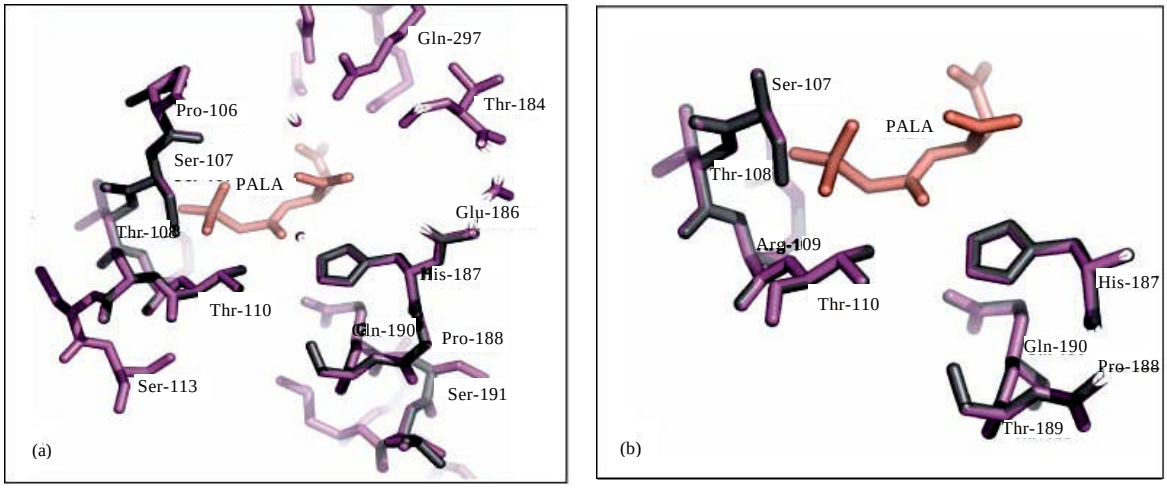

Fig. 4 (a-b): Comparison of P. Flaciparum and the Human ATCase active sites. Panel (a) pf-ATCase (purple) and the Human CAD-ATCase (grey) were superimposed as described in text. Panel (b)as in (a) but focusing on the catalytic residues by removing the non catalytic residues. The bisubstrates analogue PALA is shown (salmon)

Table 1: Grouping of representation retrieved ATCase sequences based on ATCase structural organisation modified from Wild and Wales (1990)

Organism	Class	Primary accession number	Group (Domain)	Mr (kDa)
Aeropyrum pernix	C	Q9YBD5	Archaea	18.062
Arabidopsis thaliana	"A"	P49077	Eukaryota	43.166
Archaeoglobus fulgidus	A	O30129	Archaea	17.255
Bacillus subtilis	C	P05654	Bacteria	34.170
Buchnera aphidicola	B	P57451	Bacteria	17.943
Drosophila melanogaster	CAD	P05990 and Q26376	Eukaryota	249.238
Escherichia coli	B	P00478	Bacteria	16.989
Homo sapiens (Human)	CAD	P27708	Eukaryota	242.915
Lactococcus lactis	C	Q9CF79	Bacteria	34.558
Mesocricetus auratus	CAD	Q64683	Eukaryota	18.606
Methanococcus jannaschii	B	Q58976	Archaea	35.159
Methanosarcina acetivorans	A	Q8THL3	Archaea	34.644
Mycobacterium tuberculosis	C	P71808	Bacteria	33.818
Pisum sativum (Garden pea)	"A"	Q43086	Eukaryota	42.617
Plasmodium falciparum	"?"	O15804	Eukaryota	43.251
Pyrococcus abyssi	A	P77919	Archaea	16.958
Saccharomyces cerevisiae	CA	P07259	Eukaryota	245.124
Salmonella typhimurium	B	P08421	Bacteria	16.955
Squalus acanthias	CAD	Q91437	Eukaryota	249.391
Sulfolobus acidocaldarius	C	Q55338	Archaea	34.171
Vibrio cholerae	B	Q9KP65	Bacteria	17.276
Xylella fastidiosa	B	Q9PBB8	Bacteria	34.637
Yersinia pestis	B	Q8ZB38	Bacteria	17.337

DISCUSSION

The sequence corresponding to the catalytic domain of the *pyrB* gene from *P. falciparum* was located by text searching with the ATCase EC number 2.1.3.2, a single hit of SwissProt+SpTrEMBLO15804 was found. However when a similar strategy was used to find genes encoding the regulatory subunit (*pyrI*), text searching failed to identify any *P. falciparum* genes that are annotated as ATCase regulatory sub units (*pyrI*). To convince the results, BLAST searches of the *P. falciparum* genome using the *E. coli pyrI* gene was conducted. Again this approach was also failed to identify any possible *pyrI* genes for *P. falciparum*. Hence, it can be assumed that either *P. falciparum* does not contain a *pryI* gene homolog or the homology between *P. falciparum* and *E. coli* are very low. Therefore, it is likely that the *P. falciparum* ATCase does not contain a regulatory subunit but this cannot be conclusively ruled out.

The constructed phylogenetic tree (Fig. 2) in general groups the organisms in a similar manner as the classification systems proposed by Bethell and Jones (1969) and Wild and Wales (1990), who grouped the bacteria into three classes A, B and C. This is especially true for the general grouping of prokaryotes and eukaryotes.

Figure 2 shows that the phylogenetic approach also tends to group the prokaryotes into three categories which correspond to those of as determined by enzyme morphology. However, the phylogenetic approach does not completely resolve the morphologically derived ATCase subclasses for the prokaryotic organisms (Labedan *et al*., 2004).

A similar result is also seen for the eukaryotic groups. The phylogenetic tree shows a clear eukaryotic grouping within ATCase classes (Fig. 2).

In animals, the ATCase exists as a domain of multifunctional enzyme recognized as CAD (Hemmens and Carrey, 1995). In the present study the CAD groups were found together with the CA class which is the ATCase class for yeast. This is in agreement with the classification of Wild and Wales (1990) who grouped yeast and hamster ATCase into classes CA and "A", respectively. Based on the molecular weight, class A ATCases are the biggest but these are the least comprehensively studied. The bifunctional enzyme complexes (CA) containing both ATCase and Carbamoyl Phosphatase (CPSase). Class C ATCase are much smaller and lack a regulatory domain or subunit (Vickrey *et al*., 2002; Labedan *et al*., 2004).

Interestingly *P. falciparum* and the plants *Pisum sativum* and *Arabidopsis thaliana* group distinctly from the other eukaryotic sequences (Fig. 2). The *P. falciparum* sequence sits between the eukaryotic and prokaryotic (class B) groupings making it difficult to determine which group it is more similar to. A similar phylogenetic study by Nara *et al*. (2000) also had difficulties assigning the *P. falciparum* ATCase sequence to the prokaryote or eukaryote groupings.

The reason why *P. falciparum* does not group with the eukaryotes such as *D. melanogaster*, is likely due to the fact that *P. falciparum* is the only eukaryote reported with a monofunctional ATCase (Hillier, 2001). This is in line with strong evidence that the three gene (*pyr*1, *pyr*2, *pyr*3) organization in the *P. falciparum* genome are not clustered (Krungkrai *et al*., 2003).

The present study suggests that *P. falciparum* ATCase belongs to a subclass of its own which falls between the B and CAD of the classification system of Wild and Wales (1990). It was anticipated that these structural models may be able to explain differences observed in the kinetic analysis of the mammalian and *Plasmodium* enzymes reported elsewhere in this study. Models of the three dimensional protein structures of the catalytic domain of human and *P. falciparum* ATCase were generated as described in methods based on the sequence alignment of *P. falciparum*, the human and *E. coli*.

As shown in the multiple alignments presented in Fig. 1 and 3 shows 20% highly conserved residues among the three species. This is especially true of the residues have been identified as crucial for catalysis such as those equivalent to Ser-52, Thr-53, Arg-54, His-134 and Gln-137 in *E. coli* (as reviewed by Helmstaedt *et al.* (2001), Macol *et al.* (1999) and Macol *et al.* (2001).

The α-carbon position of the residues which define the catalytic site were used to superimpose the two modelled structures with the *E. coli* structure complexed with the bisubstrate analogue N-(phosphonacetyl) aspartate, PALA (Krause *et al.* 1987). The superposition of the active site residues (Pro-106, Arg-107, Thr-108, Arg-109, Thr-110, His-187, Pro-188, Thr-189 and Gln-190) are shown in Fig. 4. It is evident, in these models that there is very little difference in the position of residues that are capable of interacting with PALA (<3Å) suggesting that the architecture of the active site is extremely conserved. However, one must be cautious when making this conclusion as both models have been constructed using the same template which may bias their similarity to the *E. coli* PALA binding site (<3Å). PALA has been well known as a potent ATCase inhibitor (Boxstael *et al.*, 2003) however, Purcarea *et al.* (2003) revealed that PALA was a relatively ineffective inhibitor of CPSase-ATCase complex. Purcarea *et al.* (2003) carried out the study on *Aquifex aeolicus* ATCase. Whether similar characteristics are also found in *P. falciparum* ATCase, need to be proven further. Hence, further studies investigating the structural biology of *P. falciparum* ATCase are still needed.

CONCLUSION

The residues sequences of *P. falciparum* ATCase in the phylogenetic tree constructed clearly positioned *P. falciparum* as Class C or "A" based on structural organization of Wild and Wales (1990). Structural models constructed shown a strong similarity between *P. falciparum* ATCase and the human ATCase. However, the structure of *P. falciparum* ATCase needs to be determined experimentally to confirm this and to assist the rational design of antimalarial drugs.

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