Chapter 1

Introduction

1.1. The Malaria Parasite, *Plasmodium falciparum*

Malaria is caused by species of the *Plasmodium* genus. It infects humans, primates, rodents as well as birds. *Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae* are the four main human parasites with the virulent *P. falciparum* implicated in the vast majority of human infections. The *Plasmodium* family belongs to a large, monophyletic group of organisms called apicomplexans. Their distinguishing feature is the presence of an apical organellar complex for invading the host cell. Apicomplexans include well known organisms such as *Cryptosporidium, Toxoplasma* and *Theileria* (Figure 1.1; Aravind *et al.*, 2003, Sherman 1998). There are some disagreements as to the origins of malaria. Most of the studies supports the idea that *P. falciparum* originated in Africa and then expanded to southeast Asia and South America but the date of this expansion varies from 10 000 years ago until a maximum of 400 000 years ago (Hartl, 2004). Different sequences tell different tales. Protein-coding genes suggest the occurrence of a rapid expansion (from a few hundred individuals) of the *P. falciparum* population 24 500-57 500 (upper limit) years ago with a probable common ancestor about 10 000 years ago (Rich *et al.*, 1998). The same protein-coding genes used by Rich *et al.* (1998) were analysed using Coalescent theory, which reported a common ancestor about 300 000-400 000 years ago from a population of about a 100 000 parasites (Hughes *et al.*, 2001). The great difference between these two estimates was narrowed down to the age and accuracy of data sets which the two groups used (Hartl, 2004). Another approach used introns in genes on chromosome 2 and 3. The introns for chromosome 2 indicated a common ancestor anything from 9 500-61 000 years ago but a study of 204 coding and non-coding regions from chromosome 3 suggested a common ancestor 100 000-180 000 years ago (Hartl, 2004). In an effort to decrease the uncertainty in the estimates of the population expansion of *P. falciparum,* it was decided to investigate the mitochondrial genome of the parasite. The *P. falciparum* mitochondrial genome is 6kb in size and inherited, as in most mammals, only from the female parent. The results showed that a rapid African expansion occurred about 10 000 years ago but some lineages are more ancient (50 000-100 000 years)

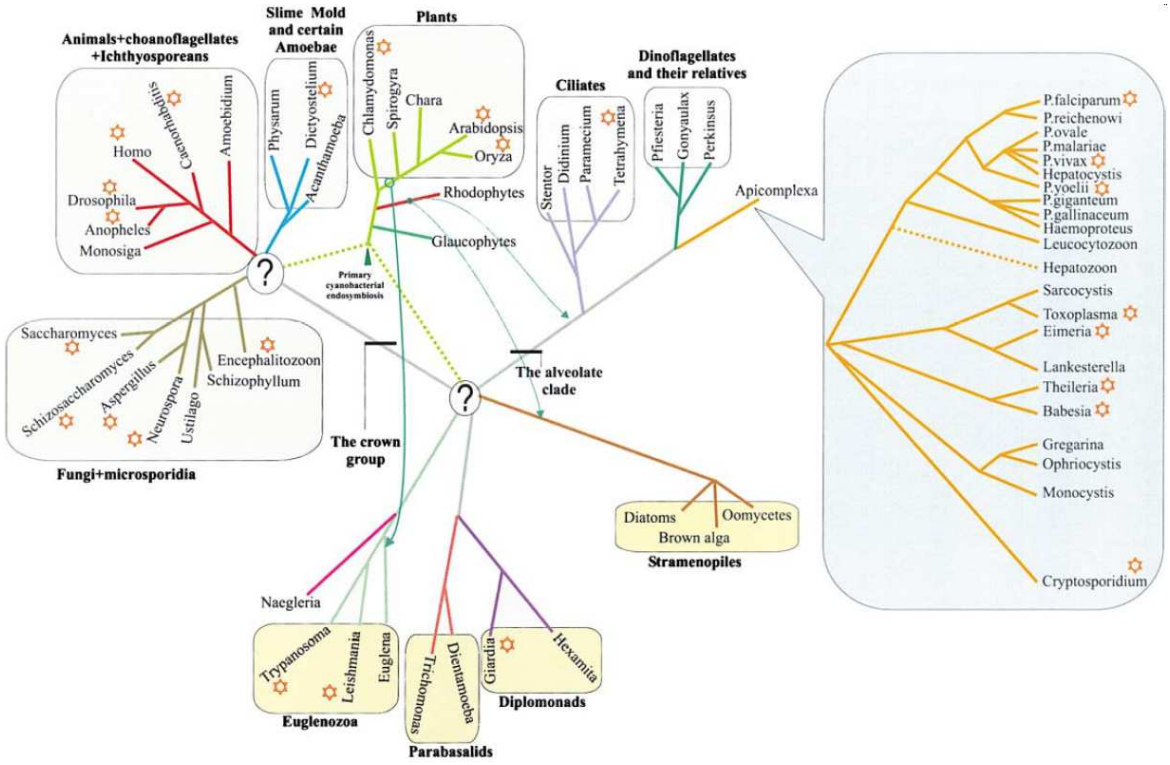

Figure 1.1: The phylogenetic context of *P. falciparum*. Question marks indicate areas where phylogentic relationships are not sorted out yet, red stars indicate organisms for which a complete genome sequence are either finished or in the final stages of processing. The thin arrows indicate secondary endosymbiotic events such as the acquisition of the chloroplast. Adapted from Aravind *et al.*, 2003.

that spread to South America and southeast Asia before the African expansion (Joy *et al.*, 2003). This data goes against the modern belief that the slave trade and Europeans introduced malaria into South America (Sherman 1998). Most of the studies on the population and genetic origins of *P. falciparum* supports the idea that a rapid expansion of *P. falciparum* occurred in Africa about 10 000 years ago.

The first historical evidence of malaria comes from Egyptian mummies and documents. Malaria symptoms were reported from Egypt (1570 B.C.), Persia (2000 B.C.), China (2700 B.C.) and by the Greeks (Homer 750 B.C.; Aristotle 384-322 B.C.; Plato 428-347 B.C.). The disease came to prominence during the Roman era and its name hails from the Italian name for "Roman fever" *mal'aria* ("bad air"). After that the disease spread slowly but surely across most of Europe and into Russia. By the early 1800's malaria had a worldwide distribution (Sherman, 1998). In the 1880's Charles L. A. Lavernan, Camillo Golgi and William G. MacCullum proved that malaria was caused by a single-celled organism which infects the erythrocytes of the human host. The end of the 1890's saw Ronald Ross and Patrick Manson prove that malaria was transmitted by the mosquito. Ross received the Nobel prize in 1902 and Lavernan received the Nobel prize in 1907 for their work on malaria (Sherman, 1998).

Plasmodium has a life cycle which is divided into three parts (Figure 1.2). Sporozoites infect the liver (the exoerythrocytic stage) where they develop into merozoites, which spread to the blood (erythrocytic stage). The merozoites develop into trophozoites, which develop into schizonts. The schizont develops

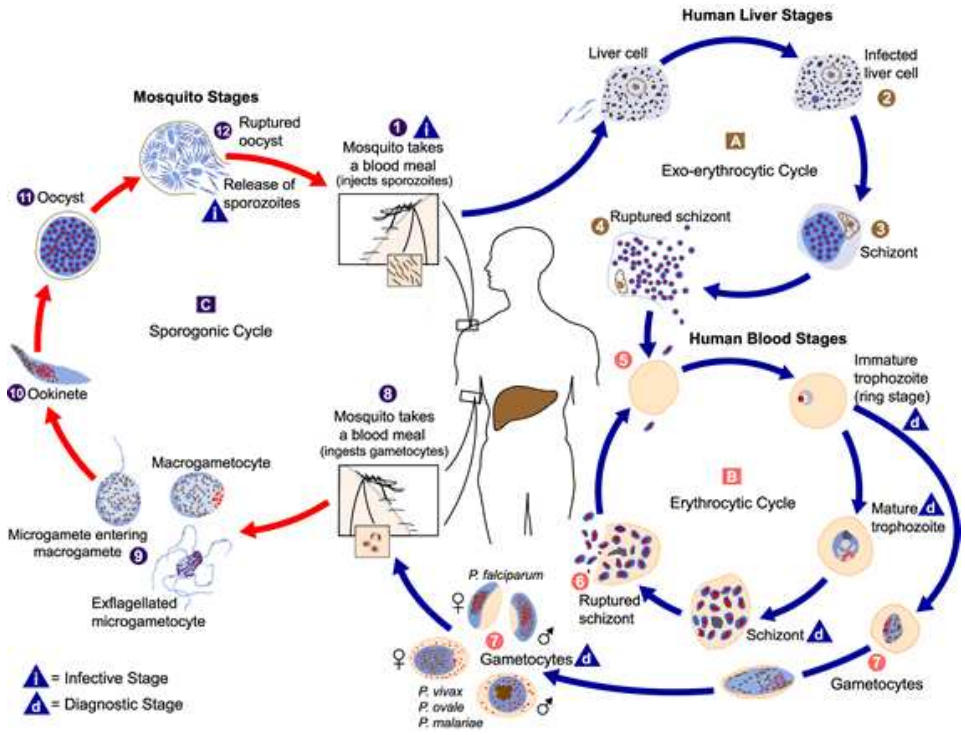

Figure 1.2: The life cycle of the malarial parasite. Source: http://www.well.ox.ac.uk/ich/images.

into trophozoites, which develop into male and female gametocytes. The gametocytes then need the mosquito to complete its life cycle by forming a zygote, which develops into sporozoites in the salivary gland of the mosquito (mosquito stage). In the case of *P. falciparum* the parasite is mostly carried by the *Anopheles gambiae* mosquito (Sherman, 1998).

Malaria is one of the most devastating diseases of the modern world. The worst affected region is sub-Saharan Africa with the worst affected countries suffering a 1.3% economic "growth penalty" each year as well as sustaining annual economic losses upwards of US$2 billion (Kilama, 2003; Figure 1.3). Each year malaria infects between 300 and 500 million people resulting in more than 1.5 million deaths, most of whom are children under the age of five (WHO Malaria fact sheet #94, http://www.who.int/mediacentre /factsheets/fs094/en/). Most of the fragile African economies cannot carry the economic and social burden of treating malaria. Alternative options to continous treatment are being considered such as vaccines, improving sanitation and living conditions and investigating low-cost, preventative methods such as bednets and raised houses (Kilama, 2003; Charlwood *et al.*, 2003). The current status of malaria vaccines will be dicussed in section 1.2.

The impact of malaria on the African economy is devastating. Although a major part of the problem is the cost of treating infections, the disease also results in a loss of income as adults are unable to work. This in turn forces adolescents to go searching for work to earn money and when infected, the same fate befalls them. The never ending cycle of infection, treatment and recovery drains the economy and hampers any attempts at economic recovery (Samba, 2004). The infection of children results in

young, female adolescents having to take care of the children. This reduces the earning capacity of the young adolescents as well as preventing the children from attending school. A strategy targeting young adolescents has been lacking, especially young females prior to conception and during and after pregnancy (Brabin *et al.,* 2005).

Some prevention strategies have been introduced but have met with limited success. The most notable was the introduction of insecticide-treated bednets. The treated bednets reduced infection of infants by 30%. The main problem with this approach is that the insecticide only lasts a few months and local communities do not have enough financial resources to obtain new insecticide (Snow *et al.,* 1997; Friedman *et al.,* 2003).

An alternative strategy is to target the malaria carrier, *Anopheles gambiae.* This type of vector control has met with limited success as *A. gambiae* has developed resistance against permethrin, the most common insecticide used (Ranson *et al.,* 2004). Dichloro-diphenyl-trichloroethane (DDT) spraying was the method of choice for years but due to the accumulation of DDT in animals its use was banned in most countries. Recently the effect of pyrethroid and organophosphate mixtures targeting *A. gambiae* proved to be feasible and may be the way forward in vector control against permethrin-resistant *A. gambiae* (Bonnet *et al.,* 2004). Vector control through limited contact between humans and parasites are hampered by the occurrence of vast, humid tropical areas in Africa.

Another problem in preventing the spread of malaria is the migration of people between areas. More and more land is being explored and opened up for economical exploitation as mines or for agriculture. This causes an influx and migration of workers resulting in malaria being carried to new areas by travellers and workers (Cruz Marques, 1987). The flux of workers between areas makes it more difficult to control the vector.

A sometimes overlooked factor in resistance is the occurrence of non-compliance with malaria treatment. This is especially prevalent where longer treatment periods (up to a week) are involved. Pinto *et al.* (2003) showed that a single 600mg dose of chloroquine is as effective as 600mg followed by two daily 450mg doses in the treatment of uncomplicated *P. vivax.* A single dose treatment will increase compliance in comparison with multiple dose treatments. The next section will focus on drug resistance in malaria and the way forward.

1.2. Drug Resistance in Malaria

The first drug used for malaria was quinine, which was isolated in 1820 from the bark of Cinchina trees in South America. Chloroquine was first synthesized in Germany and introduced at the end of World War II as a potent antimalarial. It remained the drug of choice for 40 years (Sherman, 1998). When resistance emerged after only ten years of use, the development of drugs such as mefloquine,

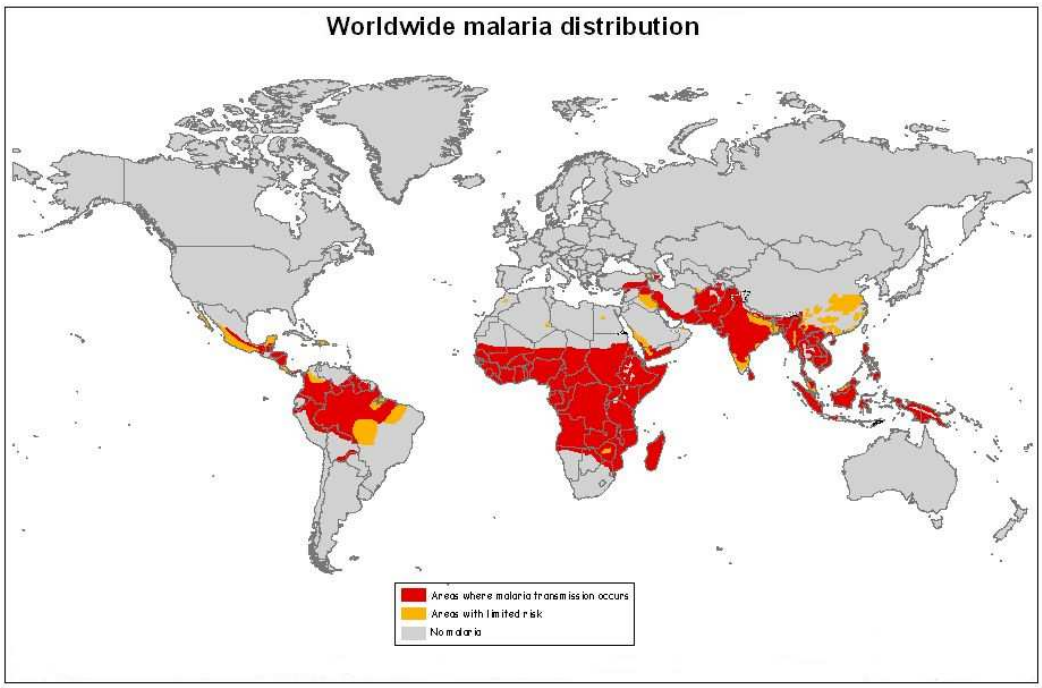

Figure 1.3: The distribution of malaria in 2002. Red shading indicates areas of malaria transmission, yellow shading indicates areas with a limited risk. Source: http://www.med.sc.edu:85/parasitology/blood-proto.htm.

sulfadoxine-pyrimethamine and artemisin derivatives were initiated. When resistance to some of the new drugs emerged, it signalled the beginning of multidrug-resistant *P. falciparum* (Wongsrichanalai *et al.,* 2002). As soon as a new drug was introduced, resistance developed within 10-20 years or in some cases even faster (Kremsner *et al.,* 2004; Table 1.1).

The emergence of multidrug-resistant parasites has complicated the treatment of malaria (Figure 1.4). Chloroquine resistance is now common in South America, Africa and southeast Asia (Sherman 1998). Sulfadoxine-pyrimethamine resistant parasites now occur in Gabon (Mawili-Mboumba *et al.,* 2001), Tanzania (Mutabingwa *et al.,* 2001), Kenya (Nzila *et al.,* 2000), Vietnam (Masimirembwa *et al.,* 1999), Brazil (Vasconcelos *et al.,* 2000), South Africa (Roper *et al.,* 2003) the Middle East (Wang *et al.,* 1997) and Thailand (Wongsrichanalai *et al.,* 2002). Mefloquine resistance occurs in Thailand and Africa (Wongsrichanalai *et al.,* 2002). Decreased quinine sensitivity occurs in South America and Africa, artesunate resistance in Thailand and dihydroartemisinin resistance in Africa (Wongsrichanalai *et al.,* 2002).

The cause of sulfadoxine-pyrimethamine resistance has been elucidated and is due to point mutations in the dihydropteroate synthase (DHPS) and DHFR enzymes, respectively (Triglia *et al.,* 1997; Yuvaniyama *et al.*, 2003). In DHFR up to four mutations confer resistance against pyrimethamine. These mutations include Ala16Val, Asn51Ile, Cys59Arg, Ser108Asn and Ile164Leu. Combinations of these mutations occur widely as well as in combination with mutations in DHPS. In DHPS there are five known resistance causing mutations which confer resistance to sulfadoxine. These are Ser436Ala/Phe,

Table 1.1: Drug introduction and resistance in malaria. Note the short times between the first introduction of the drug and emergence of resistance. Adapted from Wongsrichanalai *et al.*, 2002.

Antimalarial drug	Introduced	First reported resistance
Quinine	1632	1910
Chloroquine	1945	1957
Proguanil	1948	1949
Sulfadoxine-pyrimethamine	1967	1967
Mefloquine	1977	1977
Atovaquone	1996	1996

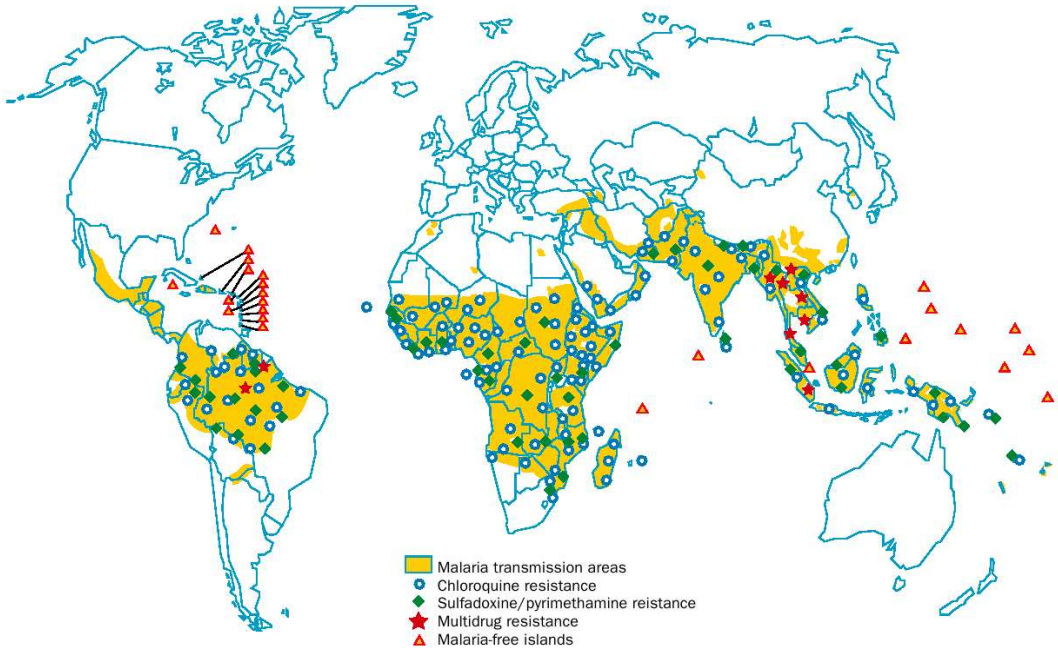

Figure 1.4: The occurrence of drug-resistant *P. falciparum*. Adapted from Wongsrichanalai *et al.*, 2002.

Ala437Gly, Lys540Glu, Ala581Gly and Ala613Ser/Thr. Up to four mutations have been observed in one genotype and these can combine with up to four mutations in the DHFR gene. This makes sulfadoxine-pyrimethamine treatment virtually useless in resistant genotypes (Wongsrichanalai *et al.*, 2002).

Triglia *et al.* (1994) established that gene amplification was not responsible for sulfadoxine resistance. Recently Nirmalan *et al.* (2004) showed that the antifolate drug target dihydrofolate reductase-thymidilate synthase (DHFR-TS) expression is upregulated in parasites when put under selective pressure by challenging the TS portion of DHFR-TS with fluoro-substituted bases. A factor which prevents antifolates from being a dependable treatment, is the occurrence of folate salvage and transport from the host by the *P. falciparum* parasite. Wang *et al.* (2004) showed that *P. falciparum* parasites have the ability to salvage pre-formed folate from the host or from growth medium. This reduces the effectiveness of

any antifolate drug as the folate pathway is circumvented. An ideal antifolate drug would block vital folate-metabolizing enzymes as well as block the transport of folate by the parasite.

The wide occurrence of resistance against the classic folate metabolism targets spurred investigations into alternative malaria targets. The ultimate solution to the malaria pandemic would be the development of a vaccine. This approach is being hampered by the lack of interest from industry and the lack of support for non-European affiliated countries. Current malaria vaccine targets include merozoite surface protein 1 and 3 and glutamine-rich protein (Kilama, 2003). Organisms that elicit immunity after a single infection are ideal for vaccine development. Unfortunately, in the case of malaria, partial immunity only develops after several years of exposure. This has been ascribed to antigenic polymorphism, antigenic switching and poor antigen immunogenicity. Polymorphisms in *P. falciparum* occur through at least three mechanisms, namely insertions/deletions of repeating units, point mutations and intragenic recombination. In MSP-1 polymorphisms have been attributed to the insertion of different numbers of a 9 bp tandem repeat into a region known as Block 2 in the gene. The insertion of these repeats coupled with intragenic recombination enables the parasite to continually change the antigenic epitopes and thus evade the host's immune system (Sakihama *et al.,* 2004; Ferreira *et al.,* 2003). Good (2005) suggested that a malaria vaccine should incorporate multiple antigens to elicit a maximal response. An alternative approach is using whole organisms to elicit an immune response (Good, 2005). One malarial vaccine used merozoite surface protein 1 and 2 (MSP-1 and MSP-2) as antigens (Genton *et al.,* 2002) but this was improved by including ring-infected erythrocyte surface antigen (RESA), MSP-1 and MSP-2 (Genton *et al.,* 2003). The studies showed an increase in the humoral response against the three antigens and shows promise as a potential vaccine against malaria.

A recently proposed drug-target is the hexose transporter of *P. falciparum.* The intraerythrocytic stages of *P. falciparum* are completely dependent on a glucose supply from the host which is taken up by a facilitative hexose transporter. The blocking of this transporter by long-chain *O*-3-hexose derivatives in cultured medium, resulted in the death of *P. falciparum* parasites. These long-chain *O*-3-hexose derivatives represent a potential new broad spectrum drug against different *Plasmodium spp.* (Joët *et al.*, 2004). Mitamura *et al.* (2003) presented the lipid metabolism of *P. falciparum* as a potential new drug target. Various approaches have now verified the existence of several *de novo* lipid synthesising pathways (glycerophospholipids, fatty acids and isoprenoids) and their study should present a few new targets in the fight against malaria (Joët *et al.,* 2003). One of the most potent antimalarials is Artemisinin which targets the sarco/endoplasmic reticulum Ca^{2+}-ATPase of *P. falciparum.* Derivatives of Artemisinins could provide a potent new antimalarial (Eckstein-Ludwig *et al.,* 2003). Vaidya (2004) highlights the vital importance of the mitochondrial and plastid genomes in the metabolism of *P. falciparum.* The mitochondrion and the plastid are vital to the parasite as their interruption was shown to be fatal for

the parasite. By studying the processes regulated by the genes of the mitochodrion and plastid genomes, new drug targets could also be identified (Vaidya, 2004).

The next section will focus on folate metabolism and the antifolates as well as their targets in *P. falciparum*.

1.3. Folates

1.3.1. Folate metabolism

Various processes in the cell require different co-factors. One of the most important co-factors is folate. Folate and its derivatives perform various functions in the cell: (1) it donates a methyl group during the conversion of homocysteine to methionine, (2) accepts a methyl group during the conversion of serine to glycine, (3) donates a methylene group to assist in synthesis of thymidilate, (4) tetrahydrofolate accepts a formimino group during the production of glutamic acid from formiminoglutamic acid, (5) donates a methyl group during the synthesis of pyrimidines and (6) can generate formate (Gilman *et al.,* 2001). DNA replication also demands pyrimidines and purines. Folate metabolism can provide the pyrimidines needed for replication.

The human host cannot produce folate and thus it is a vital ingredient in human diets. For the parasite to survive and reproduce it needs folate. Folate precursors are produced in the parasite by combining guanine triphosphate (GTP), *p*-aminobenzoic acid and glutamate (Figure 1.5). Dihydropteroate synthase (DHPS) is involved in producing 7,8-dihydropteroate, which in turn is converted to dihydrofolate via dihydrofolate synthase (DHFS). Dihydrofolate is reduced to tetrahydrofolic acid via the dihydrofolate reductase (DHFR) enzyme. Tetrahydrofolic acid is then methylated by serine hydroxymethyl transferase. The resulting methyltetrahydrofolate is used as a methyl donor in the *de novo* synthesis of pyrimidines. Methyltetrahydrofolate transfers its methyl group to deoxyuracil monophosphate to form deoxythymine monophosphate via thymidylate synthase (TS) (Gilman *et al.,* 2001).

In the *Plasmodium* species DHPS is a bifunctional enzyme together with hydroxymethyldihydropteridine pyrophosphokinase (PPPK), the enzyme which produces the substrate for DHPS (Triglia *et al.,* 1994). DHFR and TS are also linked in a bifunctional enzyme complex (Inselburg *et al.,* 1988) as well as DHFS and tetrahydrofolylpolyglutamate synthase (FPGS) (Salcedo *et al.,* 2001).

As folate is a vital ingredient for survival, a shutdown of the pathway would lead to parasite death. Various drugs target the folate pathway and these will be discussed in the next section. One of the problems with targeting the folate metabolism in *P. falciparum* is the existence of a folate salvage

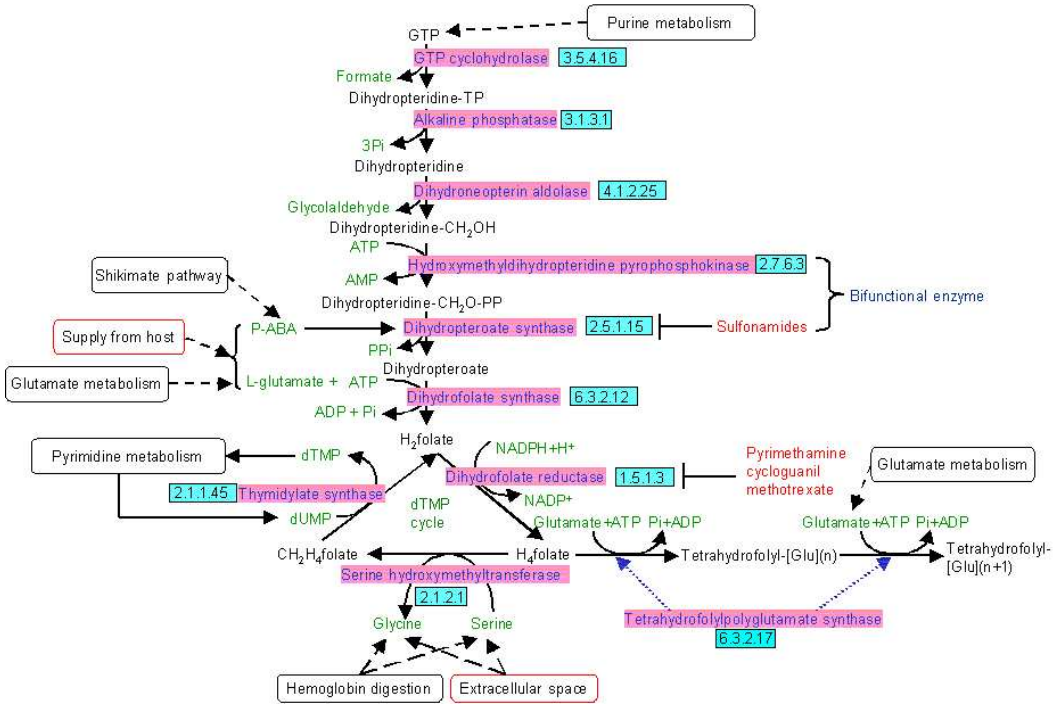

Figure 1.5: Folate metabolism in *P. falciparum*. Text in red indicates currently-used drugs and their targets in malaria. Source: http://sites.huji.ac.il/malaria.

pathway. This pathway can import folate from the medium around it (in the case of *P. falciparum*, from the erythrocyte) and thus, to a certain extent, circumvent the effect of anti-folates (Wang *et al.*, 2004).

1.3.2. Antifolates

Antifolates target certain enzymes in the folate pathway with the intention of interrupting the pathway and preventing the formation of vital compounds. Olliaro (2001) has divided the antifolates into two groups, Type-1 and Type-2. Type-1 antifolates include sulfone and sulfonamide drugs which mimic *p*-aminobenzoic acid. These drugs mainly target the DHPS enzyme and include drugs such as sulfadoxine and dapsone. Type-2 antifolates inhibit the DHFR enzyme and include drugs such as pyrimethamine, quinazolines and triazine metabolites (Olliaro 2001). The structures of the most widely used antifolates are shown in Figure 1.6.

Type-1 drugs block the synthesis of 7,8-dihydropteroate, a vital precursor in *de novo* folate synthesis, by mimicking *p*-aminobenzoic acid, a substrate of DHPS. Type-2 drugs prevent the reduction of H_2folate to H_4folate by DHFR and thus prevent the recycling of vital folate derivatives. Sulfadoxine, the most widely used antifolate type-1 drug, is usually given in combination with pyrimethamine (a type-2 drug) and is commercially known as Fansidar (Kasekarn *et al.*, 2004). One of the drawbacks of the sulfadoxine/pyrimethamine combination is the rapid emergence of resistance to both compounds. As mentioned in section 1.2 resistance to sulfadoxine and pyrimethamine is due to mutations in DHPS and DHFR,

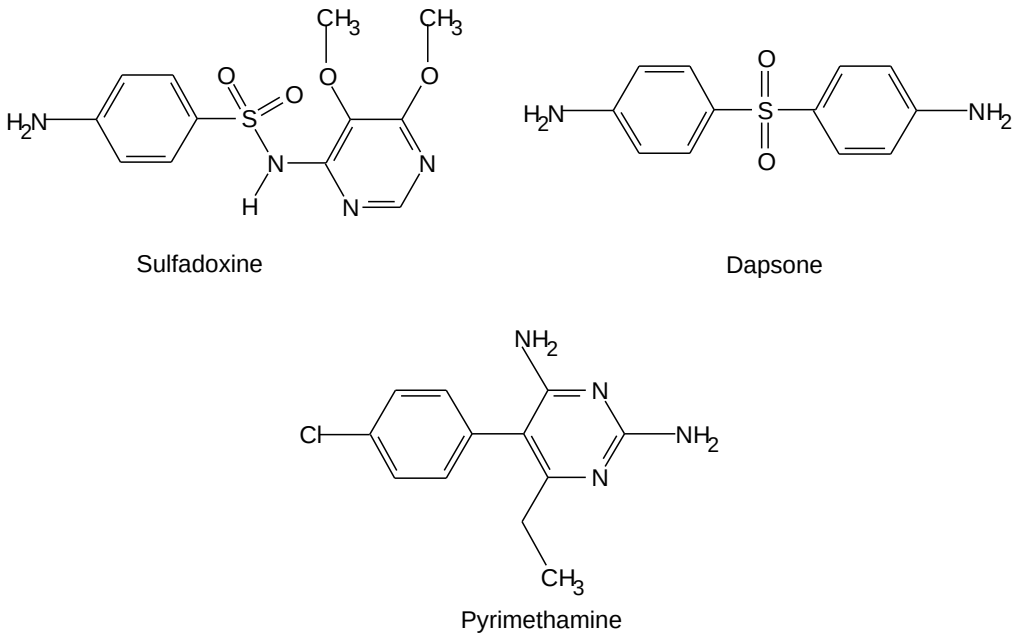

Figure 1.6: The most widely used antifolates in the treatment and prevention of malaria (Kasekarn *et al.*, 2004).

respectively. The next two sections will focus on the general structures, characteristics and mechanisms of action of PPPK and DHPS.

1.4. Dihydropteroate synthase (DHPS)

1.4.1. Structure of *P. falciparum* DHPS

The PfDHPS sequence was published in 1994 (Triglia *et al.*, 1994) and revealed that it was a bifunctional enzyme together with PPPK. The PfPPPK-DHPS enzyme complex consists of 706 amino acids with the PPPK enzyme (378 amino acids) occurring first in the sequence followed by the DHPS enzyme (328 amino acids). Triglia *et al.* (1994) localized PfPPPK-DHPS to a single copy on chromosome 8 of the *P. falciparum* genome. PfDHPS also contains an intron in the sequence (120bp in length), which contains conserved malarial splice acceptor and donor sites. PPPK-DHPS expression appears to happen throughout the life cycle of the parasite but peaks during the trophozoite stage. From immunoblots the size of PfPPPK-DHPS appears to be 68kDa (the size deduced from the amino acid sequence is 83kDa) but native enzyme isolation points to a size between 190-250 kDa, indicating that PfPPPK-DHPS may occur as a multimeric enzyme (Triglia *et al.*, 1994).

DHPS assumes the Triosephosphate Isomerase fold (TIM-barrel fold) in all known X-ray structures and thus it is realistic to expect that PfDHPS also occurs as a TIM-barrel. The next section will discuss the TIM-barrel fold.

1.4.1.1. The TIM-barrel fold

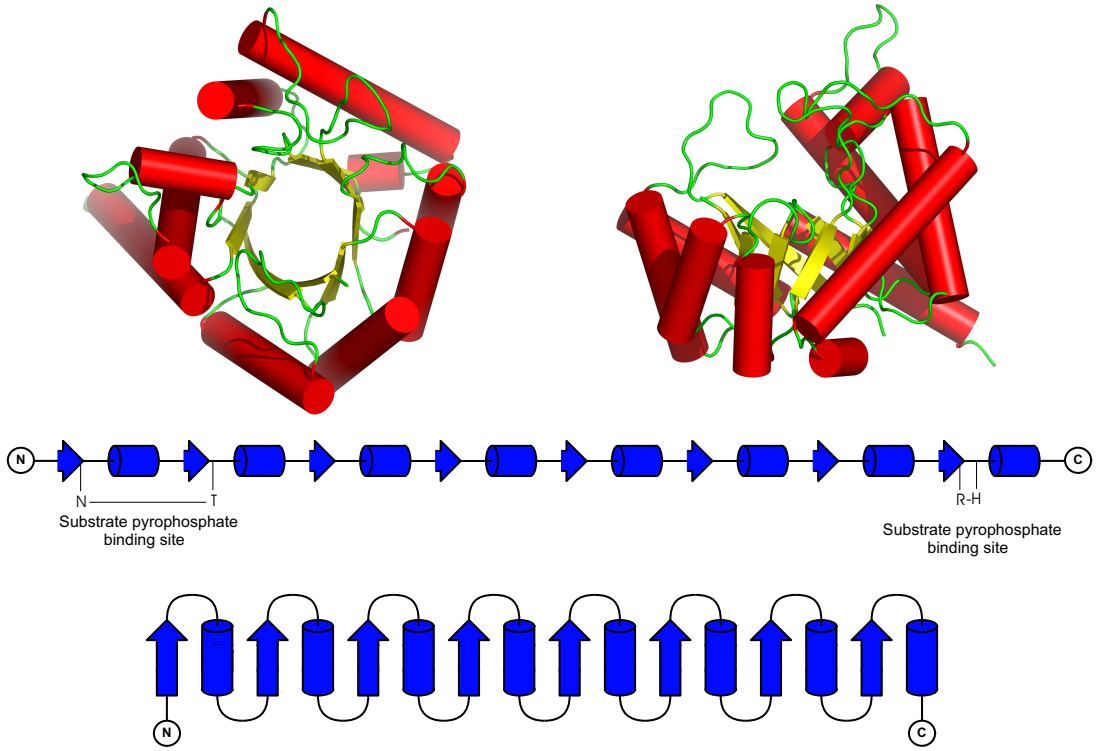

Figure 1.7: The structure of the TIM-barrel fold. Yellow indicates β-sheets, red indicates α-helices and green indicates the loops between the secondary structural elements. The middle figure shows a linear representation of the order in which the secondary structural elements follow one another and the bottom figure shows the orientation of the secondary structures in a TIM-barrel enzyme. Produced with PYMOL (DeLano, 2002; http://www.pymol.org) and TopDraw (Bond, 2003).

The TIM-barrel (β/α barrel) fold is one of the most common folds in the protein world and serves as a scaffold for various enzymatic functions. A search of the CATH database (Orengo *et al.*, 1997) revealed that at least 1870 proteins match the TIM-barrel topology (five of the six EC families are represented). Nagano *et al.* (2002) did an extensive review of TIM-barrel enzymes and found 21 homologous superfamilies and 76 families related by sequence. The TIM-barrel proteins almost always occur as enzymes (in contrast to structural proteins) and the co-factor/substrate usually includes a phosphate moiety. The active site always occurs at the C-terminal end of the barrel sheets and some members of the family exibit a structurally conserved phosphate binding site. The basic TIM-barrel topology (Figure 1.7) consists of 8 β-sheets and 8 α-helices, which fold into a barrel structure with the α-helices on the outside and the β-sheets on the inside (Nagano *et al.*, 2002). The barrels may also contain 7 or 9 β-sheets with secondary structural elements being numbered from the N-terminal end. TIM-barrels usually contain around 250 residues but may contain as little as 200 residues (Traut *et al.*, 2000). TIM-barrels also have a unique property in that the β-sheets usually exhibit a complete ring of hydrogen bonds (Wierenga, 2001). Wierenga (2001) also observed that the loops at the C-terminal end of the sheets are usually longer than those at the N-terminal end of the sheets and could thus contribute to the active site specificity.

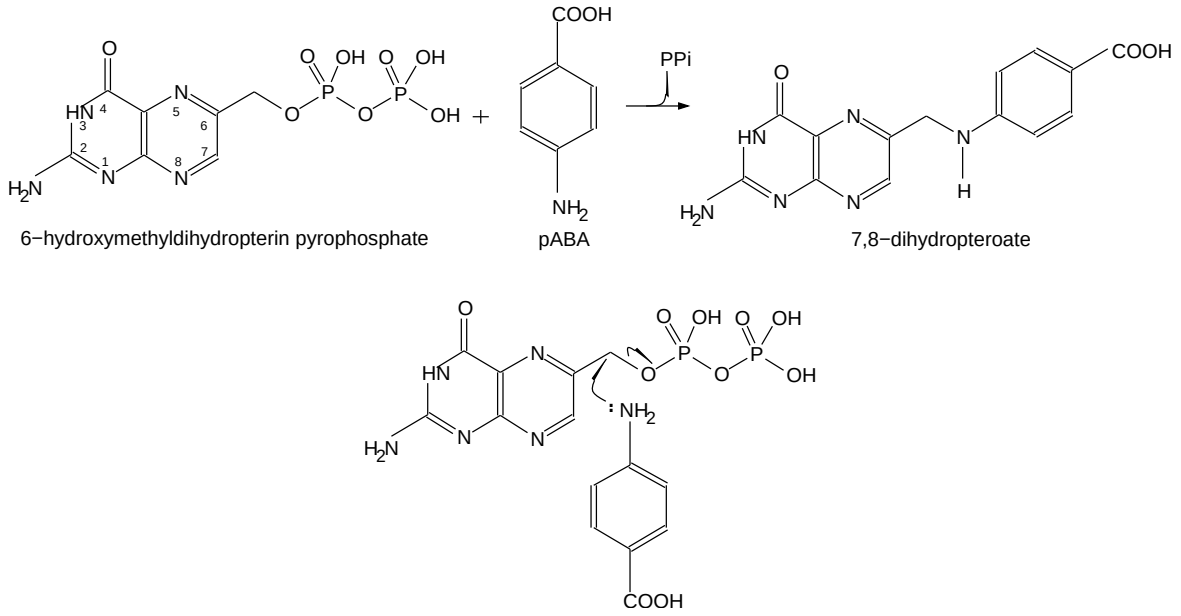

Figure 1.8: The reaction pathway of DHPS. Top: 6-hydroxymethyldihydropterin pyrophosphate (DHP) and *p*ABA condense to form 7,8-dihydropteroate with the production of one pyrophosphate group. Bottom: The mechanism of DHP and *p*ABA condensation (Baboaglu *et al.*, 2004).

The wide variety of TIM-barrel proteins led to the assumption that all TIM-barrels are evolutionary related. Lang *et al.* (2000) proposed that the original gene only coded for a half β/α barrel. This ancestral gene then underwent gene duplication and fusion to create a full β/α barrel, which underwent further gene duplication as well as functional and structural adaptations, resulting in a wide range of activities and functions. Copley *et al.* (2000) provides "statistically reliable evidence", which indicates that at least 12 of the 23 barrel superfamilies share a common origin. This data also supports the enzyme recruitment theory, which states that enzymes can be recruited between pathways, as well as supporting the idea that key metabolite-binding enzymes served as common ancestors for metabolic pathway evolution. The work done by Copley *et al.* (2000) also shows how one scaffold can adapt and fulfill various functions.

DHPS in *P. falciparum* exibits a conserved phosphate binding site and the substrate includes a phosphate group. This is consistent with the results for the DHPS family found by Nagano *et al.* (2002). The next section will discuss the function and mechanism behind *P. falciparum* DHPS.

1.4.2. Function and Mechanism of DHPS

The function of DHPS is to provide a precursor for the synthesis of folate (see section 1.3.1). Under the Enzyme Commission (EC) classification DHPS falls in subclass 2.5.1.15 (IUBMB 1992). This subclass of enzymes are involved in the transfer of alkyl or aryl groups, other than methyl groups. The mechanism of action of DHPS is based on a condensation reaction between *p*-aminobenzoic acid and 6-hydroxymethyldihydro-pterin pyrophosphate to form dihydropteroic acid (Figure 1.8) with a Mg^{2+} ion

as co-factor. The condensation reaction occurs through a SN_2 nucleophilic attack of the nitrogen on the carbon and the release of a pyrophosphate group. This reaction produces dihydropteroic acid which is used by dihydrofolate synthase to produce H_2 folate (Baboaglu *et al.*, 2004).

1.5. Hydroxymethyldihydropteridine pyrophosphokinase (PPPK)

1.5.1. Structure of *P. falciparum* PPPK

P. falciparum PPPK is part of the bifunctional PPPK-DHPS complex and contains one intron of 194 bp in length (Triglia *et al.*, 1994). PfPPPK consists of 378 amino acids and has two inserts when compared to other PPPK enzymes. Triglia *et al.* (1994) has shown that these inserts are not introns as cDNA amplification of the flanking areas correspond to the genomic DNA sequence. PPPK is connected to DHPS through a hinge region but this hinge region shows no homology to the only other known bifunctional PPPK-DHPS enzyme of *P. carinii* (Triglia *et al.*, 1994). From homology to other PPPK genes it is evident that PfPPPK folds into a Ferrodoxin-like fold (Figure 1.9; PPPK is also known as HPPK; Blaszczyk *et el.*, 2000; Hennig *et al.*, 1999; Stammers *et al.*, 1999). This will be discussed in the next section.

1.5.1.1. The Ferrodoxin-like fold

The Ferrodoxin-like fold falls in the alpha and beta class of proteins in SCOP (Hubbard *et al.*, 1999). It is described as an α/β sandwich with an antiparallel β-sheet. The Ferrodoxin-like fold consists of 5 α-helices and 4 β-sheets. The enzyme folds into a four stranded anti-parallel β-plate flanked on one side by 2 α-helices and on the other side by 3 α-helices. The secondary structures assumes a $\beta_1\alpha_1\beta_2\beta_3\alpha_2\beta_4\alpha_3\alpha_4\alpha_5$ arrangement. The $\beta\alpha\beta\beta\alpha\beta$-motif is common to other nucleoside diphosphate kinases but PPPK contains an extra three α-helices (Stammers *et al.*, 1999). Nucleoside kinases usually bind ADP between the $\alpha_2 - \beta_4$ and the $\beta_2 - \beta_3$ interconnecting loops but PPPK binds ATP between the connecting loops of $\alpha_2 - \beta_4$ and the initial loop of the C-terminal region (Stammers *et al.*, 1999). The next section will describe the mechanism behind PPPK.

1.5.2. Function and Mechanism of PPPK

According to the Enzyme Commission (EC) classification, PPPK falls under subclass 2.7.6.3. This subclass is classified as diphosphotransferases (IUBMB 1992). PPPK catalyses the transfer of a pyrophosphate group from ATP to 6-hydroxymethyl-7,8-dihydropterin (HP) to form DHP (Figure 1.10; Blaszczyk *et al.*, 2000). DHP is used by DHPS to form pteroic acid, a precursor to folate. The PPPK reaction

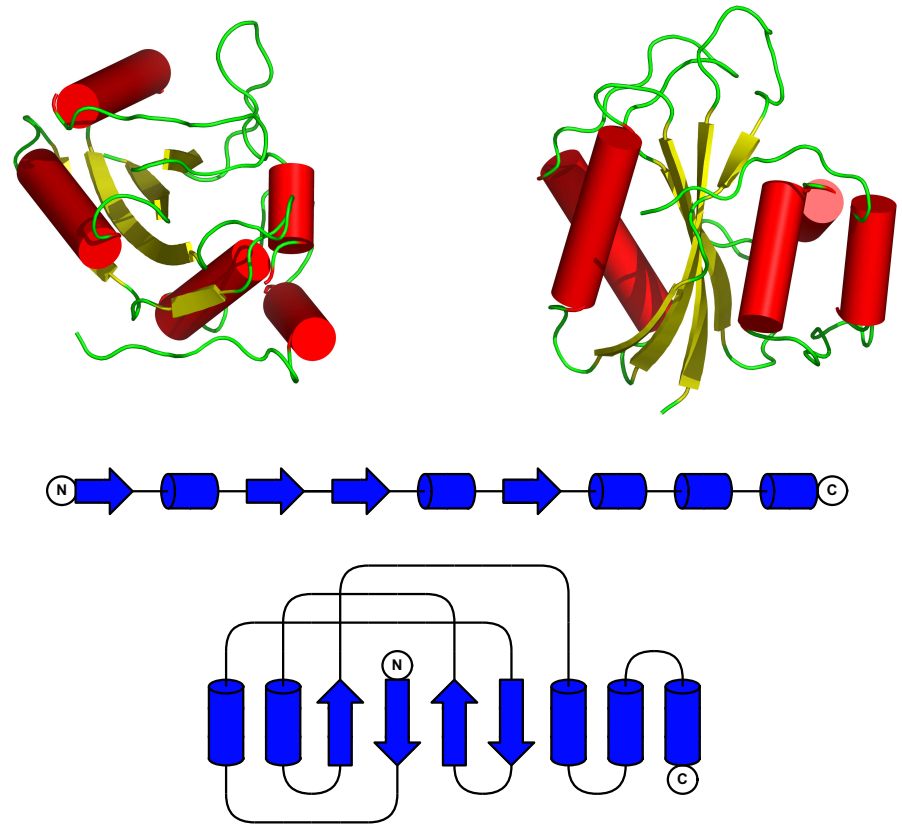

Figure 1.9: Top: The structure of the Ferrodoxin-like fold. The middle figure indicates the sequence in which the secondary elements follow one another. The bottom figure indicates the topology of the Ferrodoxin-like fold. Produced with PYMOL (DeLano 2002, http://www.pymol.org) and TopDraw (Bond 2003).

has two Mg^{2+} ions as co-factors, which helps to anchor the pyrophosphate groups during catalysis. The reaction occurs in a tightly closed enzyme with loops 1-3 closing over the active site. Loop 2 and 3 seems to close over the area where the two catalytic Mg^{2+} ions are located. In the closed structure the three loops associate with one another through a network of hydrogen bonds, which stabilises the interaction between the loops (Blaszczyk *et al.*, 2000).

1.6. Aims

The PfPPPK-DHPS complex contains one of the enzymes against which anti-folate resistance has developed. In order to investigate the mechanism behind sulfa-drug resistance a structural model of the complex is needed. Credible models should contain a reliable representation of the active sites and all the residues involved in resistance. Such models should aid explanations of the effect of resistance-causing mutations on substrate binding, drug binding and protein movement. To gain a more complete understanding of the PfPPPK-DHPS complex, the active sites as well as the interaction between the proteins and their natural substrates need to be investigated to reveal any possible similarity in ligand binding. To achieve these aims the following strategy was implemented:

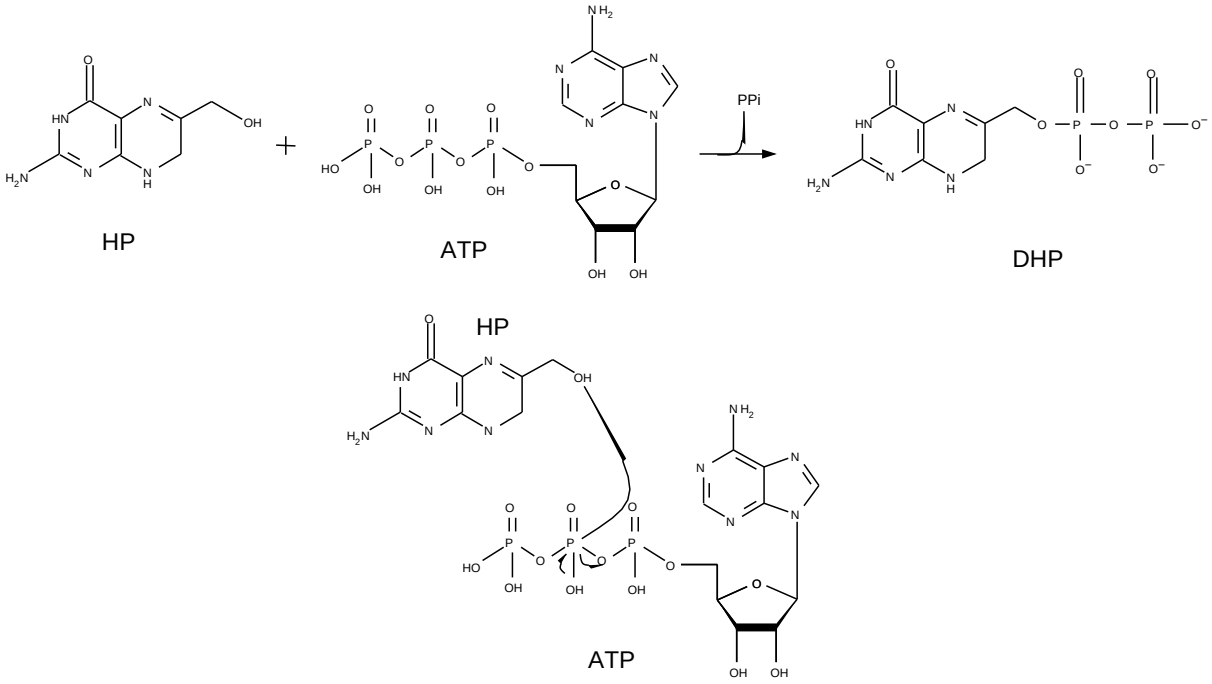

Figure 1.10: Top: The reaction pathway of PPPK. Bottom: The mechanism of PPi transfer from ATP to 6-hydroxymethyl-7,8-dihydropterin (HP) by PPPK as proposed by Blaszczyk *et al.* (2000).

- Homology models of *P. falciparum* PPPK and DHPS were constructed separately to investigate the structural characteristics of the two enzymes (**Chapter 2**).
- The natural substrates were docked into the active site of PfDHPS to investigate the interaction between PfDHPS and the substrates. The interactions between sulfa-drug resistant strains and the natural substrates were also investigated (**Chapter 2**).
- Sulfadoxine was docked into the active site of wild type DHPS, as well as the different resistant strains to investigate the mechanism behind sulfa-drug interaction in PfDHPS (**Chapter 2**).
- The effects of the mutations on the binding and movement of the substrates as well as on sulfa-drugs in the PfDHPS active site, were investigated with the use of Molecular Dynamics (**Chapter 3**).
- The extent of loop movement in PfDHPS and PfPPPK, as well as the effect of resistance-causing mutations on these loop movements, were investigated with Molecular Dynamics (**Chapter 3**).

Chapter 4 will provide a brief summary as well some concluding remarks.