CHAPTER V

DISCUSSION

In this research the method of Suckling et al. (1977) was chosen to use for the purification of dihydropteroate synthase from E. coli strain K12 ATCC 3110 because this method is rapid and a rather high purification of 730 fold can be obtained only in one step. Bartels and Bock (1983) purified the enzyme from E. coli to at least 3000 fold, however, this method is composed of many steps of purification and the enzyme obtained still shows many bands from IEF-PAGE.

The principle of the enzyme purification method of Suckling et al. (1977) is affinity chromatography. The gel (Sepharose 4B) in the affinity chromatography is activated by 2,4-dichloro-6-methoxy-s-triazine (Lang et al. 1977). The gel activated with 2,4-dichloro-6-methoxy-s-triazine shows the outstanding stability of triazinylagarose materials (Lang et al. 1977). Although the most widely used method for attaching ligands to agarose employs cyanogen bromide for the activation of the carbohydrate, the gel activated with cyanogen bromide had limited stability due to the hydrolysis of the bound ligands.

In this research, the principle of the enzyme purification by sulfonamide-Sepharose column (I) was affinity chromatography. In a column, 4-(4-aminobenzenesulfonamido) benzenesulfonylglycine, a competitive inhibitor (Suckling et al. 1977) was used for specific binding to the enzyme while the other proteins were eluted because these proteins could not bind to this inhibitor. Since

the mechanism of action of the enzyme is an ordered mechanism in which DHPP is the first substrate and PABA is the second substrate to bind to the enzyme, (Ferone and Webb, 1975; Suckling et al. 1977). Therefore DHPP was included in buffer I. DTT in buffer I prevents the oxidation of DHPP. Magnesium ions was used to enhance the binding of the enzyme with the inhibitor since Swedberg et al. (1979) reported the dependence of the dihydropteroate-forming reaction on magnesium ions. He also reported that the dialyzed enzyme extracts were inactive in the absence of magnesium ions, and the magnesium-dependent reaction could be stopped completely by the addition of ethylenediamine-tetraacetic acid (2.5 mmol/l). The spacer arm bis-(3-aminopropyl) amine was used to enhance the binding of the inhibitor to the enzyme.

The method of the enzyme purification by the sulfonamide-Sepharose column (I) was modified from the method of Suckling et al. (1977). The modification was that sodium chloride (0.5 mol/l) was added in buffer II in this research, whereas it was absent in the method of Suckling et al. (1977). The reason for the addition of sodium chloride in buffer II was given below.

Firstly, the method of Suckling et al. (1977) was tried, but it was found that the enzyme could not be eluted out from the column. Secondly, the method in which sodium chloride (0.5 mol/l) was added into both buffers was tried, but the result was unsatisfactory. 55% of the enzyme activity was eluted with buffer I and migrated with the unwanted protein. When the concentration of sodium chloride in buffer I and buffer II was reduced to 0.2 mol/l, the loss of the enzyme activity with buffer I was 31%. Thirdly, when sodium chloride (0.5 mol/l) was present only in buffer II, 106 purification fold and 33% yield were obtained.

Therefore, this method was used in this research. Finally, when the linear gradient of sodium chloride from 0 to 1 mol/l in Tris buffer (0.2 mol/l, pH 8.5) was used instead of sodium chloride (0.5 mol/l) in Tris buffer (0.2 mol/l, pH 8.5) (buffer II), the results of 20 purification fold and 43% yield were obtained. The purification fold was decreased because the unwanted protein peak in front of the enzyme peak was broadened resulting in overlapping with the enzyme peak.

Although the principle of the enzyme purification in sulfonamide-Sepharose column (I) was a specific binding between the enzyme and its inhibitor, the non-specific binding of the other proteins with the activated Sepharose also occurred. These proteins could be seen from polyacrylamide gel electrophoresis. The non-specific binding may occur from the interaction between the positively charged amino group of the spacer arms and the negatively charged proteins. The amino groups of the free spacer arms were partially protonated since the column was performed with the buffer of pH 8.5.

The concentration of PMSF used for the enzyme modification in this research was the highest concentration which could be prepared because PMSF was unlikely to dissolve in water and it had limited solubility in iso-propanol. The concentration of iso-propanol must also be kept in a certain limit, if the iso-propanol concentration was higher than that used in the experiment, the enzyme activity would be decreased.

Our results indicated that cysteine, arginine and lysine residues were important for enzyme catalysis. It was possible that these residues might exist at the enzyme active site. The optimum pH of the enzyme was 8.55, and the pKa value of sulfhydryl group of cysteine is 8.3 which is nearly equal to the optimum pH of the enzyme.

Hence, at this optimum pH, cysteine residue would react as both general acid and general base. At the optimum pH of the enzyme arginine (pKa = 12.5) and lysine (pKa = 10.8) residues would contain the positive charges while DHPP would contain the negative charges. Hence, the interaction of the opposite charges would render DHPP to bind with these amino acid residues at the active site. However, under the experimental condition in this research the pKa values of these side chains in the enzyme active site might not be the values indicated above due to the interactions of the neighbouring groups in the active site. Iwai and Kobashi (1975) reported that dihydropteroate synthase from S. indica could be modified by PMB which is the modifying agent of sulfhydryl group of cysteine. This result was agreeable to our result in which dihydropteroate synthase from E. coli could be modified by PMB. Thus, cysteine residue might be present at the enzyme active site.

Gomi and Fujioka (1982) reported that the compounds containing the amino and the carboxyl groups are capable of reacting with idoacetamide. However, our results indicated that the substrates (DHPP and PABA) and the inhibitor (sulfanilamide) containing the amino and the carboxyl groups were unlikely to react with iodoacetamide. The reason might be that the amino groups or the carboxyl group of the substrates are attached to the aromatic rings and the aromaticity might cause these groups unreactive toward iodoacetamide.

Although amino group could be modified by 2,4-pentanedione, 2,3-butanedione and phenylglyoxal (Gilbert III and O'Leary, 1975; Glazer et al. 1975; Takahashi, 1968), our results indicated that the substrates (DHPP and PABA) and the inhibitor (sulfanilamide) containing the amino groups were unlikely to react with those modifying agents.

Glazer et al. (1975) reported that, for the protein modification by iodoacetamide in the presence of mercaptoethanol, the sulhydryl groups of cysteine residues in protein react most rapidly with iodoacetamide, and the excess iodoacetamide can react further with mercaptoethanol. In this research, it was found that, in addition to iodoacetamide, PMB might react with 2-mercaptoethanol.

Gilbert III and O'Leary (1975) reported that 2,4-pentanedione does not react with phenol or with mercaptoethanol in aqueous solution at pH 9.0 for 48 hours. In this research, it was found that 2,4-pentanedione was unlikely to react with 2-mercaptoethanol. This result was agreeable to that of Gilbert III and O'Leary.

From the experiments of the enzyme protection from all modifying agents by DHPP and by sulfanilamide in the presence of DHPP, it was found that % control (E + M + R + DHPP) was higher than % control (E + M + R) or % control (E + M). This result indicated that cysteine, arginine and lysine residues may exist at the enzyme active site.

The purpose of the protection of the enzyme by sulfanilamide in the absence of DHPP was to investigate whether sulfanilamide itself in the absence of DHPP could protect the enzyme from the modifying agents. The result indicated that sulfanilamide itself could not protect the enzyme from the modification. Ferrone and Webb (1975) and Suckling et al. (1977) reported that the action of enzyme is an ordered mechanism in which DHPP is the first substrate to bind to the enzyme and PABA is the second substrate. Since sulfanilamide has the structure similar to PABA, it may be possible that sulfanilamide could not bind to the enzyme unless DHPP binds first to the enzyme. Hence, sulfanilamide itself in the absence of DHPP could not protect the enzyme from the modifying agents.

The optimum pH for dihydropteroate synthase from E. coli K12 ATCC 3110 was 8.55, and the K_m value for PABA was found to be 1.30×10^{-6} mol/l. Richey and Brown (1969) reported that the optimum pH of the enzyme is 8.5 and the K_m for PABA is 2.5×10^{-6} mol/l. The source of the enzyme is dihydropteroate synthase from E. coli B, the purification fold of the enzyme is 52.4. Roland et al. (1979) reported that the K_m value for PABA was $(0.57 \pm 0.05) \times 10^{-6}$ mol/l, and dihydropteroate synthase was partially purified from E. coli B. The enzyme was eluted from a Sephadex G-100 column with a specific activity of 1 to 2 nmol/min/mg of protein. However, the purification fold was not reported. Swedberg et al. (1979) reported that the K_m value for PABA was 5.1×10^{-6} mol/l, and dihydropteroate synthase from E. coli C strain C-167 ($uracil^-$, Str^r) was partially purified by the method of Richey and Brown (1969). The purification fold was not reported.

The Ki value for sulfanilamide obtained from this research was 4.8×10^{-5} mol/l. Roland et al. (1979) partially purified dihydropteroate synthase from E. coli B by Sephadex G-100 column, and they reported the K_i value for sulfanilamide of $(5.7 \pm 0.5) \times 10^{-6}$ mol/l.

The discrepancy of these results of the enzymatic properties (optimum pH, K_m, and K_i) may be due to the different purities of the enzyme, the different sources of the enzyme and the different conditions used in the experiments.

The Δft value and the π values were used for the indication of the hydrophobicity of p-aminobenzenesulfonamidoalkanoic acid side chains. The Δft values are more reliable than the π values because all Δft values of the amino acid side chains are obtained directly from the experiment (Nazaki and Tanford, 1971), whereas the π values of the amino

acid side chains are calculated from the π values of the side chains of the other compounds (Hansch and Coats, 1970; Leo et al. 1971). However, the Δft values and the π values of p-aminobenzenesulfonamidoalkanoic acids have similar pattern.

In this research, it was found that p-aminobenzenesulfonamidoalkanoic acids exhibiting the potent inhibitors might contain the aliphatic side chains possessing very low value of Δft or π.

Kongkiattikajorn (1984) determined the concentration required for 50% inhibition (I_{50}) of these seven p-amibobenzenesulfonamidoalkanoic acids. The crude dihydropteroate synthase from E. coli K12 ATCC 3110 was used as an enzyme source. We found that the I'_{50} values of these compounds and the K_i values have similar pattern.

The relationship between π or Δft and K_i of p-aminobenzenesulfonamidoalkanoic acids was not linear. The non-linear relationship was also observed between π and I_{50} of N^1-phenyl and N^1-pyridyl sulfonamides (Miller et al. 1972).

Foye et al. (1982) studied the effect of sodium 4-aminobenzenesulfonamidoethanethiosulfate and sodium 4-aminobenzenesulfonamidopropanethiosulfate on the enzyme activity. They concluded that an increase in the hydrophobic nature of these sulfonamides did not change the inhibitory activity against the enzyme from a sulfanilamide-resistant strain of Neisseria gonorrhoeae. This result was inconsistant with our result in which an increase of the hydrophobicity of the aliphatic side chains of p-aminobenzenesulfonamidoalkanoic acids would decrease the enzyme inhibitory activity. This inconsistency might be resulted from the different sources of the enzyme and the different kinds of sulfonamides. The thiosulfate compounds used in the experiment of Foye et al. posses the negative charges and these charges might play more important role

than the hydrophobicity in binding of the compounds to the enzyme. In addition, only two compounds were tested, therefore the relationship between the hydrophobicity and the inhibitory activity can not be concluded.

Thijsen (1977) studied the role of the hydrophobicity of the α-phenyl derivative of β-p-aminobenzoylpropionic acid in the interaction with dihydropteroate synthase from E. coli B. He found that the Ki values of α-phenyl-β-p-aminobenzoylpropionic acid and β-p-amino-benzoylpropionic acid are similar, hence, the hydrophobicity of these two compounds does not play an important role in the interaction with the enzyme.

Summary

1. Dihydropteroate synthase from E. coli strain K12 ATCC 3110 was partially purified by the following steps : crude cell-free extract, ammonium sulfate precipitation (30-70% saturation fraction), sulfonamide-Sepharose column (I) and sulfonamide-Sepharose column (II). The purification was 417 fold and 14% yield was obtained. However, the polyacrylamide gel electrophoresis pattern showed at least 6 bands of proteins. The optimum pH of the enzyme was 8.55 and the optimum temperature was 42^{o} C.

2. The active site of the enzyme might contain the amino acid residues of cysteine, arginine and lysine.

3. The K_m value for PABA was 1.30×10^{-6} mol/l. The K_i values for N-(p-aminobenzenesulfonyl) glycine, N-(p-aminobenzenesulfonyl) tyrosine, N-(p-aminobenzenesulfonyl) alanine, N-(p-aminobenzenesulfonyl) phenylalanine, N-(p-aminobenzenesulfonyl) methionine, N-(p-aminobenzenesulfonyl) leucine and N-(p-aminobenzenesulfonyl) valine were 4.0×10^{-5}, 13.8×10^{-5},

23.5×10^{-5}, 24.0×10^{-5}, 49.3×10^{-5}, 84.0×10^{-5} and 100.0×10^{-5} mol/l respectively. The K_i value for sulfanilamide was 4.8×10^{-5}, mol/l. All the compounds exhibited competitive inhibitors.

4. The $1/K_i$ values for p-aminobenzenesulfonamidoalkanoic acids had the non-linear relationship with the hydrophobicity values (Δft or π). The 1/Ki value for N-(p-aminobenzenesulfonyl) glycine in which the Δft and the π values for the amino acid side chain of this compound equal to zero was the highest value in the curve. It was found that the $1/K_i$ values were decreased when the Δft values were between 0-1500 cal/mol or the π values were between 0-1.5 units. However, the $1/K_i$ values were slightly increased when the Δft values were between 1500-2500 cal/mol or the π values were between 1.5-2.63 units. Therefore, the hydrophobicity of the compounds may play an important role in the enzyme inhibition.

Suggestion

1. It was found that the $1/K_i$ values were slightly increased when the Δft values or the π values of the amino acid side chains of p-aminobenzenesulfonamidoalkanoic acids were between 1500-2500 cal/mol or 1.5-2.63 units respectively. Thus, p-aminobenzenesulfonamidoalkanoic acids in which the Δft values or the π values of amino acid side chains were higher than 2500 or 2.63 units respectively should be selected for further study to see whether these compounds can exhibit the potent enzyme inhibitors.

2. In the experiment, only one series of inhibitors was tested. Thus, the different series of inhibitors should be tested to find out the relationship between the inhibitory activity and the hydrophobicity of the side chains. This study might lead to the general conclusion of

the effect of hydrophobicity on the enzyme activity, and the most suitable hydrophobicity might be obtained.

3. It was found that the active site of dihydropteroate synthase may contain the amino acid residues of cysteine, arginine and lysine. Therefore, the irreversible inhibitors might be obtained if the inhibitors posses the groups capable to react irreversibly with those amino acid residues in the enzyme.