MUTAGENESIS OF *PLASMODIUM FALCIPARUM* DIHYDROFOLATE REDUCTASE: LINKAGES BETWEEN ANTI-FOLATE RESISTANCE AND MUTATION AT RESIDUE 108

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ชื่อวิทยานิพนธ์	ความสัมพันธ์ระหว่างการกลายพันธุ์ของกรดอะมิโนที่ตำแหน่ง 108 ในเอ็นไซม์ไดไฮโดรโฟเลต รีดักเทส ของเชื้อ พลาสโมเดียม ฟัลซิปารั่ม และการดื้อยาแอนตี้โฟเลต
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บทคัดย่อ

จากหลักฐานข้อมูลการศึกษาที่ผ่านมา ได้บ่งชี้ถึงความสัมพันธ์ระหว่างการดื้อยาแอนตี้-โฟเลต และการกลายพันธุ์ที่ตำแหน่ง 108 จากกรดอะมิโน serine เป็น asparagine ในเอ็นไซม์ไดไฮโดรโฟเลต รีดักเทส ของเชื้อมาลาเรียพลาสโมเดียม ฟัลซิปารั่ม การศึกษานี้ต้องการที่จะเข้าใจความสำคัญของกรดอะมิโน serine ที่ตำแหน่ง 108 จึงได้นำเอายีนสังเคราะห์สำหรับเอ็นไซม์ไดไฮโดรโฟเลต รีดักเทสของเชื้อพลาสโมเดียม ฟัลซิปารั่ม มาเป็นแม่แบบในการสร้างยีนกลายพันธุ์ โดยทำให้ตำแหน่งกรดอะมิโนที่ 108 ซึ่งปรกติเป็น serine ถูกทดแทนด้วยกรดอะมิโนอื่นอีก 19 ชนิด จากผลการทดลองพบว่า ยีนกลายพันธุ์ไดไฮโดรโฟเลต รีดักเทส เฉพาะสายพันธุ์ซึ่งมีกรดอะมิโนที่ตำแหน่ง 108 เป็น lysine เท่านั้นที่ไม่สามารถแสดงออก (expression) โปรตีนในเชื้อแบคทีเรียได้ ในขณะที่สายพันธุ์กลายซึ่งมีกรดอะมิโนอื่นๆ อีก 19 ชนิด ที่ตำแหน่ง 108 สามารถแสดงออกโปรตีนในแบคทีเรียในรูปของ inclusion bodies ที่มีขนาด 27 กิโลดาลตัน เมื่อนำ inclusion bodies เหล่านี้มาทำให้คืนสภาพโดยวิธีการ refolding ในบัฟเฟอร์ที่เหมาะสม

จะสามารถทำให้เอ็นไซม์กลับคืนสู่สภาพที่สามารถเร่งปฏิกริยาได้ ผลการศึกษาได้พบว่า การกลายพันธุ์ของกรดอะมิโนตำแหน่ง 108 มีผลกระทบต่อคุณสมบัติทางจลศาสตร์ของเอ็นไซม์ และทำให้การยับยั้งการทำงานของเอ็นไซม์ด้วยสารแอนตี้โฟเลต เช่น ไพริเมธามีน และไซโคลกัวนิล เปลี่ยนแปลงไป กล่าวคือ เมื่อกรดอะมิโนที่ตำแหน่ง 108 ถูกแทนด้วย threonine และ asparagine จะทำให้การทำงานของเอ็นไซม์ลดลงถึง 50% เมื่อเทียบกับเอ็นไซม์สายพันธุ์แท้ (wild-type) ที่มี serine ที่ตำแหน่ง 108 ผลการทดลองยังได้พบอีกว่า ยีนกลายพันธุ์ของเอ็นไซม์ที่มีกรดอะมิโนตำแหน่ง 108 เป็น glycine, alanine, glutamine, cysteine, valine, leucine และ methionine จะมีอัตราเร่งของเอ็นไซม์ที่ต่ำลงมาก ในขณะที่ยีนกลายพันธุ์ของเอ็นไซม์ที่มีกรดอะมิโนตำแหน่ง 108 เป็น isoleusine, arginine, proline, aspartic acid, histidine, tyrosine, phenylalanine, tryptophan และ glutamine ไม่สามารถเร่งปฏิกริยาได้เลย

จากการศึกษาคุณสมบัติทางจลศาสตร์ของเอ็นไซม์บริสุทธิ์ที่แยกจากสายพันธุ์ 4 ชนิด ที่มีกรดอะมิโนตำแหน่ง 108 เป็น glycine, cysteine, alanine และ glutamine ตลอดจนการยับยั้งเอ็นไซม์เหล่านี้ด้วยสารแอนตี้โฟเลต เช่น ไพริเมธามีน และไซโคลกัวนิล พบว่ายีนกลายพันธุ์ของเอ็นไซม์ที่มีกรดอะมิโนตำแหน่ง 108 เป็น glutamine ให้เอ็นไซม์ที่ดื้อต่อสารแอนตี้โฟเลตทั้งสองชนิดและมีค่า K_m ต่อสับสเตรทสูง ส่วนยีนกลายพันธุ์ของเอ็นไซม์ที่มีกรดอะมิโน glycine, cysteine และ alanine ที่ตำแหน่ง 108 นั้นไม่ดื้อต่อยาเลย เมื่อทำการศึกษาจลศาสตร์ของเอ็นไซม์บริสุทธิ์ที่มีกรดอะมิโน asparagine และ glutamine ที่ตำแหน่ง 108 ได้พบว่า การยับยั้งการทำงานของเอ็นไซม์ด้วยไพริเมธามีน และไซโคลกัวนิล มีลักษณะของการยับยั้งเป็นแบบแข่งขัน (competitive inhibition) เช่นเดียวกับของเอ็นไซม์ wild-type จากข้อมูลการศึกษาสามารถสรุปได้ว่า การกลายพันธุ์จากกรดอะมิโน serine เป็น asparagine ที่พบในเชื้อมาลาเรียที่ดื้อยาแอนตี้โฟเลตในธรรมชาตินั้น น่าจะเหมาะสมและเป็นประโยชน์แก่การอยู่รอดของเชื้อมาลาเรีย ทั้งนี้เนื่องจากเอ็นไซม์กลายพันธุ์ที่ได้นั้นมีเสถียรภาพ อีกทั้งมีความสามารถต้านทานต่อยาแอนตี้-โฟเลตได้ ในขณะที่การกลายพันธุ์ไปเป็นกรดอะมิโนชนิดอื่นนั้นไม่เหมาะสมที่จะเกิดขึ้นในธรรมชาติ

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ABSTRACT

The roles of residue 108 of *Plasmodium falciparum* DHFR in conferring anti-folate resistance were investigated. Mutants containing mutations at residue 108 of synthetic plasmodial DHFR were constructed by combinatorial cassette mutagenesis, a process by which the target codon is mutated to encode for all possible amino acids. With the exception of S108K, all other DHFR mutants highly expressed DHFR as inactive inclusion bodies of molecular mass about 27 kDa in *E. coli* system. Active DHFR activity could be recovered upon refolding of the inclusion bodies. Substitution of Ser108 by other amino acids affected the kinetic parameters of the enzymes and altered the inhibitory effects against pyrimethamine and cycloguanil. Mutants with Thr or Asn at residue 108 caused approximately 50% reduction in DHFR activity as compared to the wild-type enzyme. Poor

DHFR activities were observed from mutants with Gly, Ala, Gln at residue 108, and mutants with Cyc, Val, Leu and Met showed very poor DHFR activities. Mutations at residue 108 from Ser to Ile, Arg, Pro, Asp, His, Tyr, Phe, Trp, Glu yielded inactive enzyme. The mutant DHFRs from S108G, S108C, S108A and S108Q were purified to homogeneity by methotrexate-sepharose affinity chromatography. The kinetic parameters and inhibition by antifolates of the mutant enzymes were investigated. Mutant DHFR with Ser108 to Gln mutation was highly resistant to both pyrimethamine and cycloguanil with elevated K_m values for the substrates. However, other mutant DHFRs were susceptible to the drugs. Inhibition studies also revealed that the wild-type DHFR and DHFRs from S108N and S108Q mutants were competitively inhibited by pyrimethamine and cycloguanil. The data suggested that mutation at residue 108 from Ser to Asn was more preferably selected over other amino acids in nature, presumably due to its stability and ability to confer resistance to anti-folates, while substitutions with other amino acids might not be favorable or parasite survival.

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ABBREVIATIONS

A	adenine, alanine
Ala	alanine
Arg	arginine
Asn	asparagine
Asp	aspartic acid
ATP	adenine 5'-triphosphate
Bis	N,N'-methylene-bis acrylamide
bp	base pair
BSA	bovine serum albumin
C	cysteine, cytocine
°C	degree celcius
$CaCl_2$	calcium chloride
CFU	colony forming units
CH_2H_4folate	N^5 N^{10}-methylene tetrahydrofolate
cm	centrimeter
Cyc	cycloguanil
Cys	cysteine

ABBREVIATIONS (cont.)

D	glutamic acid
DHFR	dihydrofolate reductase
dTMP	2'-deoxythymidine-5'-monophosphate
DTT	dithiothreitol
dUMP	2'-deoxyuridine-5'-monophosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
E	Aspartic acid
EDTA	ethylenediamine tetraacetic acid
F	phenylalanine
G	glycine, guanosine
g	gram
Gln	glutamine
Glu	glutamic acid
Gly	glycine
GndHCl	guanidine hydrochloride (aminomethanamidine hydrochloride)

ABBREVIATIONS (cont.)

H	histidine
HCl	hydrochloric acid
H_2folate	7,8-dihydrofolate
H_4folate	5,6,7,8-tetrahydrofolate
His	histamine
h	hour
I	isoleucine
IC_{50}	50 percent inhibitory concentration
Ile	isoleucine
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
K	lysine
kb	kilobase
k_{cat}	catalytic constant
KCl	potassium chloride
kDa	kilodalton

ABBREVIATIONS (cont.)

KH_2PO_4	potassium dihydrogen phosphate
K_i	inhibitory constant
K_m	Michaelis-Menten constant
KOAc	potassium acetate
KOH	potassium hydroxide
L	litre, lysine
Leu	leucine
Lys	lysine
M	methionine, molar
Met	methionine
$MgCl_2$	magnesium chloride
mA	milliampere
mg	milligram
min	minute
ml	millilitre
mM	millimolar
mol	mole

ABBREVIATIONS (cont.)

MTX	methotrexate
N	asparagine, Normal
NaCl	sodium chloride
NADP	β-nicotinamide adenine dinucleotide phosphate
NADPH	β-nicotinamide adenine dinucleotide phosphate, reduced form
$NaHCO_3$	sodium bicarbonate
Na_2HPO_4	disodium hydrogen phosphate
NaOAc	sodium acetate
NaOH	sodium hydroxide
NH_4OAc	ammonium acetate
NH_4OH	ammonium hydroxide
nm	nanometer
nM	nanomolar
ng	nanogram
OD	optical density
P	proline

ABBREVIATIONS (cont.)

PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
pmole	picomole
pf	*Plasmodium falciparum*
Phe	phenylalanine
Pyr	pyrimethamine
Pro	proline
psi	pound per square inch
Q	glutamine
R	arginine
RNA	ribonucleic acid
RNase	ribonuclease
rpm	round per minute
S	serine
s	second
SDS	sodium dodocyl sulphate
Ser	serine

ABBREVIATIONS (cont.)

SHMT	serine hydroxymethyl transferase
T	threonine, thymine
TBE buffer	tris-borate-EDTA buffer
TE buffer	tris-EDTA buffer
TEMED	N,N,N',N'-tetramethylenediamine
TES	N-tris[hydroxymethyl] methyl-2-aminoacetate
THF	tetrahydrofolate
Thr	threonine
Tris	tris[hydroxymethyl] aminomethane
Trp	tryptophan
TS	thymidylate synthase
Tyr	tyrosine
UV	ultraviolet
V	valine, volts
Val	valine
V_i	initial velocity
V_{max}	maximum velocity

ABBREVIATIONS (cont.)

V/V	volume by volume
W	tryptophan, watt
W/V	weight by volume
X	time
Y	tyrosine
β-ME	β-mercaptoethanol
Δ	delta, difference
Σ	molar extinction coefficient
μ	micro
/	per
%	percent

CHAPTER I

INTRODUCTION

1.1 Malaria disease

Malaria is one of the six major tropical diseases chosen by the World Health Organization's Special Programme for Research and Training in Tropical disease (TDR) as a targeted disease. Malaria is caused by protozoa parasite in the genus *Plasmodium*, and is transmitted by female *Anopheles* mosquito. Of the four species of human malaria, *Plasmodium vivax* and *Plasmodium falciparum* are the two major species commonly found in human (1, 2). The incidence of malaria infection is highly dependent upon area. People in the developing countries are facing higher risk for malaria infection than those living in other areas of the world. According to WHO's statistical estimation, there are 300-500 million clinical cases of malaria per year, 90% of them are from Africa alone and around one million deaths of African children reported each year (3, 4).

During 1940s to 1950s, a program for malaria control was initiated by a joint commitment between WHO and scientists from the endemic countries with the aim to eradicate malarial disease (5). Two major approaches have been undertaken to prevent the spreading of the disease; avoiding contact with mosquitoes and the application of chemoprophylaxis. Chemical insecticides have been applied to intervene the transmission of the disease to human host *via* female anopheles mosquitoes. The bed-net program has been initiated to supplement the effectiveness of the malaria control program (6). Parallel efforts have also been devoted to the

development of novel antimalarials and vaccines for effective treatment and prevention of malarial disease (6).

Unfortunately, the above strategies for the eradication of the disease have been confronting with problems which render the chemoprophylaxis of malaria less effective. Anopheles mosquitoes developed resistance to insecticides, and malaria parasites rapidly developed resistance to antimalarials (6). Further, malaria vaccine development remains far from straightforward due to the high variation of surface antigens of malaria parasite, and therefore no single vaccine has yet been successfully launched for wild use on a global scale (7, 8). The problems are further complicated by poor public health education, especially from people in the developing countries.

1.2 Chemotherapy and drug resistance in malaria

The principle of successful chemotherapy relies mainly on the selective toxicity of the drugs against the parasites. However, host-related factors, i.e. rate of absorption, degradation, excretion etc., and the host-parasite complex interaction are all contributing to the efficacy of the drug. It is essential that adequate concentration of drug reaches the intraerythrocytic parasite and is maintained over a critical period. To further achieve the goal of malaria eradication, it also critical that the entire host population is cleared and protected from reinfection by prophylaxis (2, 9). Quinine, the main alkaloid of *Cinchona ledgeniara* (9), has been the mainstay of antimalarial drug since its first introduced at the beginning of the 17th century. Nevertheless, due to its side-effects and toxicity, the drug was replaced by other relatively nontoxic but more effective antimalarials such as mepacrine

(in 1930s) and chloroquine (in 1940s). As a result of widespread use of chloroquine for the treatment of *P. falciparum* malaria, resistance to the drug has progressively emerged shortly after the drug was introduced. Other antimalarials including mefloquine, halofantrine, Fansidar (pyrimethamine +sulfadoxine) and combination of Fansidar with mefloquine (MSP) have been used with satisfactory cure rate at the beginning, but the cure rate was gradually dropped shortly afterward. Indeed, drug resistance to these antimalarials has already been observed and reported. The most recent potent antimalarial seems to be the derivatives of artemisinin, a sesquiterpene lactone which is an active component in the extract of a Chinese herb *Artemisia annua* (10). Table 1 summarizes the important antimalarials used in the treatment of malaria.

The widespread of drug resistant *P. falciparum* has been a major problem in many endemic countries including Thailand. Resistance to quinine was reported as early as 1910 (11, 12), whereas widespread of chloroquine resistance was reported to occur in South America and the entire area of Indochina subcontinent at the end of 1950s. Currently, resistance to Fansidar is increasing in West Africa while multidrug resistant malaria is in serious situation around Thai/Cambodia and Thai/Myanmar border area. Generally, drug resistance can occur through a number of different mechanisms: reduction of drug uptake through alteration of the permeability or transport properties of the membranes; sequestration or conversion of drug to inactive derivative before reaching its target; increasing of the transcriptional rate of the gene encoding the target protein through mutation in the promoter or regulatory region of the target gene; mutation of the structural gene to yield structurally altered protein which binds less efficiently to the drug. In certain circumstances, a blocked

metabolic pathway may be bypassed by a new route of synthesis; a combination of the above phenomena may also be observed (13). Knowledge and understanding of molecular mechanisms of drug resistance should help in designing the novel inhibitors against the resistant parasites that already developed.

Table 1 : Antimalarial drugs used to treat *Plasmodium falciparum*

Cinchona alkaloids:	Quinine
	Quinidine
4-Aminoquinolines:	Chloroquine
	Amodiaquine
4-Quinoline methanols:	Mefloquine
9-Phenanthrenemethanols:	Halofantrine
Acridine derivatives:	Pyronaridine
	Quinacrine
Qinghaosu and derivatives:	Artemisinin
	Artesunate
	Arteether
	Artemeter
DHFR inhibitors:	Pyrimethamine
	Proguanil and analogs
	Cycloguanil
	Trimethoprim
Other antibacterial drugs:	Tetracyclines
	Sulfa drugs
	Clindamycin

From reference 14

1.3 Dihydrofolate reductase as drug target

Dihydrofolate reductase (DHFR; 5,6,7,8-tetrahydrofolate-NAD^+ oxidoreductase (E.C.1.5.1.3)) catalyzes the NADPH-dependent reduction of H_2folate to H_4folate, an essential precursor of folate cofactors. The enzyme is essential as it is one of the three enzymes in the thymidylate synthesis cycle involving the *de novo* biosynthesis of 2' deoxythymidylate (dTMP) for DNA synthesis.

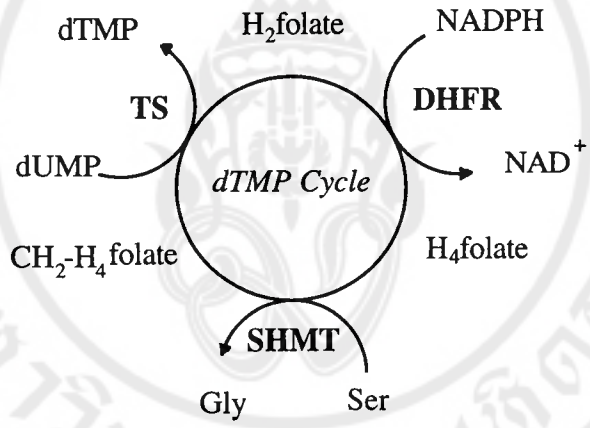


Figure 1 : dTMP synthesis cycle showing sequential reactions and metabolic relationships of DHFR, TS and SHMT (15)

Figure 1 depicts a diagrammatic representation of the sequential reactions catalyzed by the three enzymes, i.e. DHFR, TS, and SHMT. In this cycle, TS catalyzes the reductive methylation of dUMP with CH_2H_4folate to give dTMP and H_2folate. In the presence of NADPH, DHFR catalyzes the subsequent reduction of the H_2folate produced by TS to regenerate the H_4folate which will be converted by SHMT to give N^5,N^{10}-

methylenetetrahydrofolate (CH_2H_4 folate) necessary for continued dTMP synthesis (16-18).

In sources as diverse as bacteriophages, mammalian viruses, prokaryotes, fungi and vertebrates, DHFR and TS exist separately as distinct monofunctional enzymes. TS is usually a dimer of apparently identical subunits with native molecular weight ranging from 60,000 to 70,000 Da while DHFR is a monomeric protein of 18,000-22,000 Da (17, 19, 20). In many protozoa and plants, however, DHFR and TS exist on the same polypeptide as a bifunctional protein which can catalyze both reactions (21-25) with DHFR domain at the amino terminus and TS domain at the carboxy terminus. Depending upon the source, the DHFR and TS domains are separated by a junctional peptide of considerably variable length (26,27).

Pulse field gel electrophoresis analysis of *P. falciparum* chromosome indicates that the DHFR-TS gene is a single copy located on chromosome 4 (28-32). The gene is highly A+T rich (~75%) without intervening sequence (30, 33). *P. falciparum* DHFR-TS is composed of 608 amino acids with a calculated molecular mass of ~72 kDa. The protein can be divided into three separated domains; an amino-terminal DHFR domain which extends from N-terminal methionine to asparagine 218 (about 27 kDa), a junctional region extended from threonine 219 to tyrosine 322 (about 11 kDa), and a carboxyl-terminal TS domain consisting of 285 amino acids from histidine 323 to the C-terminus at residue 608 (30,33). Similar to other DHFRs thus far observed, the DHFR domain of *P. falciparum* is less conserved than the TS domain, and either DHFR or TS is more homologous to the eukaryotic than the prokaryotic enzymes. Computer prediction of the secondary structure based on the primary amino acid sequence indicates that the DHFR domain of *P. falciparum* resembles other DHFRs in gross topology

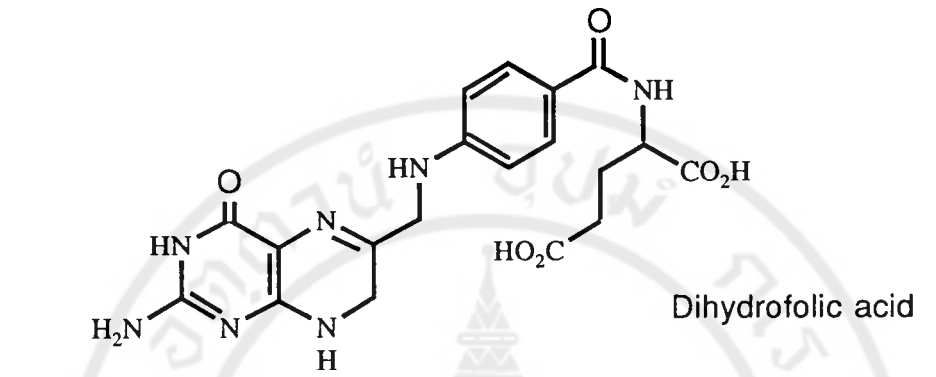
(17, 30-33); it consists of a conserved eight predominant β-strand flanked by 4 major α-helices (17), and many conserved regions are believed to participate in substrate binding (17, 30-36).

The structural difference between human and plasmodial DHFR has made DHFR an attractive target for malaria chemotherapy (37). Pyrimethamine and cycloguanil are two well-known DHFR inhibitors that have played important roles in the treatment of malaria. The effectiveness of the drug relies on the fact that it binds to parasite DHFR with much higher affinity than to the human DHFR (38-40). Figure 2 shows the chemical structure of pyrimethamine, proguanil, and cycloguanil (active metabolite of proguanil) compared to the structure of substrate H_2folate.

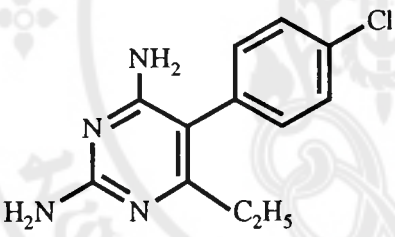
1.4 Antifolate resistance in *Plasmodium falciparum*

During the second World War, pyrimethamine and cycloguanil were the most widely used antifolates for routine malaria treatment as the drugs are relatively less toxic and thus permitting doses to be taken at much longer interval (41). Unfortunately, widespread resistance to these drugs were reported soon after the introduction of these compounds. Unlike the resistance of malaria parasites to chloroquine, in which the mechanism of resistance remains unclear, the mechanism of antifolate resistance in malaria is somewhat clearer as the mode of action of the inhibitors against the parasite is well defined. In fact, evidence which came from several studies using murine parasites as experimental models (40, 41, 44-50) suggested that resistance could arise from alteration in enzymatic properties through decreasing the affinity of the enzyme for drug. Although some have

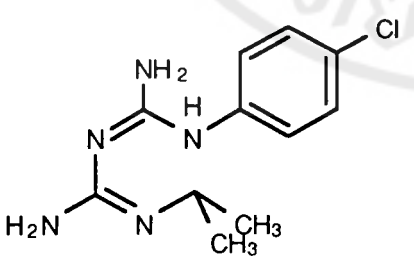
claimed to find increase in the amounts of enzyme through gene amplification (58, 59), this has never been rigorously proven.



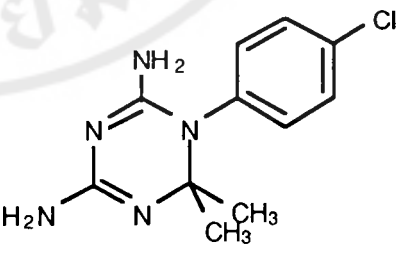
Dihydrofolic acid



Pyrimethamine



Proguanil



Cycloguanil
(active metabolite of proguanil)

Figure 2 : Structure of dihydrofolate and antifolate antimalarials

In human malaria, evidence from biochemical studies strongly suggest the structural alteration of DHFR as a possible mechanism of pyrimethamine resistance (30, 31,42, 43, 51-57); increase in K_m values for substrate (51, 52, 55, 56), and marked increase in K_i values for pyrimethamine (42, 51-53, 55-57) have been observed from enzyme obtained from pyrimethamine-resistant parasites. With the exception of Kan *et al.* (58) and Inselburg *et al.* (59), whose results showed no significant difference in K_i values for pyrimethamine between the sensitive and resistant parasites and overproduction of the target enzyme was observed. The evidence of structural alteration of DHFR is further strengthened by genetic analysis of DHFR amplified from natural isolates of different geographical origins and degrees of drug resistance. Several point mutations in the DHFR gene were observed (30-32, 53, 60-63). Alignment of the primary sequence of plasmodial DHFR with other DHFRs revealed that these residues locate within the vicinity of substrate binding site and therefore are likely to be associated with the antifolate resistant phenotype (13, 17, 31-33).

There have been a number of controversial reports on the type of inhibition of sensitive and resistant DHFRs by pyrimethamine. In *P. chabaudi*, pyrimethamine was reported to competitively inhibit the drug-sensitive DHFR whereas non-competitive inhibition was observed for the drug resistant enzyme (48). Dieckmann *et al.*, reported that pyrimethamine competitively inhibits both sensitive and resistant *P. falciparum* DHFRs (57). However, Walter *et al.*, (55) showed that pyrimethamine competitively inhibits the enzyme from sensitive parasite but non-competitively inhibits the drug-resistant DHFR, while Chen *et al.*, (56) obtained the opposite results as compared to Walter *et al.* (55). The discrepancy of the results might in part be due to the different purity of the enzyme and substrate used in the assay.

Recently, Sano *et al.*, (64) reported the competitive inhibition of pyrimethamine against the purified synthetic S108T mutant DHFR , whereas a non-competitive inhibition by pyrimethamine was observed for the purified synthetic *P. falciparum* DHFR-TS (65).

The gene encoding *P. falciparum* DHFR-TS was cloned in 1987 (33). Evidence based on analysis of the DNA sequences of DHFR from the sensitive and resistant strains of *P. falciparum* suggest that point mutations of the DHFR might be responsible for antifolate resistance in *P. falciparum*. The observation that Ser or Thr at residue 108 of pyrimethamine-sensitive DHFR was replaced by Asn in pyrimethamine-resistant enzymes is consistent from various reports (30-32, 42, 51-57, 60, 62). Further, additional mutations of Asn-51 to Ile, Cys-59 to Arg and Ile-164 to Leu have been observed in strains with higher levels of pyrimethamine resistance (30-32, 52, 53, 61-63). A double mutant with Ser-108 to Thr and Ile-164 to Met has also been reported from pyrimethamine-resistant parasite obtained by chemically induced mutation (60).

Recently, the importance of residues 51, 59, 108 and 164 in antifolate binding was further shown by using a synthetic gene (52, 64-66). The results from such study suggested that residue 108 was a critical and primary site of mutation leading to antifolate resistant phenotype, and that higher levels of antifolate resistance were sequentially acquired through mutations at residues 51, 59 and 164 (66). Evidence from analysis of Plasmodial DHFR also revealed the association of residue 16 with cycloguanil resistance. Instead of the known Ser-108 to Asn mutation found in pyrimethamine resistant parasite, a double mutation of Ser-108 to Thr and Ala-16 to Val was observed in cycloguanil-resistant parasite (28, 30, 31, 53, 61-63, 67). Interestingly, no single mutation of Ala to Val at residue 16 has

been reported in natural isolate. Based on the available evidence and the alignment of Plasmodial DHFR with DHFRs from other organisms, point mutations of the DHFR are likely to be a factor contributing to antifolate resistance in *P. falciparum*. Mutations of DHFR at other residues, i.e. Phe-223 to Ser (28, 67), Asp-54 to Asn (28, 67) and Ile-164 to Met (60) have also been reported together with Ser-108 to Thr in experimentally induced clones with pyrimethamine resistant phenotype (60).

Although the structure of *P. falciparum* DHFR-TS is not currently available and has yet to be solved, molecular modeling of the parasite enzyme based on the known DHFR structures reported from *E. coli* (36) *Lactobacillus spp.* (68, 69) and eukaryotes (70) could provide some insights into possible consequences of point mutations on antifolate resistance in *P. falciparum* (13, 17). Alignment of plasmodial DHFR to other DHFRs of known structure reveals that Ser/Thr-108 in plasmodial enzyme is equivalent to Thr-45 in *L. casei*, Thr-46 in *E. coli*, and Thr-56 in eukaryotes. The residue is conserved and lined in the middle of the short C α helix which is predicted to involve substrate binding. It is therefore not surprising that mutation of plasmodial DHFR at residue 108 would affect the inhibitor binding. The Cys-59 of *P. falciparum* DHFR is analogous to Arg-31 in *L. casei*, Lys-32 in *E. coli*, and Gln-35 in eukaryotes. The residue falls in the αB helix region, a face of which forms a part of the substrate binding pocket. The Asn-51 (analogous to Leu-23 in *L. casei*, Leu-24 in *E. coli*, and Leu-27 in eukaryotes) was predicted to lie at the amino-terminal extremity of the αB helix, while Ile-164 and Ala-16 are located in the A-β strand and E-β strand, respectively. The fact that these amino acids are conserved and locate in the vicinity of the active cavity of the enzyme, mutation of these residues might therefore directly or indirectly affect the binding of the enzyme to either

substrate or inhibitors (13, 17, 30-33). Other point mutations of *P. falciparum* DHFR, i.e. Asp-54 and Phe 223, were also reported. The residue Asp-54 of *P. falciparum* DHFR is analogous to Asp-27 of *E. coli* DHFR (71). Mutation of Asp-57 to Asn in *E. coli* DHFR causes severe depression of DHFR activity due to the critical role of this residue in substrate binding and catalysis (13, 17, 28, 67).

Recently, the linkages between point mutation(s) and antifolate-resistance in malaria were investigated, the results of which were summarized in Table 2 (28, 30, 31, 32, 53, 60, 61, 63, 66, 67). It is noteworthy from this study that the DHFR from all naturally occurring antifolate resistant parasites contains Ser to Asn mutation at residue 108. The question can therefore be raised as to why nature has to select Ser to Asn mutation at residue 108 for acquiring drug-resistant phenotype, and whether or not other amino acids at this residue could contribute to drug-resistance or affect the survival of the parasite. To address the above questions and to further understand the molecular level of the linkages between mutation at residue 108 of *P. falciparum* DHFR and antifolate resistance, a synthetic gene of *P. falciparum* DHFR described previously (52) will be exploited. Residue 108 of the synthetic DHFR gene will be replaced by all possible amino acids through cassette mutagenesis, and the kinetic and inhibition by antifolates of the mutant enzymes will be investigated. The information gained from such investigation would help explaining the role(s) of residue 108 in contributing to the stability of the mutant enzymes and the resistance to antifolate. A clearer explanation on the role of residue 108 could be obtained once the X-ray crystallographic structure of the enzyme is solved, and could lead to the design of new

effective antifolate with low propensity for the development of drug resistance.

Table 2 : Amino acid change in DHFR domain of *P. falciparum* : Correlation with drug resistance

Parasite strains	Drug susceptibility		Amino acid residue							References
	Pyr	Cyc	16	51	54	59	108	164	223	
3D7, SL/D6, FC27	S	S	A	N	D	C	S	I	F	30, 31, 53, 61
HB-3, Hond1, PA2, Camp	R^{+}	R^{+}	A	N	D	C	**N**	I	F	28, 30, 31, 53, 61, 63
N51I*	S	R^{+}	A	**I**	D	C	S	I	F	66
7G8, It.D12	R^{++}	R^{++}	A	**I**	D	C	**N**	I	F	30, 31, 53, 61, 63
C59R*	R^{+}	R^{++}	A	N	D	**R**	S	I	F	66
V-1, K-1	R^{++}	R^{++}	A	N	D	**R**	**N**	I	F	30, 32, 61
W2, Dd2	R^{++}	R^{++}	A	**I**	D	**R**	**N**	I	F	31
I164L*	S	R^{+}	A	N	D	C	S	**L**	F	66
CsI-2	R^{+++}	R^{+++}	A	N	D	**R**	**N**	**L**	F	30, 61
V1/S	R^{++++}	R^{++++}	A	**I**	D	**R**	**N**	**L**	F	63
A16V*	S	R^{+++}	**V**	N	D	C	S	I	F	66
FCR3, UPA, FAC8, FCB, It.G2F6	S	R^{++++}	**V**	N	D	C	**T**	I	F	28, 30, 31, 53, 61, 63
T9/94/300.300	R^{+}	nr	A	N	D	C	**T**	**M**	F	60
FCR3/D4-D7** FCR3/D8 (ii)**	R^{++}	nr	**V**	N	D	C	**T**	I	**S**	28
FCR3/D8 (i)**	R^{++}	nr	**V**	N	**N**	C	**T**	I	**S**	28, 67

Drug susceptibility varied among laboratories. Degree of drug resistance was arbitrarily defined by the numbers of plus symbol.

nr : no report

* : Synthetic pfDHFR mutants which have not been found in the nature.

** : Mutants obtained by chemical induction.

Bold letters are the amino acid residues mutated.

CHAPTER II

OBJECTIVE

Analysis of the gene encoding DHFR from a variety of *P. falciparum* with different geographical origins and degrees of drug resistance had led to the identification of point mutations which are potentially associated with antifolate resistance in malaria. Mutation at amino acid residue 108, from Ser (AGC) to Asn (ACC), is believed to be the primary event which, along with other point mutations, leads to higher levels of antifolate resistance in malaria. In order to further understand the molecular basis of antifolate resistance with regard to the mutation at residue 108, an approach was undertaken to generate mutants having all possible amino acids at residue 108 using the synthetic DHFR gene earlier made in our laboratory and combinatorial cassette mutagenesis. The mutant DHFRs were expressed, purified and the kinetic properties as well as the inhibition of the enzymes by antifolates, i.e. pyrimethamine (Pyr) and cycloguanil (Cyc) were investigated. The knowledge resulting from this study should lead to better understanding of linkages of residue 108 to antifolate resistance in malaria and suggest approaches to drug development effective against drug resistant parasites.

CHAPTER III

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Synthetic DHFR Gene of *Plasmodium falciparum* (pfDHFR)

Using combinatorial cassette mutagenesis, the synthetic gene encoding *Plasmodium falciparum* DHFR was mutated to generate mutants with all possible amino acids at residue 108 of the DHFR domain. The mutagenesis and manipulation of the gene were all performed in pUC-based vector. The list of mutants showing codons, amino acids at residue 108, and name of constructs were summarized in Table 3.

3.1.2 Bacterial Strains

Escherichia coli DH5α (F^-ϕ80d*lac* ZΔM15Δ (*lac* ZYA-*arg* F) U169 *deo* R *rec* A *end* A1 *hsd* R17 (r_K^-, m_K^+) *sup* E44λ^-*thi* -1 *gyr* A96 *rel* A1 was obtained from Life Technology, Inc. and was generally used as host cell for gene manipulation and amplification of recombinant plasmid pUC-pfDHFR.

Escherichia coli BL21(DE3)pLysS (F^- *hsd* S_B (r_B-, M_B-) *omp* T *gal* (λ*c It s* 857 *ind* 1 *Sam* 7 *nin* 5 *lac* UV5-T7 gene 1 pLysS) was obtained from Novagen Madison, WI. and was used as expression host for gene constructed in pET vector series.

Table 3: List of mutants with residue 108 mutations

No.	Amino acid at residue 108	Codon usage	Name of constructs
1	Serine	AGC	Wild type
2	Asparagine	AAC	S108N
3	Isoleucine	ATC	pUC-pfDHFR(S108I)
4	Threonine	ACC	S108T
5	Glycine	GGC	pUC-pfDHFR(S108G)
6	Cysteine	TGC	pUC-pfDHFR(S108C)
7	Arginine	CGC	pUC-pfDHFR(S108R)
8	Proline	CCC	pUC-pfDHFR(S108P)
9	Alanine	GCC	pUC-pfDHFR(S108A)
10	Aspartic acid	GAC	pUC-pfDHFR(S108D)
11	Valine	GTC	pUC-pfDHFR(S108V)
12	Histidine	CAC	pUC-pfDHFR(S108H)
13	Leucine	CTC	pUC-pfDHFR(S108L)
14	Tyrosine	TAC	pUC-pfDHFR(S108Y)
15	Phenylalanine	TTC	pUC-pfDHFR(S108F)
16	Lysine	AAG	pUC-pfDHFR(S108K)
17	Methionine	ATG	pUC-pfDHFR(S108M)
18	Tryptophan	TGG	pUC-pfDHFR(S108W)
19	Glutamic acid	GAG	pUC-pfDHFR(S108E)
20	Glutamine	CAG	pUC-pfDHFR(S108Q)

3.1.3 Plasmid Vector

pUC18 was used as vector for manipulating the synthetic DHFR gene. pET-17b was obtained from Novagen Madison, WI. and was used as expression vector to express the synthetic mutant DHFRs.

3.1.4 Chemicals and Reagents

All chemicals and reagents used in this study were of analytical grade or the highest purity commercially available. Restriction endonucleases and other modifying enzymes were from New England Biolabs, Bethesda Research Laboratories, or Boehringer Mannheim. The wizard plasmid purification kit was purchased from Promega. MTX-sepharose CL-6B (~ 1 μmol/ml gel) used for affinity purification of pfDHFRs was prepared according to the method described by Meek *et al.* (72). H_2folate was prepared from folic acid according to the method of Friedkin *et al* (73). Pyrimethamine, NADPH were purchased from Sigma. Cycloguanil was a gift from Burroughs Wellcome Co.

3.2 METHODS

3.2.1 Isolation and purification of pUC-pfDHFR

Plasmids were purified using Wizard Miniprep DNA purification system. The principle is based on the selective binding of DNA to a proprietary silica-based resin in the presence of chaiotropic agent and guanidine hydrochloride. Proteins and RNA were removed by excessive washing and DNA was eluted with low salt buffer or water in the final step.

A single colony of *E. coli* DH5α harboring pUC-pfDHFR was grown overnight at 37°C with shaking in Luria Bertani (LB) broth (1% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract and 1%NaCl) supplementing with 100 μg/ml of ampicillin. Aliquots of overnight culture (~1.5 ml) were dispensed into microtubes and the cells were pelleted by centrifugation at 10,000 rpm for 5 min. The supernatant was discarded and the cell pellets

were resuspended in 200 µl of buffer solution (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 100 µg/ml RNaseA). The cells were lysed by addition of 200 µl of lysis solution (1% SDS in 0.2 M NaOH) and mixed thoroughly by inverting the tubes several times until the cell suspension was clear. Aliquot of 200 µl of neutralization solution (2.55 M KOAc, pH 4.8) was added, mixed thoroughly by inverting the tubes several times followed by centrifugation at 10,000 rpm for 15 min to obtain clear supernatant for further purification of plasmid DNA.

To set up the Wizard miniprep column for DNA purification, a 3-ml disposable syringe barrel without plunger was attached to the luer-lock extension of the Wizard minicolumn. The minicolumn with syringe barrel was then inserted into the vacuum minifold using the opposite end of the minicolumn. The clear supernatant prepared above was mixed with 1 ml of Magic minipreps DNA purification resin, mixed by inversion, and the resin/DNA mix was applied into the syringe barrel/minicolumn. Vacuum was applied to suck the resin/DNA mix passing through the syringe. The minicolumn was washed with 2 ml of column-wash solution (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM EDTA, 50% ethanol) and the vacuum was continuously applied for additional 1-2 min to dry the resin. Next, the syringe barrel was removed and the minicolumn was transferred to a new microcentrifuge tube. Residual of column-wash solution was removed by centrifugation at 10,000 rpm for 20 sec. Plasmid DNA was then eluted by applying 50 µl of distilled water or Tris-EDTA buffer (10 mM Tris-Cl/1 mM EDTA, pH 8.0) to the column, incubated at room temperature for 1 min and spun at 10,000 rpm for 30 sec. One minicolumn was used for each plasmid sample. The DNA obtained was determined by measuring the absorbance at 260 nm and the concentration calculated by the equation as follows:

$$\text{Concentration of double stranded DNA} = \frac{OD_{260} \text{x dilution factor}}{20} \ \mu g/\mu l$$

3.2.2 Plasmid analysis by agarose gel electrophoresis

Agarose gel (0.8%) was prepared by dissolving 0.8 g of ultrapure agarose (SEAGEM) in 100 ml of 1XTBE buffer (89 mM Tris, 89 mM boric acid and 2 mM EDTA, pH 8.0). The gel suspension was heated until completely dissolved. After the gel was cooled to approximately 50°C, it was poured into a casting chamber equipped with comb. The gel was allowed to be harden at room temperature for approximately 30 min before electrophoresis was carried out.

DNA samples were mixed with 1/3 volume of sample dye (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol). Aliquots of the dye containing samples and the DNA markers were loaded into the wells and electrophoresis was carried out at 80-100 volts using 1xTBE as buffer. After electrophoresis, the gels were stained with ethidium bromide (~0.5 μg/ml) for 15 min with gentle shaking and destained with distilled water until the background of the gel was clear. The bands of DNA in the gel were visualized under UV transluminator and the size of the DNA fragments was calculated using the DNA standard markers.

3.2.3 Restriction analysis of vector and recombinant plasmids

The pET-17b vector and pUC-pfDHFR recombinant plasmids were digested with two restriction enzymes, i.e. *Nde*I and *Hind*III. Approximately 1 µg DNA was first restricted with *Nde*I (3-5 units of enzyme/µg DNA) in 10 µl reaction containing 1XNEbuffer4 (10XNEbuffer4 composes of 20 mM Tris-acetate, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM DTT, pH 7.9 at 25°C). The reaction was incubated at 37°C for 2 h and the digested DNAs were analyzed by 0.8% agarose gel electrophoresis. The linearized pET-17b and pUC-pfDHFR recombinant plasmids were expected to be ~3.3 and 3.6 kb, respectively. A second digestion of the linearized plasmids with *Hind*III was performed after the first digestion was complete. Digestion with *Hind*III was performed using 1XNEbuffer2 (10XNEbuffer2; 10 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, pH 7.9 at 25°C) at 37°C for 2 h. The digested plasmids were then analyzed by 0.8% agarose gel electrophoresis. A linearized DNA band of ~3.3 kb is expected from *Nde*I/*Hind*III double digestion of pET-17b, whereas double digestion of pUC-pfDHFR constructs is expected to yield an excised fragment of about 0.7 kb. The bands of interest were cut out from the gel with razor blade and recovery of DNA in the gel was performed by electroelution (see Section 3.2.4).

3.2.4 Recovery of DNA fragments by electroelution

The DNA fragments from Section 3.2.3 were recovered from the agarose gel by electroelution using ISCO Electrophoretic sample concentrator (Model 1750) according to protocol recommended from the manufacturer. At the bottom of both large and small electroelution cups, dialysis membranes were carefully placed and secured with the o-ring. The

gel fragments were chopped into small pieces and put into the large electroelution cup, filled the cups and electroelution chamber with 0.5xTBE. Electrophoresis was carried out using constant voltage 100 V for 3 h. At the end of electroelution, DNA in the gel (cathode) was recovered on the dialysis membrane placed at the bottom of the small cup (anode). A 30 sec of reversed voltage was applied to facilitate recovery yield of DNA. After removal of 0.5xTBE buffer, the DNA (~200 µl) was recovered from the small cup and transferred to a microfuge tube. The recovered DNA was precipitated by addition of 20 µl of 3 M NaOAc, pH 4.8 and 440 µl of cold absolute ethanol, placed in -80°C freezer for 15 min or -20°C for 1 hr and spun at 10,000 rpm for 15 min. The DNA pellet was then washed twice with ~0.5 ml of cold 70% ethanol, dried in Speed Vac, resuspended in 20 µl of TE buffer, and used in the subsequent experiments.

3.2.5 Ligation of pfDHFR fragments to linearized pET-17b vector

The purified pfDHFR fragments and linearized *Nde*I/*Hind*III double digested pET-17b vector from the section 3.2.4 were used in the ligation experiments. The standard ligation reaction comprised of DNA fragment and vector (3:1 ratio), 1Xligation buffer (10Xligation buffer; 0.5 M Tris HCl, pH 7.6/100 mM $MgCl_2$/10 mM ATP/10 mM DTT/50% (w/v) polyethylene glycol 8000), 1 U of T_4 ligase (Gibco: BRL; 1 U/µl), and distilled water in a final volume of 10 µl. Control reaction contained all the components except for the pfDHFR fragment. The ligation reactions were incubated overnight at 14°C, and were used to transform *E. coli* cells in the subsequent experiments.

3.2.6 Introduction of recombinant plasmids into *Escherichia coli*

3.2.6.1 Preparation of Competent *Escherichia coli* cell

Competent cells of *E. coli* strain DH5α and BL21(DE3)pLysS were prepared using the $MgCl_2$-$CaCl_2$ method as described by Mandel and Higa (74). A single colony of *E. coli* was inoculated into 3 ml of LB broth and grew overnight at 37°C with shaking. For *E.coli* BL21(DE3)pLysS, chloramphenicol (50 μg/ml) was added in the LB broth to maintain the pLys plasmid in the cell. The overnight cultures were used as inoculums for preparation of the competent cells. Generally, the cultures were started with 0.5% inoculum and the cells were grown at 37°C with shaking until OD_{600} is about 0.3-0.4. At this point, the cells were transferred to the prechilled sterile polypropylene tube and centrifuged to pellet the cells at 3,000 rpm for 7 min at 4°C. After the supernatant was discarded, the cell pellets were gently resuspended with 1/4 of the original culture volume of 0.1 M cold $MgCl_2$. The cell suspension was kept on ice for 30 min and then centrifuged at 3,000 rpm for 7 min to pellet the cells. After removing the supernatant, the cell pellets were resuspended with 1/5 of the original culture volume of 0.1 M cold $CaCl_2$, and was kept on ice for 1 h to establish the competency. At the end of the incubation period, the cells were pelleted by centrifugation at 3,000 rpm for 7 min, the supernatant was discarded, and the cell pellets were resuspended in 1/20 of the original volume of ice cold steriled storage solution containing 14% glycerol in 0.1 M $CaCl_2$. The cells were dispensed into prechilled sterile polypropylene tubes and kept in -80°C freezer until use.

To determine the competency of the cells, standard plasmid of known concentration was used to transform the competent cells prepared. The

number of transformants grown on the LB plates supplemented with antibiotics were then counted, and the transformation efficiency was calculated using the following equation:

$$\text{Transformation efficiency} = \frac{\text{CFU control plate x } 10^3 \text{x dilution factor}}{\text{amount of used plasmid (ng)}}$$

CFU = colony forming unit

3.2.6.2 Transformation of plasmids into competent cells

The frozen competent cells prepared as described in Section 3.2.6.1 were thawed on ice and aliquots of 100 µl were dispensed into the prechilled Falcon tubes (17x100 mm polypropylene tube, Falcon 2059). To conduct the transformation, plasmid (10-100 ng) was carefully added to the thawed competent cells. The cell/plasmid suspension was gently mixed by tapping the tube, incubated on ice for 30 min, followed by heat shock at 37°C for 20 sec. After heat shock, the cells were immediately placed on ice for 2 min and 0.9 ml of LB broth was added. The cell suspensions were incubated at 37°C with shaking for 1 h, plated on LB agar supplemented with 100 µg/ml of ampicillin, and incubated at 37°C. Transformed cells grew as separated colonies on LB agar/ampicillin plates after overnight incubation were selected for further characterization.

3.2.7 Small scale isolation of recombinant plasmid

Plasmid isolation by rapid alkaline lysis miniprep method was performed as described (75). A colony of *E. coli* harboring recombinant plasmid was inoculated into 5 ml LB broth containing 100 µg/ml of ampicillin. After overnight incubation at 37°C with shaking, the cells were pelleted by centrifugation at 10,000 rpm for 5 min and the supernatant discarded. The cell pellet was resuspended in 100 µl of TE buffer, pH 7.4 containing 100 µg/ml of RNaseA, mixed thoroughly by vortex mixer, and 200 µl of freshly prepared lysis buffer (1% SDS in 0.2 N NaOH) was added. The suspension was gently mixed by inversion several times until clear viscous solution was obtained. The solution was left at room temperature for 10 min prior to neutralization by addition of 150 µl of 3 M KOAc pH 4.8. This was followed by mixing thoroughly, and centrifuged at 10,000 rpm for 15 min to precipitate the white protein-chromosomal DNA complexes. The clear supernatant was transferred to a new microtube and the plasmid was precipitated by addition of two volume of cold absolute ethanol. After incubation at -80°C for 15 min (or -20°C for 1 h), the DNA was pelleted by centrifugation at 10,000 rpm for 15 min, washed twice with cold 70% ethanol to remove any salt residual and dried in a SpeedVac concentrator. The plasmid was then dissolved with appropriate volume of TE buffer and the concentration was determined either by estimation on agarose gel electrophoresis or by spectrophotometric measurement at OD_{260} nm. The plasmids isolated were characterized with respect to size and restriction map and were transformed into appropriate *E. coli* cells for propagation and/or manipulation.

3.2.8 Expression and preparation of inclusion bodies

A single colony of *E. coli* BL21(DE3)pLysS harboring the recombinant pET-pfDHFR plasmid on LB/ampicillin plate was inoculated in 5 ml LB broth containing 100 μg/ml of ampicillin and grew overnight at 37°C with shaking. The culture was used to inoculate 500 ml of LB/ampicillin broth. The culture was then incubated at 37°C with shaking until the OD_{600} was ~0.5-0.6. At which time the culture was added isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM. After additional 3 h of growth at 37°C with shaking, the cells were harvested by centrifugation at 6,000 rpm for 10 min at 4°C. The cell pellets were washed twice with cold phosphate buffered saline (PBS: 10 mM phosphate buffer, pH 7.4/0.85% NaCl), and partial purification of the inclusion bodies in the cells was then performed according to the method of Lin and Cheng (76).

Briefly, the washed cell pellets were resuspended in 50 ml bufferA (20 mM Tris-Cl, pH 7.5/20% sucrose/1 mM EDTA) and incubated on ice for 10 min. After incubation, 50 ml cold distilled water was added and the cell suspension was placed on ice for additional 10 min prior to centrifugation to pellet the spheroplasts at 6,000 rpm for 30 min. The resulting spheroplasts were resuspended in 10 ml buffer P (5 mM EDTA in PBS buffer) and were subjected to disruption by French Pressure Cell set at 18,000 psi. The lysate was treated with DNaseI (40 μg/ml) and RNaseTI ($1.3x10^2$ unit/ml), incubated at room temperature for 10 min prior to addition of 20 ml of buffer P. After centrifugation at 6,000 rpm for 30 min, the inclusion bodies were obtained, and were washed three times with buffer W at 4°C (25% sucrose, 5 mM EDTA, 1% TritonX 100). The inclusion bodies were considered partially purified materials and were stored at -20°C for subsequent experiments.

3.2.9 Unfolding and refolding of inclusion bodies

Inclusion bodies obtained from expression of pET-pfDHFR constructs as described in Section 3.2.8 were solubilized at a final protein concentration of approximately 0.025 mg/ml in buffer A (20 mM potassium phosphate buffer pH 7.0, 0.1 mM EDTA, 10 mM DTT) containing 0.2 M KCl and 6 M guanidine HCl. This was gently stirred at 4°C for 3 h or until the inclusion bodies were completely dissolved. Refolding of the solubilized inclusion bodies was achieved by 10-fold rapid dilution in buffer A containing 0.2 M KCl and 20% glycerol; dropwise addition of the unfolded proteins into the stirring refolding buffer was achieved by peristaltic pump. Using this method, DHFR activity could be recovered after 3 h of refolding. To ensure complete refolding of the proteins, the refolding process was generally left overnight. Any aggregates formed were pelleted by centrifugation at 6,000 rpm for 15 min. The clear supernatant containing refolded DHFR was transferred to new container for further purification by affinity chromatography.

3.2.10 Purification of synthetic Plasmodial dihydrofolate reductase

Methotrexate-Sepharose CL-6B (~1 µmol/ml gel) was prepared by linking Sepharose CL-6B resin with methotrexate by chemical reaction according to the method described by Meek *et al.* (72). The affinity resin was exploited for purification of both wild-type and mutant DHFRs. A column packed with Methotrexate-Sepharose CL-6B (1.5x6.0 cm) was pre-equilibrated with buffer A (see Section 3.2.9) containing 0.2 M KCl/20% glycerol. The refolded enzyme (~400 ml) obtained from inclusion bodies of

500 ml culture was circulated through the column using the flow rate of 0.8 ml/min. The column was washed with ~100 ml buffer A containing 0.5 M KCl/20% glycerol to remove nonspecifically bound proteins, followed by ~100 ml buffer A containing 50 mM KCl/20% glycerol. The DHFR bound to the column was eluted by applying 50 ml of 4 mM H_2folate in buffer B (50 mM TES pH 8.0/0.1 mM EDTA/10 mM DTT/50 mM KCl/20% glycerol). To improve the yield of the elution step, a portion of 4 mM H_2folate in buffer B (20 ml) was first applied into the column. After overnight equilibration, elution was resumed with the remaining 30 ml of the elution buffer. Fractions (4 ml) were collected and active fractions with DHFR activity were pooled and concentrated by Centriprep 10 concentrator (Amicon). The concentrated enzyme was passed through NAP-25 (Pharmacia) pre-equilibrated with buffer A (20 mM potassium phosphate buffer, pH 7.0/0.1 mM EDTA/10 mM DTT/20% glycerol) to remove H_2folate. The purified enzyme was stored at -20°C until use.

3.2.11 Preparation of dihydrofolate (H_2folate)

H_2folate, used as substrate for DHFR assay, was prepared by chemically reduction of folic acid with sodium dithionite in the presence of β-mercaptoethanol according to the method of Friedkin *et al.* (73).

Folic acid (1.2 g) was resuspended in 100 ml of 1 M β-mercaptoethanol in a beaker wrapped with aluminum foil to protect from light, and the pH was adjusted to about 7.4 by dropwise addition of 1N KOH. As the pH approaching 7.4, the folic acid began to dissolve. When a clear yellowish solution was obtained, sodium dithionite (12 g) was added and the solution was placed on ice. The solution was continuously stirred for 45-60

min, at which time folic acid was reduced and dihydrofolate (H_2folate) appeared as precipitate. Collection of the precipitate was achieved by passing the solution through scintered glass funnel preinstalled to a suction flask connected to vacuum. The precipitate was extensively washed with ice-cold 1 mM HCl/150 mM β-mercaptoethanol, and resuspended in the same ice-cold solution. Aliquots of H_2folate suspension (~1 ml) were dispensed into 2 ml Wheaton serum bottles, capped with rubber stoppers and sealed with aluminum seals by hand-operator crimper prior to store at -80°C for further use.

A working solution of H_2folate was prepared freshly on the day of use. The stock H_2folate kept at -80°C was thawed at room temperature, transferred to the microcentrifuge tubes, and centrifuged at 10,000 rpm for 1-2 min to pellet the H_2folate. After removal of clear supernatant, approximately 1000 µl of 1XDHFR buffer (50 mM TES; pH 7.0/ 1 mM EDTA/ 75 mM β-mercaptoethanol) was added to the H_2folate pellet, mixed thoroughly, and the H_2folate suspension was dissolved by dropwise addition of 2 N NaOH. The concentration of H_2folate was calculated from the absorbance at 282 nm using Σ_{282} = 28,400 $M^{-1}cm^{-1}$(77) as shown by the following equation:

$$\text{Concentration of } H_2\text{folate} = \frac{OD_{282} \times \text{dilution factor}}{28{,}400} \quad M$$

The purity of the H_2folate prepared was determined enzymatically using the DHFR assay as described in Section 3.2.13 with the following

modifications; a final concentration of 50 μM H_2folate and slightly increased in amount of enzyme were used to ensure the detection of complete utilization of substrate H_2folate. The change in absorbance at 340 nm (ΔOD_{340}) was used to calculate the amount of pure H_2folate in the reaction. The percentage of substrate purity was then calculated from the concentration of H_2folate determined enzymatically versus that determined spectrophotometrically as shown in the following equation:

$$H_2\text{folate concentration} = \frac{\Delta OD_{340} \times 10^6}{12{,}300} \quad \mu M$$

$$\%\ \text{purity of } H_2\text{folate} = \frac{[H_2\text{folate}]\ \text{determined enzymatically}}{[H_2\text{folate}]\ \text{determined spectrophotometrically}} \times 100$$

3.2.12 Preparation of NADPH

Stock solution of NADPH , prepared by dissolving NADPH powder in 1XDHFR buffer, was measured for its absorbance at 340 nm and the concentration was calculated using Σ_{340} = 62,900 $M^{-1}cm^{-1}$(78). A working solution of 2 mM in 1XDHFR buffer was then prepared. Aliquots of 1 ml were dispensed into 1.5 ml microcentrifuge tubes and were kept at -80°C until use.

3.2.13 Determination of dihydrofolate reductase activity

DHFR was determined spectrophotometrically by measuring the rate of reduction of absorbance at 340 nm. The enzyme activity was calculated using Σ_{340} = 12,300 $M^{-1}cm^{-1}$ (79). One unit of activity is defined as the amount of enzyme which catalyzes the reduction of one μmole of substrate per minute at 25°C. The standard assay reaction (1 ml), performed in 1-cm path length cuvette, composes of 1XDHFR buffer, 100 μM H_2folate, 100 μM NADPH, 1 mg/ml of BSA and the approximately 0.01 units of enzyme. Unless specified, the reaction was initiated with substrate H_2folate. Blank contained all components except for enzyme.

3.2.14 Kinetic analysis of Plasmodial DHFRs

The kinetic properties of the pfDHFRs were determined using standard condition as described in Section 3.2.13. To determine the K_m value for H_2folate, the enzyme activity was assayed at varying concentrations of H_2folate from 0.8 to 100 μM (0.8-200 μM for S108Q mutant DHFR), while keeping the concentration of NADPH constant at 100 μM. Likewise, determination of K_m value for NADPH was performed by varying the NADPH concentrations from 0.8 to100 μM while keeping the concentration of H_2folate constant at 100 μM. Approximately 0.001-0.005 units of the enzyme were used in each assay. Kinetic parameters were calculated using a software "K-soft" on Macintoch computer for a non-linear least square fit of the data to the Michaelis-Menten equation (80).

3.2.15 Inhibition of Plasmodial DHFRs by antifolates

The inhibition constants (K_i) for antifolates Pyr and Cyc of mutant DHFRs were determined. The stock solution of Pyr and Cyc were prepared in DMSO and the concentrations were determined using Σ_{272} = 7,320 $M^{-1}cm^{-1}$ (81) for Pyr and Σ_{241} = 13,183 $M^{-1}cm^{-1}$ (82) for Cyc at pH ~ 2.0. Determination of DHFR activity was performed using standard DHFR assay reaction which comprised of 100 µM of NADPH, 100 µM of H_2folate and approximately 0.005-0.01 units of enzyme in the presence of varying inhibitor concentrations. The inhibition constants (K_i) were calculated from a non-linear least square fit program with the assumption that Pyr and Cyc are competitive inhibitors of DHFRs. One hundred percent of DHFR activity was calculated from the enzyme activity without inhibitors, and the 50% inhibitory concentration (IC_{50}) was defined as the concentration of inhibitor which inhibited the activity of enzyme by 50%. The IC_{50} could also be calculated from the following equation:

$$IC_{50} = K_i (1+([S]/K_m))$$

when K_i = Inhibitory constants of the inhibitor (µM)

[S] = H_2folate concentration in the assay (100 µM)

K_m = Michaelis-Menten constant for H_2folate of the enzyme used in the assay system

Double reciprocal plots illustrating the type of inhibition of mutant pfDHFRs by Pyr and Cyc were performed by determination of DHFR activities at varying concentrations of H_2folate as described in Section 3.2.14, but in the presence of various constant concentrations of inhibitors.

The data were analyzed and plotted using software "K-soft" on a Macintoch computer.

3.2.16 Titration of Plasmodial DHFRs by methotrexate

Methotrexate (MTX), a tight and stoichiometric binding inhibitor of DHFR, was exploited for titration of Plasmodial purified DHFRs. A stock solution of MTX was prepared by in DMSO and the concentration was determined spectrophotometrically using Σ_{302} = 22,000 $M^{-1}cm^{-1}$ (83) at pH ~13. To perform the titration, the amounts of DHFR were varied (0-26.4 pmole) while keeping the amount of MTX constant at 5 pmoles. The activities of the enzyme, both with and without MTX in the assay reaction, were plotted against the amounts of enzyme used. The amounts of active enzyme were calculated by extrapolating the straight line of the slope from assay reactions containing 5 pmoles MTX (84).

3.2.17 Determination of protein concentration

The concentrations of protein samples were determined using colorimetric method as described by Read and Northcote (85). To prepare the protein assay reagent, 100 mg of Serva Blue G (Serva Research Grade 35050) was dissolved in 94 ml of absolute ethanol and stirred at room temperature with light protection (covering with aluminum foil) until the dye was completely dissolved. To the dye solution was added 200 ml of 85% (w/v) ortho-phosphoric acid and the solution was continuously stirred for additionally 3 h prior to adjusting the final volume to 2 L with water. The dye

solution was filtered through Whatman filter paper (No. 1) and kept at room temperature in amber bottle for further use.

To determine the protein concentration, aliquots of samples (varied from 10-100 µl) were added to 1 ml of the protein reagent. The solution was then mixed thoroughly, incubated at room temperature for 10 min and the absorbance at 596 nm was measured against the reagent blank to which the same volume of distilled water was added instead of sample. The protein concentrations were calculated from a standard curve generated by using bovine serum albumin as standard protein.

3.2.18 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dedecyl sulfate (SDS) polyacrylamide electrophoresis (4% stacking gel and 12% separating gel) was performed according to the method described by Laemmli (86). Protein samples were solubilized with sample-solubilizing buffer composed of 4% SDS, 20% glycerol, 10% β-mercaptoethanol and bromophenol blue in 0.125 M Tris-Cl pH 6.8. The samples were boiled in boiling water for 3 min prior to loading onto the wells. Electrophoresis was carried out from cathode to anode at 30 mA constant current using running buffer which composes of 0.025 M Tris, 0.19 M glycine, pH 8.5, and 0.1% SDS. Electrophoresis was stopped when the dye front of bromophenol blue reached the bottom of the gel. The gel was then removed from the glass plates and stained in Coomassie staining solution (0.125% Coomassie Brilliant Blue R250, 50% methanol and 10% acetic acid) with gentle shaking at room temperature for about 1-2 h, followed by destaining in destaining solution (5% methanol, 10% acetic

acid) with several changes until the background of the gel was clear. Low molecular weight standard markers (Bio-Rad) were phosphorylase B (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4).

CHAPTER IV

RESULTS

4.1 Cloning of Synthetic Plasmodial DHFR (pfDHFR)

A series of Plasmodial DHFR mutants were constructed in pUC-based plasmid by combinatorial cassette mutagenesis to replace Ser at residue 108 by all other 19 common amino acids. The sequences of codon corresponding to amino acid residue 108 were verified by DNA sequencing. The 693 bp *Nde*I/*Hind*III DHFR fragments from each mutant were excised, gel purified, and ligated with *Nde*I/*Hind*III predigested pET-vector to give recombinant mutants. Figure 3 shows the restriction analysis of the representative clones on 0.8% agarose gel. Digestion of the recombinant plasmids by *Nde*I/*Hind*III resulted in excision of the 693 bp pfDHFR fragment. The clones were transformed into *E.coli* BL21(DE3)pLysS for further expression.

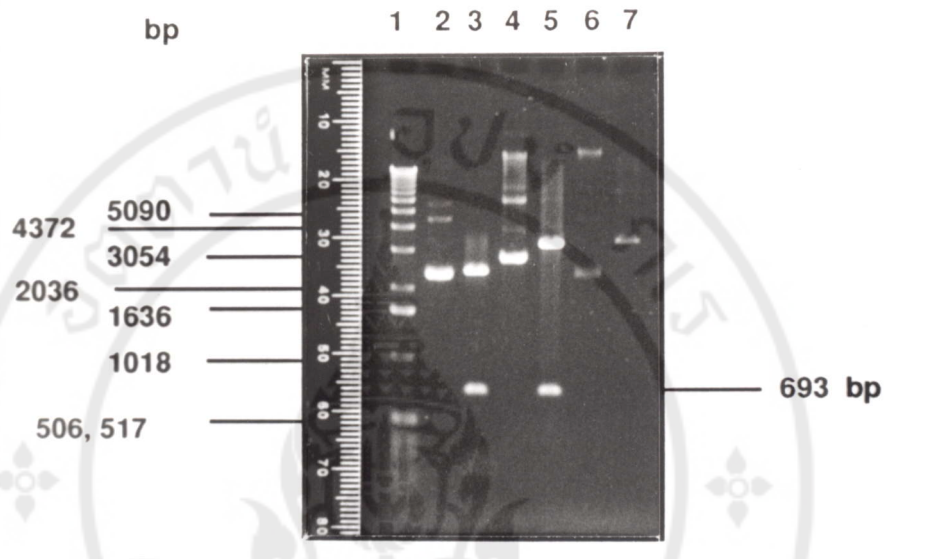


Figure 3 : 0.8% agarose gel electrophoresis of *Nde* I/ *Hind* III digestion of the plasmids containing pfDHFR was stained with ethidium bromide and visualized under the short wave-length UV.

Lane 1 : 1Kb ladder standard size markers

2 : undigested pUC-pfDHFR

3 : *Nde* I/ *Hind* III digested pUC-pfDHFR

4 : undigested pET-pfDHFR

5 : *Nde* I/ *Hind* III digested pET-pfDHFR

6 : undigested pET-17b

7 : *Nde* I/ *Hind* III digested pET-17b

4.2 Expression of pfDHFR in *E.coli* BL21(DE3)pLysS

Expression of pET-pfDHFR mutants was performed as described in Section 3.2.8. *E.coli* BL21(DE3)pLysS cells harboring recombinant pET-pfDHFRs were grown at 37 °C and expression of pfDHFR was achieved by IPTG induction. The induced cells were harvested, solubilized in SDS sample buffer and SDS-PAGE was carried out to monitor the expression of pfDHFR. Figure 4 illustrates SDS-PAGE of the *E.coli* cells harboring pET-pfDHFR mutant plasmids. With the exception of S108K (Figure 4, lane15), replacement of Ser 108 with the remaining amino acids resulted in mutants which expressed DHFR which could be visualized as intense bands of molecular mass 27 kDa (Figure 4, lane 3-14 and lane 16-22). Similar to that observed in the wild-type construct, the DHFRs from these mutants were expressed as insoluble inactive inclusion bodies, and required subsequent refolding in appropriate condition to recover the active enzyme activity.

Figure 4 : 12% SDS-PAGE of cell lysate of *E.coli* BL21(DE3)pLysS harboring pET-pfDHFR mutants which expressed pfDHFRs of as 27 kDa. The gels were stained with Coomassie blue.

Lane 1 : Standard low molecular weight protein markers

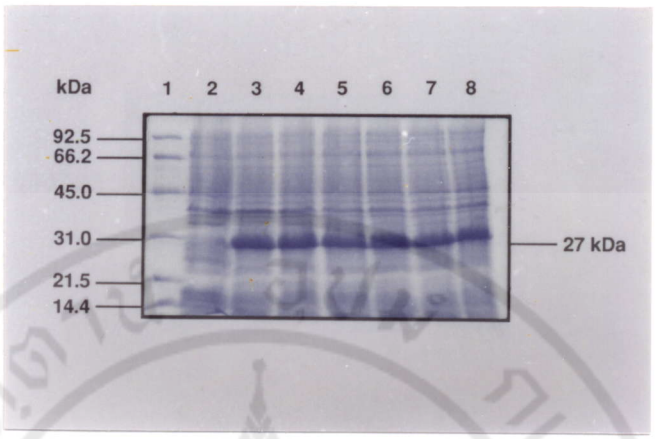
2 : *E.coli* BL21(DE3)pLysS harboring pET-17b

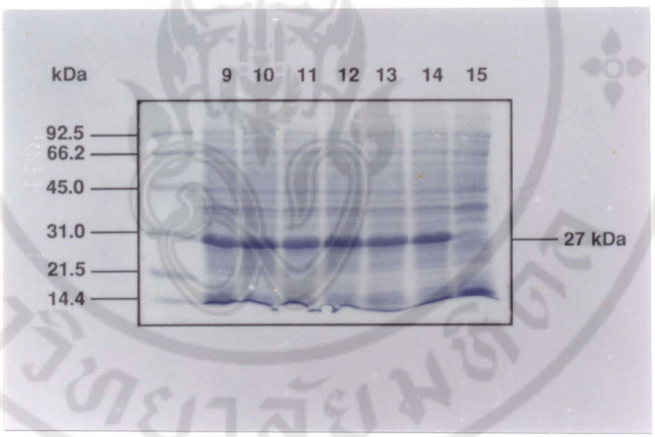
3 : *E.coli* BL21(DE3)pLysS harboring pET-pfDHFR wild-type)

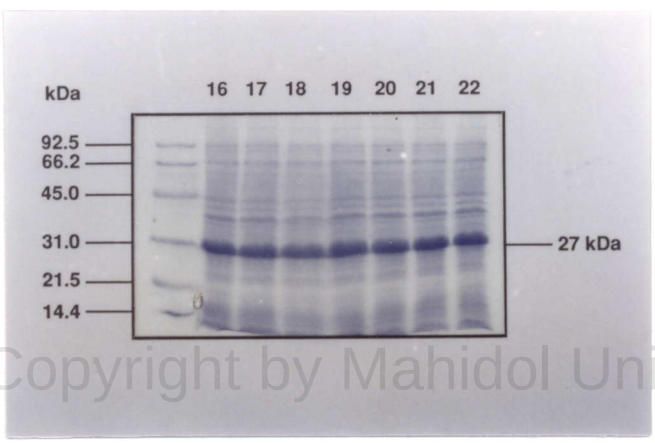
4-14 : *E.coli* BL21(DE3)pLysS harboring pET-pfDHFR (S108G, A, V, L, I, P, T, C, M, N, Q)

15 : *E.coli* BL21(DE3)pLysS harboring pET-pfDHFR (S108K)

16-22 : *E.coli* BL21(DE3)pLysS harboring pET-pfDHFR (S108F, Y, W, R, H, D, E)







4.3 Determination of DHFR activities from mutant DHFR constructs

Inclusion bodies prepared from 250 ml culture of each of pfDHFR mutants, except that from S108K mutant, were refolded and the activities of DHFR were determined spectrophotometrically. The results were summarized in Table 4. Replacement of Ser 108 by Asn and Thr to give S108N and S108T mutants, respectively caused approximately 50% reduction in DHFR activity as compared to the wild-type enzyme. Poor DHFR activities were observed from mutants with Ser 108 being replaced with Gly, Ala, Gln (3.5, 2.4, 4.6 μmole/min/mg, respectively). Further decrease in DHFR activities was observed from mutants containing Cys, Val, Leu and Met at residue 108 (1.2, 0.7, 1.0, 0.6 μmole/min/mg, respectively), whereas mutants with Ile, Arg, Pro, Asp, His, Tyr, Phe, Trp, Glu at residue 108 yielded an inactive enzyme which did not show detectable enzyme activity.

Table 4 : Determination of DHFR activities from wild-type and mutant pfDHFRs

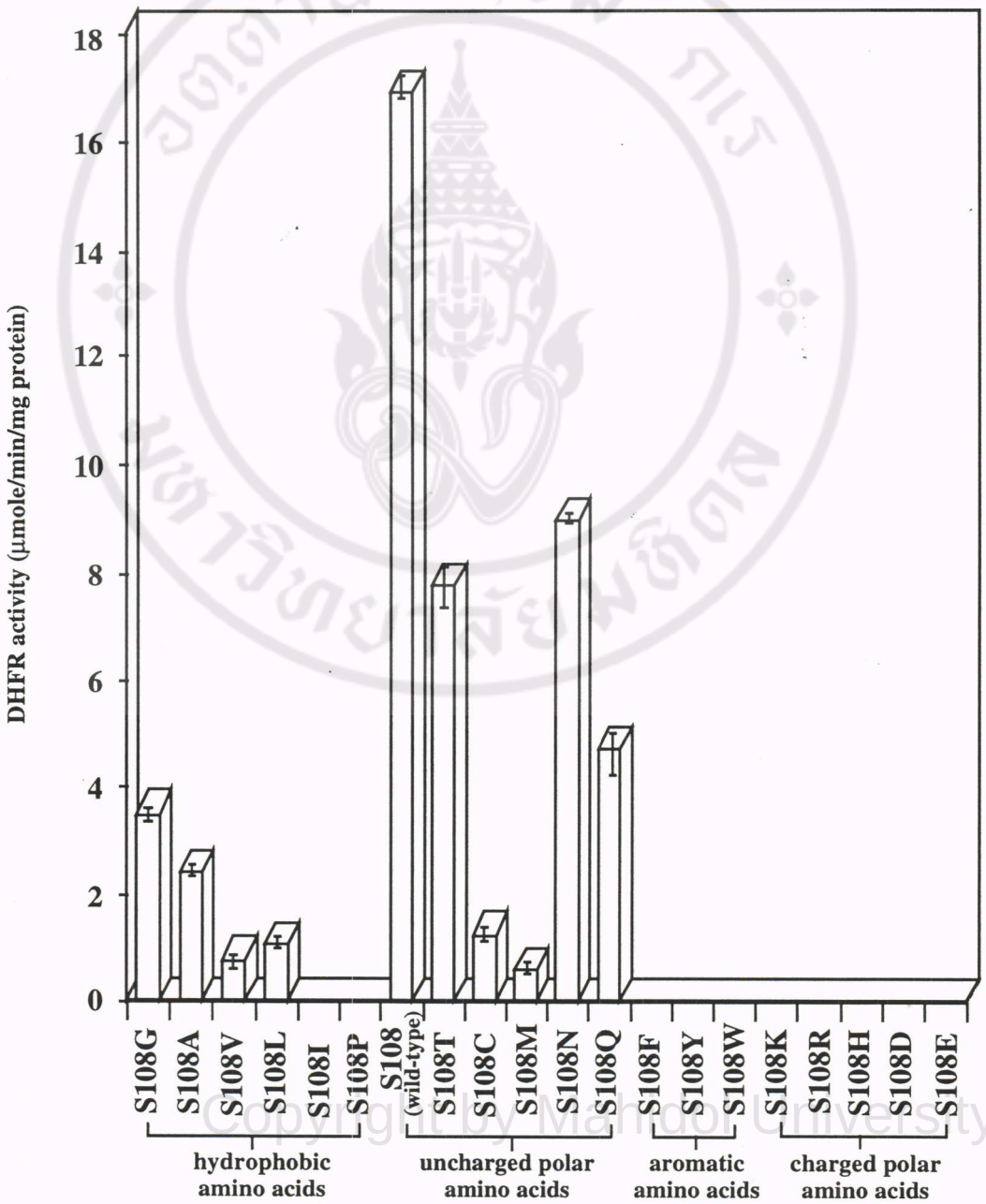
Type of amino acid	Mutant (codon) [a]		Specific activity μmole/min/mg [b]
hydrophobic amino acid	S108G	(GGC)	3.50±0.09
	S108A	(GCC)	2.38±0.03
	S108V	(GTC)	0.73±0.03
	S108L	(CTC)	1.01±0.03
	S108I	(ATC)	nd
	S108P	(CCC)	nd
uncharged-polar amino acid	wild-type	(AGC)	17.05±0.30
	S108T	(ACC)	7.79±0.21
	S108C	(TGC)	1.23±0.04
	S108M	(ATG)	0.56±0.02
	S108N	(AAC)	9.09±0.17
	S108Q	(CAG)	4.63±0.30
aromatic amino acid	S108F	(TTC)	nd
	S108Y	(TAC)	nd
	S108W	(TGG)	nd
charged-polar amino acid	S108K	(AAG)	-
	S108R	(CGC)	nd
	S108H	(CAC)	nd
	S108D	(GAC)	nd
	S108E	(GAG)	nd

a Refolding enzyme was prepared from inclusion bodies of 250 ml culture.

b The reaction was assayed the activity in 1 ml reaction containing concentration of H_2folate as 100 μM at 25°C by initiating with H_2folate. The specific activities of the enzyme (mean ± SD) were average values from five determinations.

nd not detected

Figure 5 : Determination of activity of wild-type pfDHFR and mutant pfDHFRs at residue 108



4.4 Purification of Mutant pfDHFRs

Four DHFR mutants having Ser 108 replaced by Gly, Cys, Ala and Gln were chosen for MTX-Sepharose CL-6B affinity purification and further characterization. Table 5 shows representative data from the affinity purification of DHFR (S108Q) mutant. Inclusion bodies of mutant DHFR (S108Q) prepared from a 500 ml culture were refolded in 400 ml refolding buffer (see Section 3.2.10). A total protein of about 28 mg with total DHFR activity of 320 units was obtained from the refolding material. The enzyme was purified about 3 folds with 55% yield by MTX-Sepharose CL-6B. Further gel filtration by NAP-25 column increased the fold purification to about 7, but the yield of the enzyme was reduced to 39%. As a final result, a total protein of 1.5 mg was obtained (Table 5). Purification of other DHFR mutants, i.e. S108G, S108C, and S108A, were also achieved by using MTX-Sepharose CL-6B affinity resin with 10-30% yield and 2-10 fold purification. SDS-PAGE of the purified DHFRs from S108Q, S108G, S108C, and S108A mutants revealed single band of molecular mass ~27 kDa (Figure 6).

The purified enzymes from section 3.2.10 (S108G, S108C, S108A, S108Q) were kept, in the presence of 20% glycerol, at -20°C to test for the stability. Under this storage condition, all enzymes were stable for at least 5 months, except S108Q which showed some reduction in activity after 7 months (Figure 7).

Table 5 : Purification of mutant pfDHFR (S108Q) by MTX-Sepharose CL-6B chromatography

Purification step	Protein (mg/ml)	Activity [b] (μM/min/ml)	Volume (ml)	Total protein (mg)	Total activity (μM/min)	Specific activity (μM/min/mg protein)	% Yield	Fold
Refolding [a]	0.07	0.8	400	28.0	320	11.4	100	1
MTX-column	0.09	3.5	50	4.5	175	38.9	55	3.4
Gel filtration (Nap-25)	0.19	15.5	8	1.5	124	82.7	39	7.3

[a] Enzyme was prepared by refolding of inclusion bodies from 500 ml culture.

[b] Activity of DHFR was determined at 25°C by standard DHFR assay in 1 ml reaction.

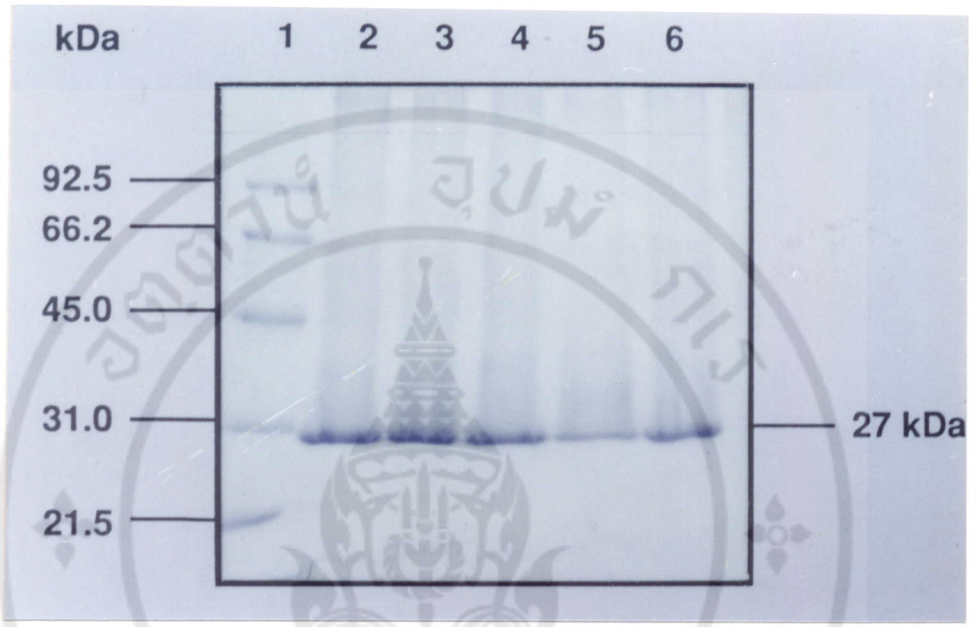


Figure 6 : 12% SDS-PAGE of purified mutant pfDHFRs stained with Coomassie blue.

Lane 1 : Standard low molecular weight protein markers

2 : purified wild-type DHFR

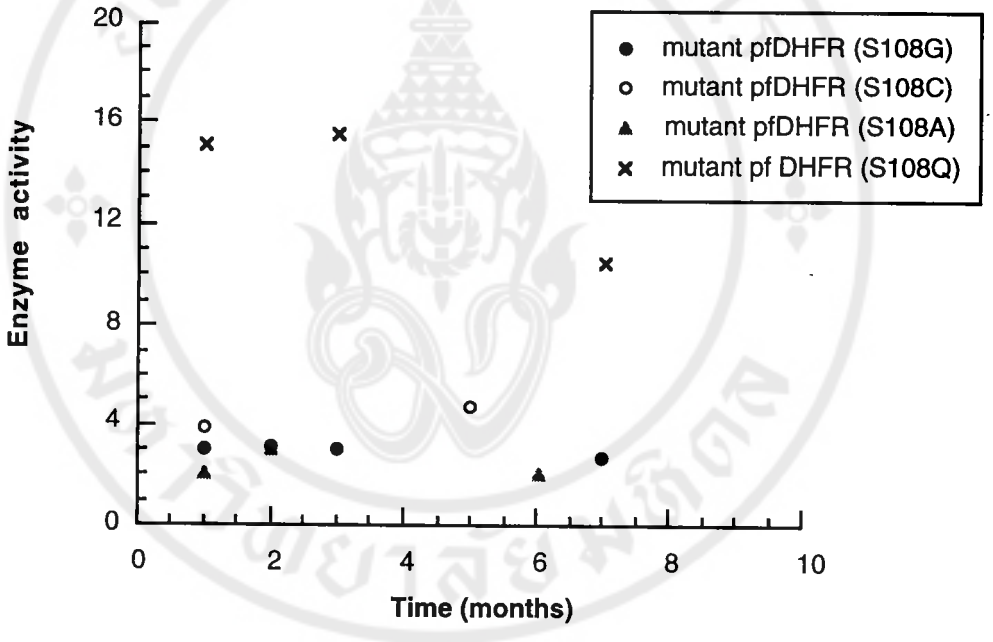
3 : purified mutant DHFR (S108G)

4 : purified mutant DHFR (S108C)

5 : purified mutant DHFR (S108A)

6 : purified mutant DHFR (S108Q)

Figure 7 : Stability of purified mutant pfDHFRs at -20°C



4.5 Kinetic properties of purified mutant pfDHFRs

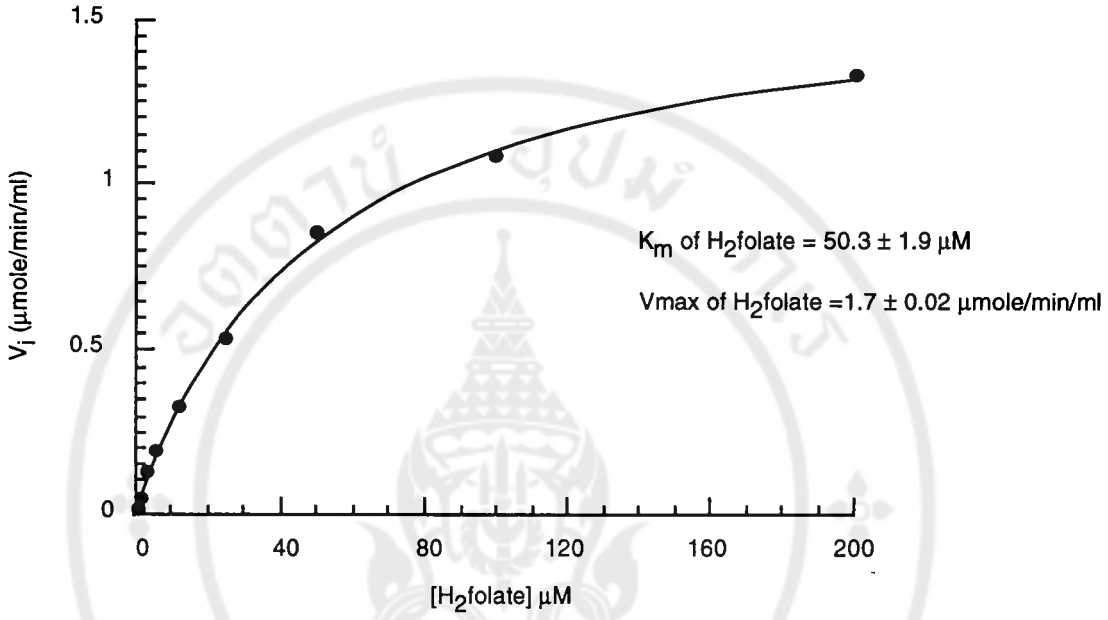
The kinetic properties of the purified DHFRs from S108G, S108C, S108A, and S108Q mutants were investigated and summarized in Table 6. Figure 8 shows the representative plots of initial velocity of the purified DHFR from S108Q mutant against varying concentrations of H_2folate and NADPH.

The K_m values for H_2Folate of the purified DHFRs from most mutants (Table 6 and Figure 9) were not significantly different from that previously reported for the wild-type DHFR (52); the K_m values for H_2Folate of S108A and S108T mutant DHFRs (~14 μM) were comparable to the wild-type enzyme (~13 μM), whereas slightly increased in the K_m values for H_2folate were observed for DHFRs from S108C, S108G and S108N mutants (~20-25 μM). However, S108Q mutant yielded DHFR with increased in K_m value for H_2Folate by a factor of 4 (Table 6 and Figure 9). The K_m values for NADPH of S108G, S108C, and S108A (~8-9 μM) were comparable to S108N mutant (~7 μM). Mutation of Ser 108 to Gln yielded S108Q mutant which gave enzyme with K_m value for NADPH (~14 μM) comparable to the S108T mutant (~17 μM), but was about 3-fold increased as compared to the wild-type enzyme (~4.7 μM) (Table 6 and Figure 9).

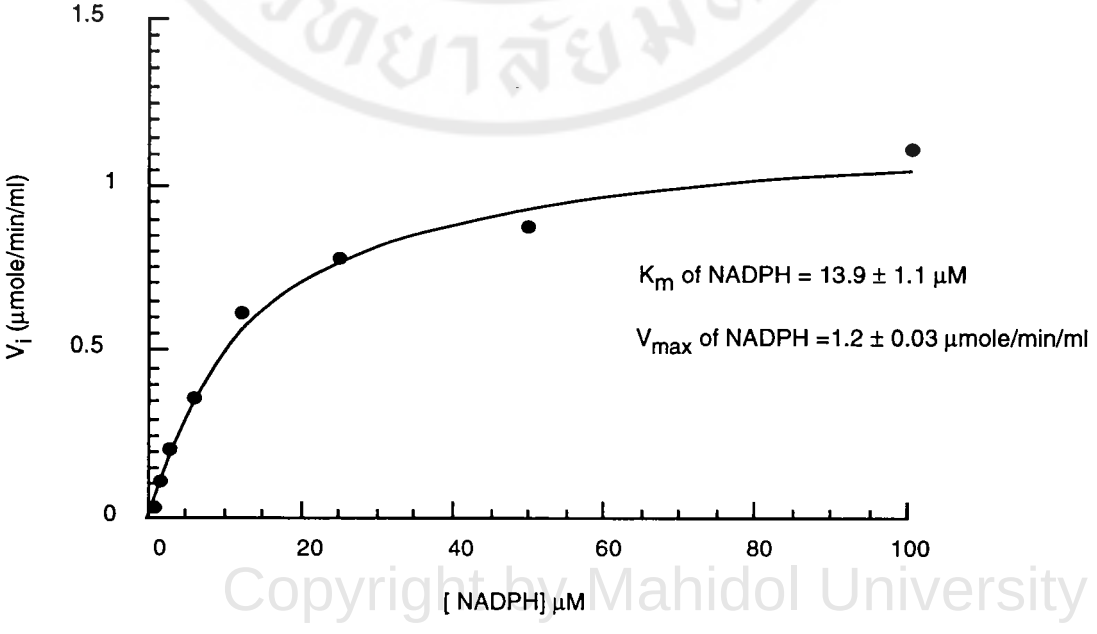
Replacement of residue 108 from Ser to Thr (S108T) and from Ser to Gln (S108Q) decreased the catalytic activity (k_{cat}) of the enzyme by approximately 50% as compared to the wild-type enzyme. Mutants with S108A and S108G mutations resulted in enzymes of which the k_{cat} values were reduced to about 21 sec^{-1}. The k_{cat} value for S108C mutant DHFR was further decreased to about 12 sec^{-1}, some 7-fold less active as compared to the wild-type enzyme (Table 6 and Figure 9).

The catalytic efficiency (k_{cat}/K_m) values of the mutant DHFRs were calculated and compared with the wild-type enzyme (Table 6). Replacement of Ser108 by Asn resulted in 50% decrease in catalytic efficiency of the enzyme; the k_{cat}/K_m values for S108N mutant enzymes was 3.7×10^{-6} $M^{-1}sec^{-1}$ as compared to the 6.9×10^{-6} $M^{-1}sec^{-1}$ for the wild-type enzyme. A decrease in k_{cat}/K_m values was observed for S108T, S108A, and S108G mutants (2.7×10^{-6}, 1.5×10^{-6}, 1.1×10^{-6} $M^{-1}sec^{-1}$, respectively). Replacement of Ser108 by Cys and Gln caused dramatic decrease in the catalytic efficiency of the enzyme (Table 6).

Figure 8 : Determination of kinetic parameters for H_2folate and NADPH of purified mutant pfDHFR (S108Q)



A) Michaelis-Menten plot of initial velocity against varied concentrations of H_2folate



B) Michaelis-Menten plot of initial velocity against varied concentrations of NADPH

Table 6 : Kinetic parameters of purified wild-type and purified mutant pfDHFRs

Enzyme type	K_m (μM)		k_{cat} [a]	k_{cat}/K_m [b]
	H_2folate	NADPH	(sec^{-1})	($M^{-1}sec^{-1}$)
wild-type (S108)[c]	12.9 ± 4.9	4.7 ± 1.3	88.4	6.9×10^{-6}
S108N[c]	24.7 ± 9.3	6.9 ± 2.1	92.0	3.7×10^{-6}
S108T[d]	13.5 ± 1.6	17.1 ± 2.6	36.0	2.7×10^{-6}
S108G	20.0 ± 0.8	8.7 ± 0.6	22.6	1.1×10^{-6}
S108C	22.8 ± 0.9	9.2 ± 1.1	12.0	0.5×10^{-6}
S108A	13.9 ± 1.5	8.9 ± 1.0	21.1	1.5×10^{-6}
S108Q	50.3 ± 1.9	13.9 ± 1.1	37.0	0.7×10^{-6}

[a] Data obtained from the average of two determinations.

[b] Data were calculated by dividing the k_{cat} by the K_m for H_2folate.

[c] Data from Ref 52

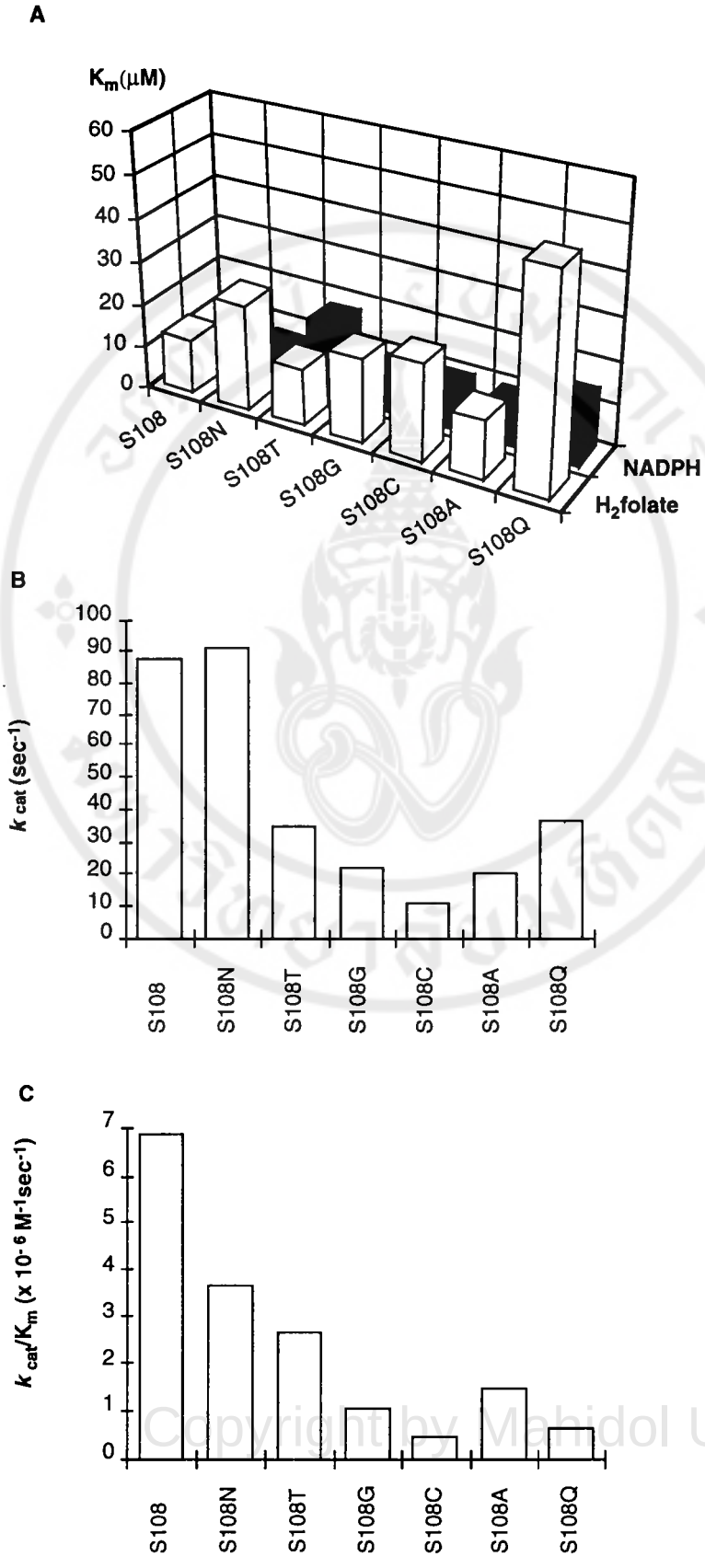
[d] Data from Ref 66

Figure 9 : Kinetic parameters for purified wild-type pfDHFR and purified mutant pfDHFRs at residue 108

A) Michaelis-Menten constant (K_m) vaule for H_2folate and NADPH

B) k_{cat} of purified wild-type pfDHFR and purified mutant pfDHFRs at residue 108

C) k_{cat}/K_m of purified wild-type pfDHFR and purified mutant pfDHFRs at residue 108



4.6 Inhibition of mutant DHFRs by antifolates

The inhibition of mutant DHFRs by pyrimethamine and cycloguanil was investigated by determination of the enzyme activities in the presence of varying concentrations of the inhibitors and the results are summarized in Table 7, Figure 10 and 11. The K_i values for both pyrimethamine and cycloguanil of the mutant DHFRs with S108T, S108G, S108C, S108A were comparable to that observed for the wild-type enzyme. Mutant DHFR with Ser 108 to Gln mutation (S108Q) was resistant to both pyrimethamine and cycloguanil; the K_i values for pyrimethamine and cycloguanil were 95 and 158 nM, respectively.

Both pyrimethamine and cycloguanil competitively inhibited the wild-type and mutant DHFRs. Figure 12 show the double reciprocal plots illustrating typical competitive inhibition by pyrimethamine and cycloguanil of the wild-type and mutant enzymes S108N and S108Q.

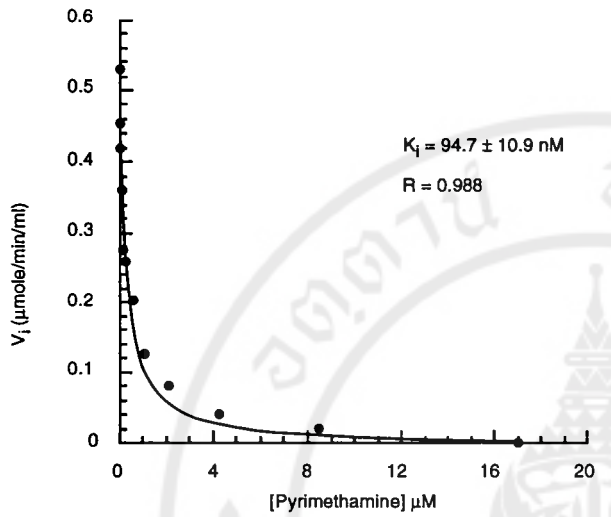
Figure 10 : Inhibitory curve of antifolate inhibitor for purified mutant pfDHFR (S108Q)

A.1) Inhibitory curve of initial velocity (V_i) against variable concentration of pyrimethamine inhibitor

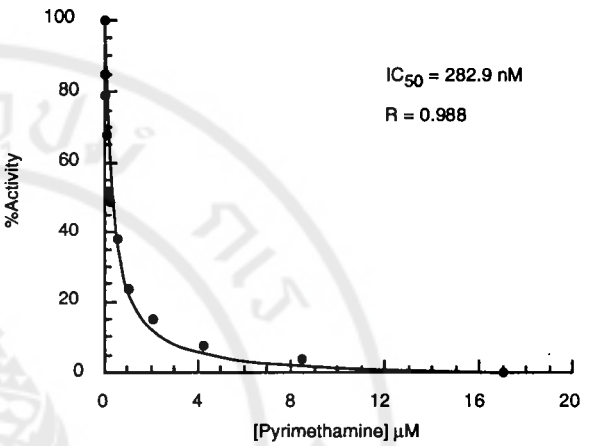
A.2) Inhibitory curve of percentage of enzyme activity against variable concentration of pyrimethamine inhibitor

B.1) Inhibitory curve of initial velocity (V_i) against variable concentration of cycloguanil inhibitor

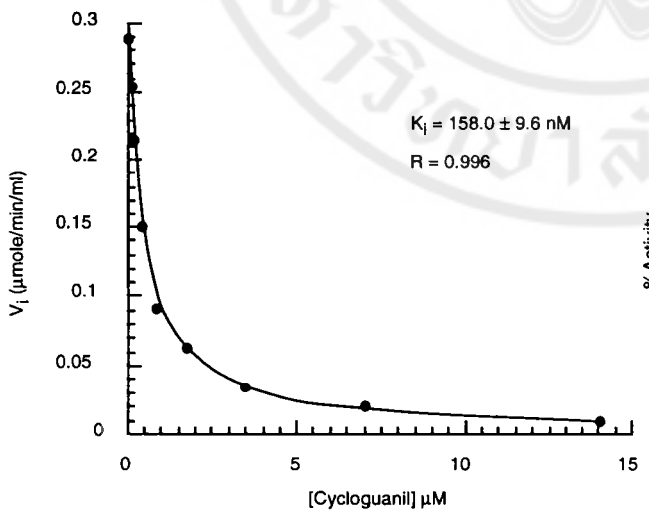
B.2) Inhibitory curve of percentage of enzyme activity against variable concentration of cycloguanil inhibitor



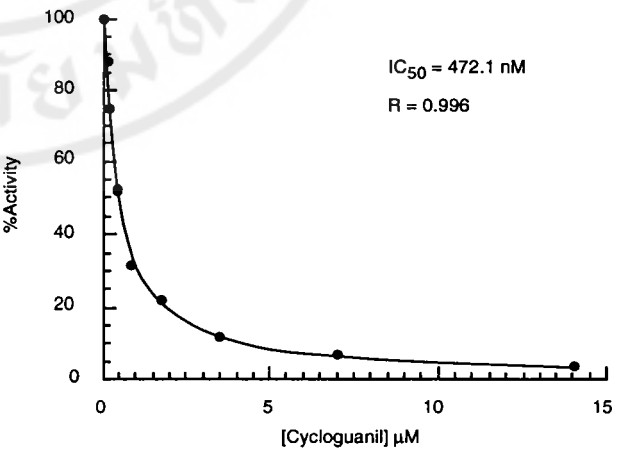
A.1) Inhibitory curve of initial velocity (Vi) against variable concentration of pyrimethamine inhibitor



A.2) Inhibitory curve of percentage of enzyme activity against variable concentration of pyrimethamine inhibitor



B.1) Inhibitory curve of initial velocity (Vi) against variable concentration of cycloguanil inhibitor



B.2) Inhibitory curve of percentage of enzyme activity against variable concentration of cycloguanil inhibitor

Table 7 : The inhibitory effect of antifolate inhibitors; pyrimethamine and cycloguanil on purified wild-type and purified mutant pfDHFRs

Enzyme	Pyrimethamine		Cycloguanil	
	K_i (nM) [a]	IC_{50} (nM) [b]	K_i (nM) [a]	IC_{50} (nM)[b]
wild-type (S108)[c]	1.5 ± 0.17	12.9	2.6 ± 0.3	22.8
S108N[c]	13.4 ± 3.8	67.6	14.7 ± 1.7	74.3
S108T[d]	1.4 ± 0.2	11.5	1.6 ± 0.2	13.7
S108G	7.1 ± 1.0	42.6	17 ± 1.7	102.0
S108C	5.9 ± 0.1	31.8	2.7 ± 0.4	14.5
S108A	3.9 ± 0.8	31.9	2.0 ± 0.3	16.4
S108Q	94.7 ± 10.9	282.9	158.0 ± 9.6	472.1

The enzyme activity was assayed in 1 ml reaction containing 100 µM of H_2folate at 25°C.

[a] Data were reported as mean±SD from two determinations.

[b] Data were calculated by equation in Section 3.2.15.

[c] Data from Ref 52

[d] Data from Ref 66

Figure 11 : Inhibitory effect of antifolate compounds for purified mutant pfDHFRs

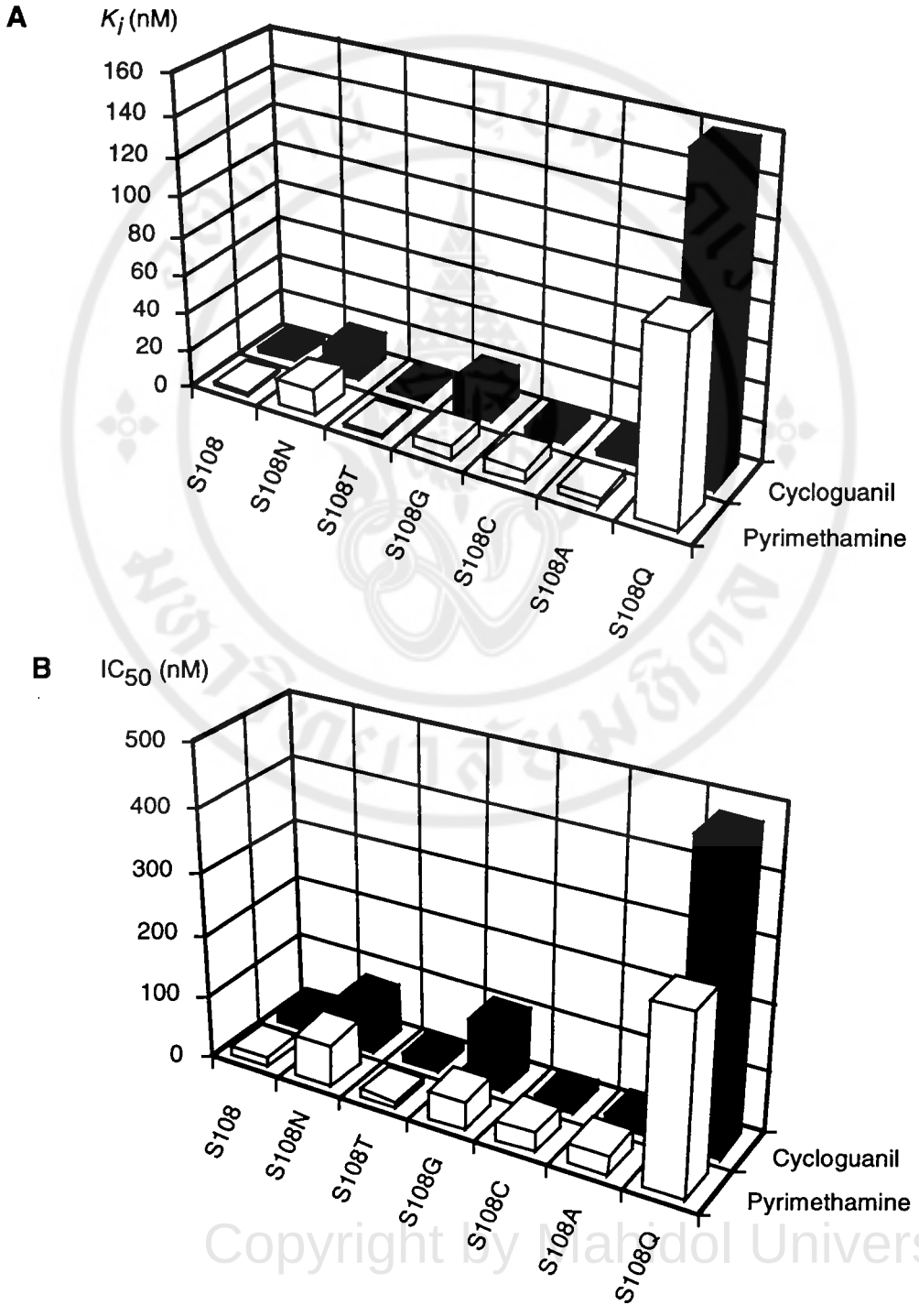


Figure 12 : Inhibition of purified wild-type and purified mutant pfDHFRs (S108N, S108Q) by antifolates Pyr and Cyc

The reciprocal plots between the initial velocities (v_i) at various concentrations of antifolates (Pyr and Cyc) and the concentrations H_2folate were illustrated using purified wild-type, S108N and S108Q mutant DHFRs.

A. Inhibition of purified wild-type pfDHFR by Pyr

B. Inhibition of purified wild-type pfDHFR by Cyc

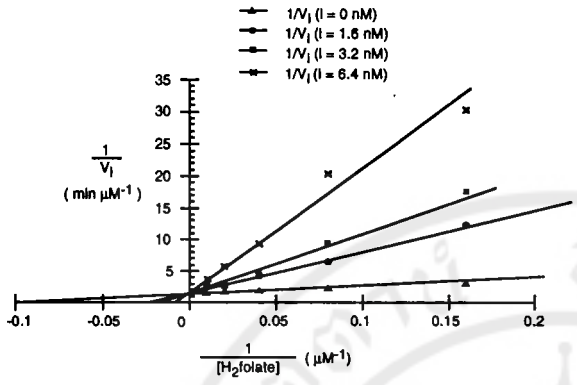
C. Inhibition of purified mutant pfDHFR (S108N) by Pyr

D. Inhibition of purified mutant pfDHFR (S108N) by Cyc

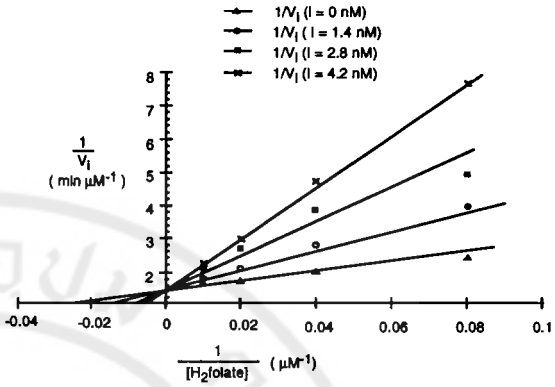
E. Inhibition of purified mutant pfDHFR (S108Q) by Pyr

F. Inhibition of purified mutant pfDHFR (S108Q) by Cyc

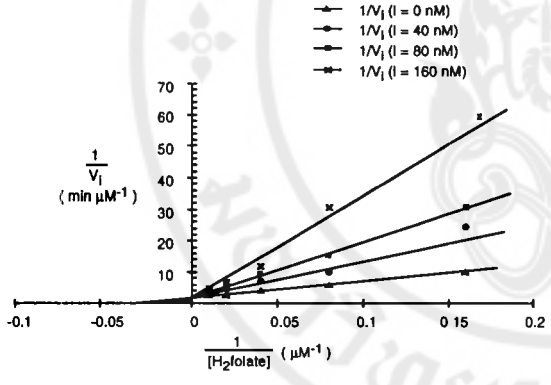
A. Pyrimethamine inhibition of wild-type pfDHFR (S108)



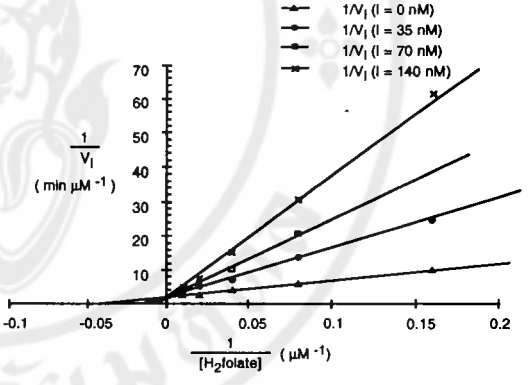
B. Cycloguanil inhibition of wild-type pfDHFR (S108)



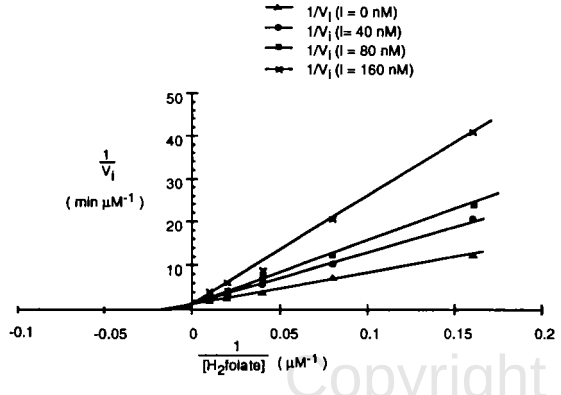
C. Pyrimethamine inhibition of mutant pfDHFR (S108N)



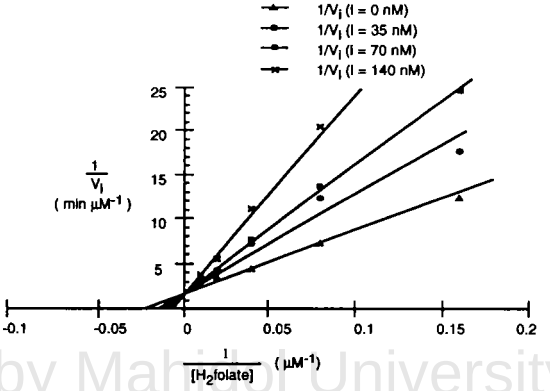
D. Cycloguanil inhibition of mutant pfDHFR (S108N)



E. Pyrimethamine inhibition of mutant pfDHFR (S108Q)



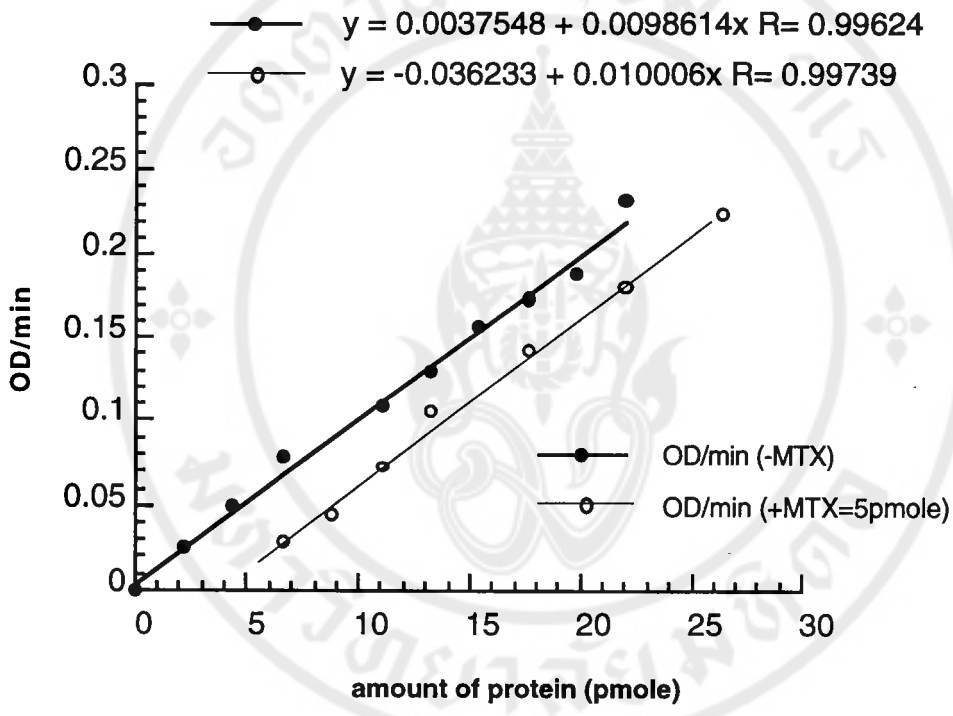
F. Cycloguanil inhibition of mutant pfDHFR (S108Q)



4.7 Determination of active enzyme

The purified pfDHFR (S108Q) was titrated the active form as described in section 3.2.16. The MTX titration curve was shown in figure13. The result indicated the rate of reaction in the case of control (-MTX) was proportional to the enzyme concentration and a straight line through the origin. In the case of presence 5 pmole MTX, the slope of line was the same as control but it passed through the X-axis at approximately 5 pmole.

Figure 13 : Metrotrexate titration curve of purified pfDHFR(S108Q)



CHAPTER V

DISCUSSION

Previous works on the analysis of *P. falciparum* DHFR sequences have provided evidence suggesting point mutations of DHFR as possible mechanism of antifolate resistance in malaria (30-32, 42, 51-57, 60, 62). Ser to Asn mutation at residue 108 is a common mutation observed among antifolate resistant mutants and therefore is believed to be a key residue associated with drug resistance. In prokaryotic (36, 68, 69) and eukaryotic (70) DHFRs, the importance of the amino acid equivalent to residue 108 of *P. falciparum* DHFR is well documented. The X-ray structures of DHFRs from these organisms clearly demonstrate the contribution of hydroxyl group from Thr in forming hydrogen bonding with the adenine mononucleotide 5' phosphate moiety of the NADPH cofactor and van der Waals interaction to the C_6 of nicotinamide rings. As a conclusion from these lines of evidence, it was suggested that the presence of polar amino acid at this position is critical for weak bond formation between enzyme and substrate.

To further understand the role of residue 108 in *P. falciparum* DHFR with regard to enzyme stability and in conferring antifolate resistance, a synthetic gene encoding *P. falciparum* DHFR recently described (52) was exploited for the study. The synthetic gene is best suited for this purpose as it is interspersed with numerous restriction sites and therefore permits facile mutagenesis. Mutation at residue 108 was achieved by cassette replacement of a 35/41 bp *Pst* I-*Cla* I fragment with oligonucleotide duplex which contained degenerated codon (AGCT)(AGCT)(GC) at residue 108. By this approach, a single mutagenic DNA cassette generated a mixture of 32

codons encoding all 20 possible amino acids at residue 108. The desired mutants were screened by DNA sequencing.

The mutants with residue 108 substituted by all other 19 different amino acids, along with the wild type, were initially examined for their expression in *E. coli*. With the exception of S108K mutant from which no expression could be detected, the remainders expressed DHFR as insoluble inclusion bodies of molecular mass 27 kDa (Figure 4). The failure to express DHFR from S108K was unexpected, but the DNA sequences between the *Pst* I-*Cla* I sites were at least reanalysed to assure the correct sequence of the construct. Whether the S108K mutant required other suitable conditions for expression or Ser to Lys mutation led to production of unstable protein which, for some unknown reasons, affect the expression in *E. coli* remains to be further investigated. Nevertheless, no experimental information was available to explain the above observation.

The formation of inclusion bodies is not an unusual phenomenon. Foreign proteins overproduced in *E. coli* often aggregate and precipitate as insoluble and biologically inactive materials in the cytoplasm of the host cells as a result of the formation of incorrect intra- and inter-molecular disulfide bonds (87). In order to obtain the functional protein from the inclusion bodies, it is necessary to solubilize the inclusion bodies using appropriate denaturants and reducing agents. Subsequent renaturation of the protein with suitable conditions will promote the formation of correct intra- and inter-molecular disulfide bonds. In *P. falciparum* DHFR, the low expression of DHFR-TS bifunctional protein in *E. coli* was presumably associated with the high AT content of the gene (33) or the toxicity of the gene product to *E. coli* cells (88). The presence of many cysteine residues especially those around the N-terminus end of the DHFR domain might be

another internal factor which facilitates the formation of inclusion bodies during translational process in *E. coli*.

The remainding nineteen mutants which expressed DHFRs as insoluble inactive protein were further characterized. Refolding of the inclusion bodies from these mutants yielded DHFR activities, but at relatively low levels compared to the wild-type enzyme. Two 108 mutants (S108N and S108T) yielded DHFRs with ~50% reduction in enzyme activity; seven mutants (S108G, S108A, S108Q, S108C, S108V, S108L, and S108M) gave poor DHFR activity; and nine mutants (S108I, S108R, S108P, S108D, S108H, S108Y, S108F, S108W and S108E) yielded undetectable DHFR activity (Table 4). It is noteworthy that the mutations at residue 108 of natural occurring *P. falciparum* isolates are from Ser (AGC) to Asn (AAC) or Thr (ACC), both of which occur at the second base of the codon. A single-base change at the second base of the codon might be crucial for active enzymes for parasite to survive or might just be a coincident phenomenon. Theoretically, second-base mutations of codon AGC which encodes for Ser would also yield AAC (Asn), ACC (Thr) and ATC (Ile). Indeed, Asn (AAC) has been observed in moderately pyrimethamine resistant parasites, and Thr (ACC) was found to be coexisted with A16V mutation in cycloguanil resistant parasites (28, 30, 31, 53, 61-63, 67). The observation that Ser (AGC) to Ile (ATC) mutation failed to produce active DHFR implied that second-base mutation might not be crucial for the development of drug-resistant parasites. Instead the data suggested the importance of the type of amino acids which would affect the structural stability of the enzyme.

The availability of DHFR mutants having residue 108 replaced by all possible amino acids has also allowed further investigation on the effect contributed by first-base mutation. Ser (AGC) could undergo first-base

mutation to CGC (Arg), GGC (Gly) and TGC (Cys) whereas Thr (ACC) would result in CCC (Pro), GCC (Ala) and TCC (Ser) which is the wild-type sequence. Characterization of the mutant enzymes with first-base mutation showed detectable DHFR activity from three out of five mutants (S108C, S108G, S108A), albeit at low activity, while the remaining two mutants (S108P and S108R) yielded inactive enzyme (Table 4). The failure to yield active enzyme might be due to the conformational interference from the side chain of the amino acids introduced in these mutants. The observation that the enzymes derived from the first-base mutation showed very poor or undetectable activity might also provide explanation as to why these mutants were not found in natural isolates.

The amino acid at residue 108 was crucial for enzyme catalysis. Considerable evidence from X-ray crystallographic studies of prokaryotic (36, 68, 69) and eukaryotic (70) DHFRs revealed that the polarity contributed by the hydroxyl group of Thr, a residue equivalent to Ser 108 of *P. falciparum* DHFR, is essential for the formation of hydrogen bonding to the adenine mononucleotide 5' phosphate moiety of the NADPH cofactor and van der Waals interaction with C_6 of the nicotinamide rings. Therefore, it is likely that the polarity contributed from the uncharged polar amino acids at residue 108 might be essential for the catalytic capability of the enzyme.

Four mutants (S108C, S108G, S108A and S108Q) were selected for further characterization due to their relatively higher level of DHFR activity as compared to other mutants (Table 4). Although these mutants do not exist in nature, there was nothing unusual with regard to the kinetic properties of the mutant enzymes except for S108Q mutant which showed high K_m values (~50 μM) for H_2folate (Table 6). The enzymes from all four mutants had 3-4 fold decrease in k_{cat} values as compared to the wild-type

enzyme, and had catalytic efficiency (k_{cat}/K_m) values comparable to those observed from mutant DHFRs with multiple mutations, i.e. C59R+S108N, N51I+C59R+S108N, C59R+S108N+I164L, or N51I+C59R+S108N+I164L described earlier (66). Both S108C (AGC to TGC) and S108G (AGC to GGC) are DHFR mutants with first-base mutations. Although the DHFR activity could be detected from the two mutants, the activities were very poor as compared to other mutants found in nature. As evidenced by its sensitivity toward antifolates, mutation of Ser (AGC) to Cys (TGC) did not provide any advantage to the parasites with respect to the survival in the presence of drug pressure in nature. Interestingly, the DHFR from S108G mutant was moderately resistant to Pyr and Cyc with IC_{50} values of the inhibitors slightly higher than that observed from DHFR of S108N mutant. Given the kinetic and inhibition data obtained, it is predicted that the S108G mutant could survive in nature and therefore raised the possibility of whether continuous use of high concentrations of Cyc in the treatment of malaria would lead to natural selection of Cyc-resistant *P. falciparum* through S108G mutation.

The S108A mutant containing Ser (AGC) to Ala (GCC) mutation requires two-base change within the same codon. Assuming a single-base mutation occurred at a frequency of approximately 10^{-6} - 10^{-7}, a mutation with two-base change would be anticipated to occur at extremely low frequency in nature, i.e. 10^{-14} or lower. The nonexistence of S108A mutant in nature was further supported by the following observations: firstly, the catalytic efficiency of the mutant DHFR was very poor and might not be sufficient for the survival of the parasites; secondly, the mutant enzyme was found to be sensitive to both Pyr and Cyc, the properties which were not advantageous to parasite survival under the environment of drug pressure.

Of most interest seems to be the S108Q mutant of which Ser (AGC) at residue 108 was replaced by Gln (CAG). Although the K_m for H_2folate of the S108Q mutant enzyme was about 4-fold higher than that observed from the wild-type enzyme, the increased in IC_{50} for antifolates Pyr and Cyc seemed to be advantageous to parasite survival and therefore parasite containing this mutation is likely to be able to survive in nature. However, the fact that Ser to Gln mutation requires triple-base change within the same codon makes Ser to Gln mutation an improbable path for mutant generation with even much lower frequency as compared to the double-base mutation, a situation which might explain the nonexistence of S108Q mutant in natural isolates.

Based on the data from the DHFR mutants at residue 108 thus far studied, serine (AGC) to asparagine (AAC) mutation was likely to be the most favorable mutation in nature. This might be supported in part by the observations that the kinetics and antifolate resistant properties of the DHFR from S108N mutant favored the survival of the parasite . Further, the G-to-A change of the second base in the codon of Ser is a transitional mutation which is commonly found in nature, and might therefore be another factor contributing toward the more frequent observations of S108N mutant in nature.

The availability of pure monofunctional *P. falciparum* DHFR has also permitted detailed characterization of the enzyme with respect to the type of inhibition by Pyr. Although the drug has been assumed to inhibit *P. falciparum* DHFR competitively, there have been controversial reports on the type of binding of Pyr to the sensitive and resistant enzyme (55-57, 64, 65). Dieckmann *et al* reported that Pyr competitively inhibits bifunctional DHFR-TS from both sensitive and resistant parasites (57), whereas Walter *et*

al showed that Pyr competitively inhibits enzyme from sensitive parasites and non-competitively inhibits the enzyme from resistant parasites (55). A contradictory result to that observed by Walter *et al* was subsequently reported by Chen *et al* (56). More recent work from Sano *et al* (64) reported that Pyr competitively inhibits synthetic DHFRs with wild-type sequence and S108T mutation while Prapunwattana showed that Pyr non-competitively inhibits the synthetic bifunctional DHFR-TS (65). In the present study, Pyr was clearly shown to competitively inhibit the synthetic DHFRs with wild-type sequence as well as two mutants with S108N and S108Q mutations (Figure 12). The discrepancy on the type of inhibition of *P. falciparum* DHFR by Pyr is not clearly understood. However, the variable patterns reported might be due in part to the quality of the enzyme used in different laboratories or might reflect the structural differences of the monofunctional versus bifunctional enzymes. Further experiments are needed in this case to understand the molecular level of inhibition of the enzyme by Pyr and Cyc.

In conclusion, the synthetic gene of *P. falciparum* DHFR domain has been a valuable system for the understanding of molecular mechanism of drug resistance, especially the roles contributed from point mutations of certain residues of *P. falciparum* DHFR domain. In the present work, we focused our studies on residue 108 as previous studies revealed that mutation of this residue was linked to Pyr resistance in malaria. By combinatorial cassette mutagenesis, mutants having residue 108 substituted by all 20 different amino acids were constructed and the DHFRs expressed were characterized. The results from this study suggested that mutation of Ser to Asn at residue 108 of the DHFR domain was presumably the best choice of point mutation in generation of Pyr-resistant phenotype. Moreover, the data from this study also predicted that mutant with S108G mutation

could possibly exist and that *P. falciparum* mutant containing S108G might be naturally selected under continuous use of high doses of Cyc. Although a clearer role of residue 108 must await the crystal structure of *P. falciparum* DHFR to be solved in the near future, the information gained from the present study would be useful in the interpretation of data from the X-ray structure of the enzyme, and provide ideas which could lead to the design of new antifolates with low propensity to develop drug resistance in the malaria parasite.

CHAPTER VI

SUMMARY

1. The synthetic gene encoding *Plasmodium falciparum* DHFR was exploited in the construction of DHFR mutants containing all possible amino acids at residue 108.

2. With the exception of S108K mutant which showed no expression, all other mutants expressed DHFRs as inactive inclusion bodies of molecular mass ~27 kDa. The inclusion bodies could be refolded to recover the DHFR activities.

3. Substitutions of Ser 108 of the DHFR with uncharged polar amino acids (Thr, Cys, Met, Asn, Gln) and hydrophobic amino acids (Gly, Ala, Val, Leu) yielded mutants with detectable DHFR activity. On the other hand, substitutions of Ser 108 with amino acids having charged polar side chain (Arg, Asp, His, Lys, Glu), amino acids having aromatic side chain (Phe ,Tyr, Trp), and amino acids with hydrophobic side chain (Ile, Pro) yielded mutants which gave inactive DHFRs.

4. Pyrimethamine and cycloguanil competitively inhibited the wild-type, S108N and S108Q mutant DHFRs.

5. Mutation of Ser to Asn at residue 108 of *P. falciparum* DHFR yielded an enzyme which was stable and resistant to antifolates, the properties which might be of favorable for parasite survival under selection by drug pressure.

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