CHAPTER I

INTRODUCTION

1.1 Properties of Dihydropteroate Synthase

Dihydropteroate synthase (E.C.2.5.1.15) is an enzyme catalyzing the formation of 7,8-dihydropteroate (DHP) from 2-amino-4-hydroxy-6-hydroxymethyl-7, 8-dihydropteridine pyrophosphate (DHPP) and p-amino-benzoic acid (PABA) in the biosynthetic pathway of tetrahydrofolate (Brown, Weisman and Molnar, 1961; Weisman and Brown, 1964; Shiota and Disrealey, 1961; Richey and Brown, 1969). One carbon derivatives of tetrahydrofolate participate in a wide variety of one-carbon transfer reactions for the metabolism of amino acids, purines, and pyrimidines. The reaction catalyzed by dihydropteroate synthase is shown in figure 1.

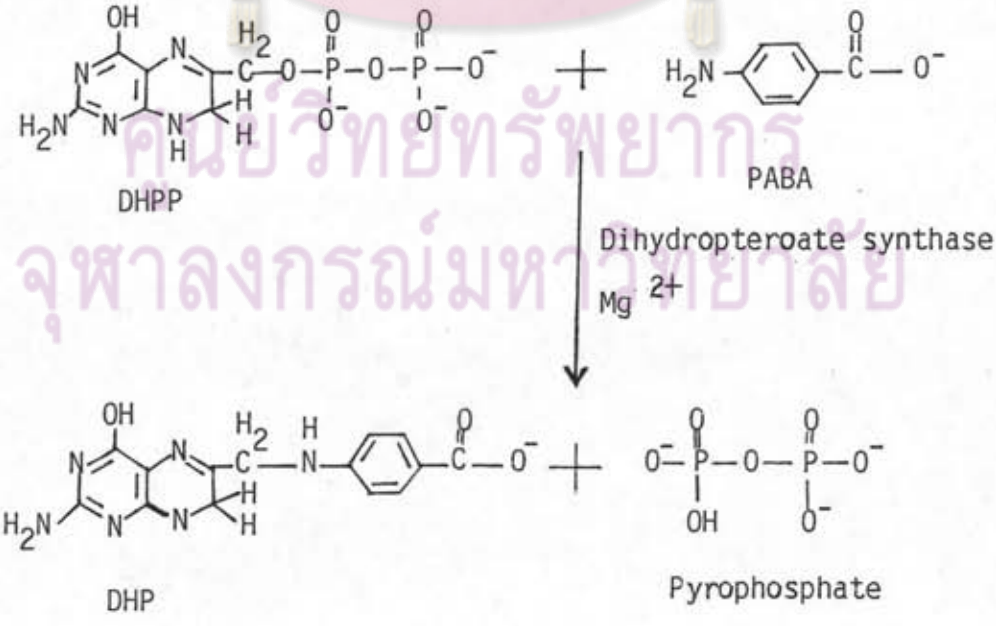


Figure 1 The enzymatic reaction catalyzed by dihydropteroate synthase

Table 1 Characteristics of dihydropteroate synthase from various microorganisms

Source	Molecular weight	Optimum pH	K_m for PABA (mol/l)
E. coli (Richey and Brown, 1969)	Approx. 50,000	8.5	2.5×10^{-6}
Veillonella strain V_2 (Shiota et al., 1964)		8.5	$(1.58\text{-}3.75) \times 10^{-4}$
S. indica (Iwai and Kobashi, 1975)	43,000	9.3	7.1×10^{-6}
P. berghei (Mc Collough and Maren, 1974)		8.5	2.8×10^{-6}
N. gonorrhaeae strain 7134 (Ho et al. 1974)			1.6×10^{-3}
N. meningitidis strain M-166 (Ho et al. 1974)			1.4×10^{-3}
D. pneumoniae strain R 6 (Ortiz, 1970)	75,000-95,000	8.0-8.2	3.3×10^{-6}
E. coli B S 5206 (Roland et al., 1979)			$(0.57 \pm 0.05) \times 10^{-6}$
E. coli mutaflor (Bock et al. 1974)	Approx. 45,000		
E. coli C strain C-167 (uracil$^-$, Str^r) (Swedberg et al. 1979)			5.1×10^{-6}

reports of Walter and Königk (1974), Iwai and Kobashi (1975), Richey and Brown (1969), Suckling, Sweeney and Wood (1977), Sai-Ubol (1983), and Bartels and Bock (1983). Walter and Königk (1974) purified the enzyme from Plasmodium chabaudi to 986 purification fold by using the following steps : crude extract, ammonium sulfate precipitation, chromatography on CM-cellulose, chromatography on hydroxyapatite, chromatography on DEAE-cellulose, chromatography on Sephadex G-200 and chromatography on DEAE-Sephadex. Iwai and Kobashi (1975) purified the enzyme from S. indica to 3.6% yield and 286 purification fold by using the following steps : crude extract, 50-70% ammonium sulfate fraction, chromatography on DEAE-cellulose and chromatography on Sephadex. Richey and Brown(1969) purified the enzyme from E. coli B to 52.4 purification fold by using the following steps : crude extract, RNase-treated extract, 20-70% ammonium sulfate fraction, 20-60% ammonium sulfate fraction, chromatography on Sephadex, chromatography on DEAE-cellulose and chromatography on dialyzed DEAE-cellulose. Suckling et al. (1977) purified the enzyme from E. coli to 180 fold only in one step by passing the crude enzyme through sulfonamide-Sepharose column (affinity column) in which the gel was activated by cyanogen bromide. The purification could be improved to 730 fold if the inhibitor and the spacer arm were attached to Sepharose by an s-triazine derivative rather than by the activation of the Sepharose with cyanogen bromide. Sai-Ubol (1983) purified the enzyme from E. coli K 12 ATCC 3110 to 209 fold and 7.74% yield by using the following steps : crude extract, 20-60% ammonium sulfate fraction and sulfonamide-Sepharose column (affinity column) in which the gel was activated by cyanogen bromide. Bartels and Bock (1983) purified the enzyme from E. coli to at least

3000 fold by using a Sephadex G-100 gel filtration, the affinity column of oxidized folate-Sepharose 4B and the hydrophobic interaction column of phenyl-Sepharose CL-4B; however, this purified enzyme shows at least 4 main bands and 10 side bands from ultra thin-layer isoelectric focusing polyacrylamide gel electrophoresis (IEF-PAGE).

1.3 Modification of Amino Acid Residues in Protein by the Modifying Agents

1.3.1 Iodoacetamide

Iodoacetamide has been known as the sulhydryl modification reagent. However, it can also modify lysine, methionine and histidine residues in proteins (Glazer et al. 1975). Iodoacetamide reacts with lysine residue at alkaline pH values at which lysine residue is partially unprotonated. Iodoacetamide reacts mostly with methionine residue at pH less than 4. Iodoacetamide reacts mostly with histidine residue at near neutral pH (Glazer et al. 1975).

1.3.2 p-Chloromercuribenzoic Acid (PMB)

PMB is an organic compounds of mercury. It was reported that it can react with the sulfhydryl group of cysteine residue in protein (Boyer, 1954; Benesch and Benesch, 1962; Deme, Trautmann, and Chatagner, 1971). The reaction of thiols with mercury resulting in highly undissociated mercaptides has been used extensively for the estimation of the amount of the sulhydryl groups both in small molecular thiols and in proteins (Boyer, 1954; Benesch and Benesch, 1962; Deme et al. 1971).

1.3.3 2,3-Butanedione

2,3-Butanedione reacts with the amino group of arginine side chains (Riordan, 1973; Glazer et al. 1975). Riordan (1973) studied the modification of arginine, lysine, and an equimolar mixture of these two compounds at pH 7.5 by 2,3-butanedione. He reported that (under the same conditions) lysine is not modified, and its presence does not interfere with the modification of arginine by 2,3-butanedione. His work with the modification of carboxypeptidase A by 2,3-butanedione based on amino acid analysis also supported that 2,3-butanedione modifies only arginine residues of the enzyme. However, Takahashi (1968) stated that 2,3-butanedione appears to react with ε-amino groups of lysine residues. Glazer et al. (1975) also summarized that 2,3-butanedione readily reacts with amino groups at the relatively high concentrations required for complete derivatization of the protein.

For the specificity of 2,3-butanedione, Riordan (1973) reported that the spectra of native and butanedione-modified carboxypeptidase in the region from 260 to 300 nm are identical, and this result would tend to rule out the possible reaction of 2,3-butanedione with phenylalanine, tyrosine, or tryptophan.

1.3.4 Phenylglyoxal

Phenylglyoxal reacts with the amino group of arginine side chains (Glazer et al. 1975, Takahashi, 1968). Takahashi (1968) also reported the specificity of reaction of phenylglyoxal with arginine. Of all amino acids, only histidine, cysteine, tryptophan, asparagine, glutamine, glycine, and lysine react with phenylglyoxal to the extent of more than 10% in 24 hours at pH values between 5.5 and 9.0. These

amino acids react with phenylglyoxal much more slowly than arginine. Lysine is almost unreactive at pH 5.5 and 7.0, however, it can react with phenylglyoxal to the extent of 20% at pH 8.0 and 9.0 in 24 hours.

1.3.5 2,4-Pentanedione

Gilbert III, and O'Leary (1975) studied the modification of enzyme by 2,4-pentanedione. It was found that 2,4-pentanedione reacts with primary amines at pH 6-9 to form the enamines (N-alkyl-4-amino-3-penten-2-ones). Selective modification of lysine and arginine side chains in proteins can readily be achieved with 2,4-pentanedione. The modification of lysine is favoured at pH 7 or by short-time reaction at pH 9.0, and the modification of arginine is favoured by long-time reaction at pH 9.0.

Gilbert III, and O'Leary (1975) investigated the possible reactions of 2,4-pentanedione with other amino acid side chains of proteins by studying the reactions of 2,4-pentanedione with the appropriate model compounds. It was found that 2,4-pentanedione does not react with phenol or with mercaptoethanol in aqueous solution at pH 9.0 for 48 hours. Gilbert III, and O'Leary (1975) concluded that 2,4-pentanedione is specific for lysine modification; however, it can modify arginine very slowly at higher pH.

1.3.6 Phenylmethylsulfonylfluoride (PMSF)

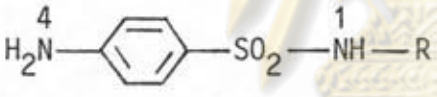
Glazer et al. (1975) reported that the derivatives of sulfonyl fluoride react only with cysteine and serine. The chemical evidence for the reaction of PMSF at cysteine residue in papain is indirect. However, the strong chemical evidence for the attack of PMSF at serine

residue in chymotrypsin (Gold and Fahrney, 1964) has been confirmed by the X-ray crystallographic structure of tosyl-chymotrypsin prepared from tosyl fluoride.

1.4 Mechanism of Action of Sulfonamide Drugs

The relationship of the chemical structure of medicinal agents to their biological activity is a fundamental problem of science, and sulfonamides have played a leading role in our current understanding of this relationship.

Sulfonamides are derivative of sulfanilamide (p-aminobenzene-sulfonamide). The general chemical structure and the numbering system of sulfonamides are shown below :



p-Aminobenzenesulfonamide is first synthesized as a dye for industrial use (Gelmo, 1908). Later, Heidelberger and Jacobs (1919) prepared one azosulfonamide and found that this compound has anti-bacterial property. Domark (1935a, 1935b) showed that a dye, 2',4'-diaminoazobenzene-4-sulfonamide (Prontosil), can be used as a therapeutic agent for the treatment of Pneumococcus and Streptococcus hemolyticus infections. Woods (1940) showed for the first time that synthetic p-aminobenzoic acid completely reverses the bacteriostatic activity of sulfonamide in vitro against many bacteria, and he postulated that sulfonamide interferes with the utilization of PABA in the enzyme systems necessary for the growth of bacteria through its similar structure. The antagonist effect has also been demonstrated

in vivo (Mc Illwain, 1942; Muir, Shamleffer and Jones, 1942). From the study of the inhibitory action of sulfonamides on enzymes in folate-synthesizing systems in bacteria (Shiota et al. 1964; Ho et al. 1975; Ho et al. 1974; Thijssen, 1973; Brown, 1962; Ortiz and Hotchkiss, 1966; Mc Cullough and Maren, 1973), protozoa (Ferone, 1973; Mc Cullough and Maren, 1974), and plants (Mitsuda and Suzuki, 1968), it is concluded that sulfonamides show their bacteriostatic activity by competing with PABA for the binding sites on dihydropteroate synthase.

Bell and Roblin (1942 and 1943) found a parabolic relation between bacteriostatic activity of sulfonamide and their respective pK_a values, and they advanced the theory that the bacteriostatic activity increases with the negative character of the sulfonyl group of a N^1-substituted sulfonamide derivative. Both ionic and molecular species are responsible for the total activity. Breuckner (1943) and Cowles (1942) proposed an alternative explanation that the sulfonamide molecule exclusively in the ionized form is responsible for the bacteriostatic action, but only the unionized form can readily penetrate the bacterial cell. However, the Bell and Roblin theory is supported by Thijssen (1974) who reported that some non-acidic N^1,N^1-dialkyl sulfonamides inhibit DHP synthesis in cell-free extract and these compounds show higher affinity for dihydropteroate synthase than their ionizeable monosubstituted parent compounds.

Seydel (1968) summarized the structure specificity of the activity of sulfonamides as the following :

1. The action of sulfonamides is antagonized by PABA containing an amino group in the 4 position of the sulfone group.

2. Exchange of the amino group of sulfonamides by H-, HO-, RO-, HOOC-, $H_2N-SO_2^-$, alkyl, or halogen substituents causes loss of activity or results in compounds which are not antagonized by PABA. N^4-derivatives are active if the free amino group is obtainable either hydrolytically or enzymatically.

3. Exchange of the benzene ring for other ring systems decreases the inhibitory activity.

4. Sulfonamides substituted in the benzene ring are mostly inactive; however, there are exceptions.

5. Exchange of the SO_2 NH group for NH_2-, -CN, $-SO_3H$, $-AsO_3H_2$, $-NH-CO-CH_3$, or $-NO_2$ results in inactive compounds. The activity maintains in some cases, however, it decreases if the sulfonamide group is exchanged for $-SO_2H$, $-SO_2C_6H_4-R$, $-SO-C_6H_4-NH_2$, $-S-S-C_6H_4-NH_2$, $-SO-SO-C_6H_4-NH_2$, $-S-(CH_3)_2$, $-SO-CH_2-C_6H_4-NH_2$, $-CO-NH_2$, $-CO-C_6H_4-R$, or $-PO(OH)_2$.

6. Simultaneous variation at the amino and sulfonamido group described in 2 and 5 results in in vivo active compounds.

Sulfonamide not only can compete with PABA for the binding site on the enzyme, but it also reacts with DHPP to form an analog. Bock et al.(1974) studied the action of [^{35}S] sulfamethoxazole in both cell-free and whole cell systems, and they were able to detect a radioactive product which shows identical R_f value with that of the chemically synthesized analog, N^1-3-(5-methylisoxazolyl)-N^4-(7,8-dihydro-6-pterinylmethyl) sulfanilamide. Swedberg et al.(1979) showed that dihydropteroate synthase from E. coli is able to catalyze the formation of a dihydropteroate analog from DHPP and sulfathiazole. Roland et al.(1979) showed that dihydropterin-sulfonamides are product

inhibition of dihydropteroate synthase; however, to obtain substantial inhibition of this enzyme by dihydropterin-sulfonamides in vivo, higher concentrations of these compounds are required than those which are attainable intracellularly. Therefore, the inhibition by dihydropterin-sulfonamides of dihydropteroate synthase may not be physiologically significant.

Sulfonamide not only can be used by itself alone for the treatment of bacterial infection, but it also can be used together with another antimicrobial agent for a better treatment. The examples are the adjunctive therapy of sulfonamide with pyrimethamine in the treatment of toxoplasmosis (Boyd, 1983) and the adjunctive therapy of sulfamethoxazole with pyrimethamine in the treatment of chloroquine-resistant falciparum malaria (Osol, 1980).

1.5 Hydrophobicity and Hydrophobicity of Amino Acid Side Chain

Nazaki and Tanford (1971) measured the hydrophobicity of the amino acid by solubility measurement. From solubility data, they calculated the free energy transfer of the solutes from water to aqueous ethanol and dioxane solutions. The free energy of transfer (ΔFt) has been defined as the change in the chemical potential of solute i in going from water to any other solvent, at the same mole fraction, at the limit of infinite dilution. The hydrophobicity of amino acid side chains (Δft) is obtained by subtraction of the ΔFt for glycine from ΔFt for the amino acid in question. The values are shown in table 2.

Table 2 Hydrophobicity scale for amino acid side chains.

Amino acid	Side chain group	Δft (cal/mol)
Glycine	-H	0
Alanine	$-CH_3$	500
Methionine	$-CH_2-CH_2-S-CH_3$	1300
Valine	$-CH(CH_3)-CH_3$	1500
Leucine	$-CH_2-CH(CH_3)-CH_3$	1800
Tyrosine	$-CH_2-C_6H_4-OH$	2300
Phenylalanine	$-CH_2-C_6H_5$	2500

Leo et al. (1971) defined the term "hydrophobic substituent constant (π) as :

$$\pi_X = \log P_X - \log P_H$$

P is the partition coefficient of a compound.

P_X is the derivative of a parent molecule, P_H.

The π values for the amino acid side chain of p-aminobenzene-sulfonamidoalkanoic acids are shown in table 3.

Fujita and Hansch (1967) Studied the correlation of the biological activity and hydrophobic substituent constant (π) of many sulfonamides. They found that the π values of meta and para derivatives of sulfanilanilides are correlated with the inhibitory activity of the compounds against Pneumococcus, Friedländer's Bacillus

Table 3 π value for the amino acid side chain of p-aminobenzenesulfonamidoalkanoic acids

p-Aminobenzenesulfonamidoalkanoic acids	Side chain group	π(1)
N-(p-aminobenzenesulfonyl) glycine	-H	0.00
N-(p-aminobenzenesulfonyl) alanine	$-CH_3$	0.50
N-(p-aminobenzenesulfonyl) methionine	$-CH_2-CH_2-S-CH_3$	1.45
N-(p-aminobenzenesulfonyl) valine	$-CH_2-CH_3$ CH_3	1.30
N-(p-aminobenzenesulfonyl) leucine	$-CH_2-CH-CH_3$ CH_3	1.80
N-(p-aminobenzenesulfonyl) tyrosine	$-CH_2-C_6H_4-OH$	1.96
N-(p-aminobenzenesulfonyl) phenylalanine	$-CH_2-C_6H_5$	2.63

(1) The π values are reported by Hansh and Coats (1970) and Leo et al.(1971).

and E. coli. The π values of substituted N^1-benzoylsulfanilamides are correlated with the inhibitory activity of the compounds against gram-negative E. coli and gram positive Mycobacterium smegmatis. The hydrophobicity of all the sulfonamides except for the para derivatives of sulfanilanilides plays a definite role in the inhibitory acitivity of the compounds. The π values of the para substituents of sulfanilanilides play practically no role in the inhibitory activity against Pneumococcus.

Miller et al.(1972) studied the relationship between the hydrophobic substituent constant (π) of the two series of substituted sulfonamides (N^1-phenyl and N^1-pyridyl) and MIC (minimum inhibitory concentration) values for the inhibition of the growth of E. coli. They also studied the relationship between the π values and I_{50} (concentration required for 50% inhibition) values for the inhibition of dihydropteroate synthase from E. coli. They found that there is no linear relationship between π and MIC or I_{50}. Foye, Kauffman and Suttimool (1982) studied the inhibitory effect of sodium 4-aminobenzenesulfonamidoethanethiosulfate, sodium 4-aminobenzenesulfonamidopropanethiosulfate and 4-aminobenzenesulfonamidopropyl bromide on dihydropteroate synthase from sulfonamide-resistant Neisseria gonorrheae. They found that an increase in hydrophobic nature of the sulfanilamide structure does not increase the inhibitory activity against this enzyme.

1.6 The Research Purpose

p-Aminobenzenesulfonamidoalkanoic acids, a group of sulfanilamide derivatives, were chosen for study in this research because each compound contains the carboxyl group which enhances the solubility of the compound in water. In addition, the hydrophobicity values of the side chains of these compounds are reported; therefore, the relationship between the hydrophobicity and the inhibitory activity against dihydropteroate synthase can be studied.

The purposes of this research are :

1.6.1 To study the inhibitory activity of p-aminobenzenesulfonamidoalkanoic acids against dihydropteroate synthase partially

purified from E. coli so that the role of the hydrophobicity on the inhibitory activity of the compounds may be obtained.

1.6.2 To study the modification of amino acid side chains in dihydropteroate synthase so that the possible nature of the enzyme active site may be obtained.