Sulfonylurea is a Non-competitive Inhibitor of Acetohydroxyacid Synthase from *Mycobacterium tuberculosis*

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Received July 5, 2006

Key Words : Acetohydroxyacid synthase, *Mycobacterium tuberculosis*, Inhibition mechanism, Sulfonylurea

Acetohydroxyacid synthase (AHAS; EC 2.2.1.6) catalyses the first step in the biosynthesis of branched-chain amino acids (BCAAs).[1] AHAS catalyzes the condensation of two molecules of pyruvate to form 2-acetolactate in the biosynthesis of valine and leucine or the condensation of pyruvate and 2-ketobutyrate to form 2-aceto-2-hydroxybutyrate in the biosynthesis of isoleucine (Fig. 1). AHAS consists of two subunits, the catalytic subunit and the regulatory subunit. The enzymatic activity of AHAS is produced from the catalytic subunit,[1,2] while the regulatory subunit affects feedback regulation and activation of the catalytic subunit. AHAS requires three cofactors for activity: thiamin diphosphate (ThDP), divalent metal ion (usually Mg^{2+}), and flavin adenine dinucleotide (FAD).[3,4] The first two cofactors are typical for the ThDP-dependent enzymes in the catalysis.[5]

Plants and many microorganisms have the capacity to synthesize the BCAAs needed for their survival from inorganic precursors. The BCAAs belong to the essential amino acids, and animals must obtain these compounds from their diet because they lack the full biosynthetic machinery for the BCAAs. Therefore, AHAS is an attractive target enzyme for development of herbicides and antimicrobial drugs. Until now, however, there has been little interest in the development of AHAS inhibitors as antimicrobial agents because most organisms would be able to overcome the effects of these inhibitors by obtaining the BCAAs from their environment. Recently, it has been shown that BCAAs auxotrophic strains of *Mycobacterium tuberculosis* are attenuated in mice because of the inability to use the BCAAs from their host.[6] *Mycobacterium tuberculosis* is the pathogen of tuberculosis which is a chronic disease and a major threat to the human population even in the developed world because of the spread of strains with multiple drug resistance.[7] In 1998, Grandoni *et al.* showed that selected sulfonylurea herbicides effectively inhibit the growth of several *M. tuberculosis* strains.[8] Sulfonylureas and imidazolinones are known to efficiently inhibit plant AHAS[9] and are used as herbicides that have been well characterized and are currently available for use on crops.[10]

In 2005, we purified and characterized recombinant AHAS from *M. tuberculosis* H37Rv strain.[11] The K_m and k_{cat} values of the catalytic subunit of AHAS from *M. tuberculosis* were 2.76 mM and 3.19 s^{-1}, respectively.[11] We also tested the inhibition potency using ten sulfonylureas and four imidazolinones. Among the 14 tested compounds, only pyrazosulfuron ethyl (PSE, IC_{50} = 0.87 μM), primisulfuron methyl (PSM, IC_{50} = 4.19 μM), sulfometuron methyl (SMM, IC_{50} = 4.79 μM), metsulfuron methyl (MSM, IC_{50} = 5.96

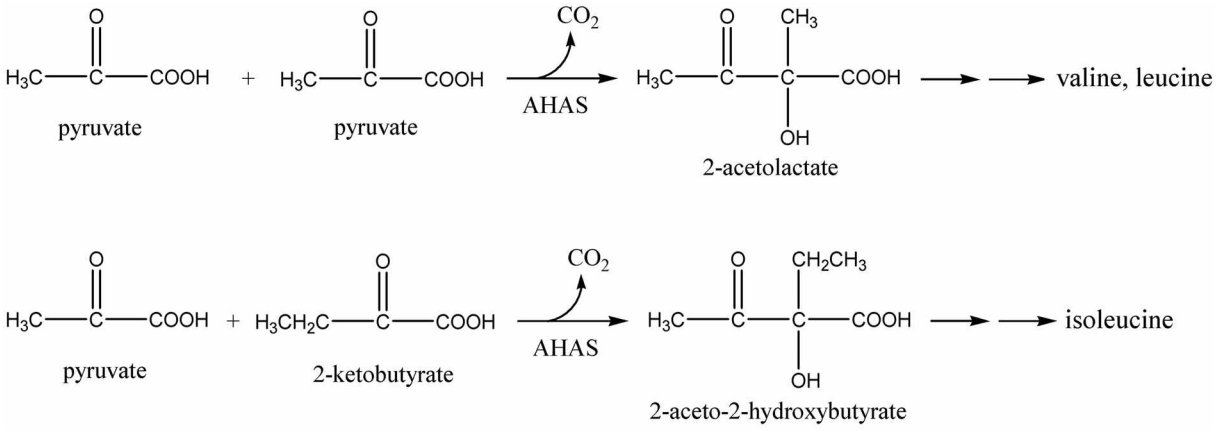

Figure 1. Reactions catalyzed by acetohydroxyacid synthase.

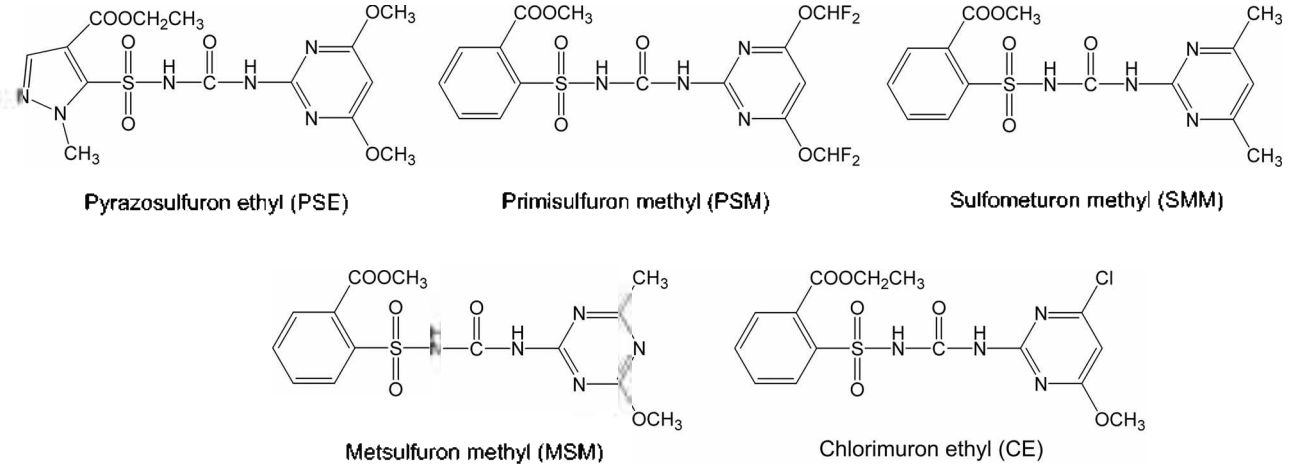

Figure 2. Structures of sulfonylurea tested for inhibition study.

μM), and chlorimuron ethyl (CE, IC_{50} = 8.97 μM) inhibited more than 80% of the activity at 40 μM.[9] In this study, we have further analyzed the inhibition mechanism for these five potent inhibitors (Fig. 2). The basic structure of the sulfonylurea herbicides is X-SO_2-NH-CO-NH-Y, where X is usually a substituted phenyl group and Y is a substituted pyrimidine or triazine ring. Although the structures of sulfonylureas can vary considerably, most consist of an ortho-substituted aromatic ring linked to either a meta di-substituted pyrimidine or triazine ring. Initial rates of the catalytic subunit of acetohydroxyacid synthase from *M. tuberculosis* (mAHAS) in the presence of herbicides were measured as a function of the concentration of pyruvate at fixed inhibitor concentrations. All sulfonylurea herbicides tested in this experiment were non-competitive inhibitors for the catalytic subunit of *M. tuberculosis* AHAS. All data including initial rates were fitted to noncompetitive inhibition equation (1) using the BASIC program designed by the algorithms of Cleland[12] and are shown in the form of a double-reciprocal plot (Fig. 3). The solid lines in Figure 3 shows the linear fit lines of the calibrated initial rates calculated from the BASIC program of Cleland. These fittings yielded the inhibition constants like as the following values: PSE (K_{is} = 3.6 ± 0.8 μM, K_{ii} = 7.1 ± 0.9 μM), PSM (K_{is} = 9.4 ± 3.3 μM, K_{ii} = 30.7 ± 5.2 μM), SMM (K_{is} = 1.9 ± 0.5 μM, K_{ii} = 11.6 ± 3.1 μM), MSM (K_{is} = 9.0 ± 2.9 μM, K_{ii} = 66.7 ± 17.7 μM), CE (K_{is} = 2.7 ± 0.7 μM, K_{ii} = 9.8 ± 1.7 μM). At any sulfonylurea concentration in the non-competitive inhibition, a portion of the enzyme will remain as nonproductive AHAS-pyruvate-sulfonylurea complex form. So, we could observe in the Figure 3. that the V_{max} in the presence of sulfonylurea is less than the V_{max} observed in the absence of sulfonylurea. The K_m value is not changed by the addition of sulfonylurea because, at any sulfonylurea concentration, the enzyme forms that can combine with pyruvate have equal affinities for pyruvate.

In general, sulfonylureas have been reported to act non-competitively as in the inhibition of *Arabidopsis* AHAS by chlorsulfuron,[13] *barley* AHAS by chlorsulfuron,[14] and *E.coli* AHAS I,II,III by sulfometuron methyl and chlorsulfuron.[15,16] However, nearly competitive inhibition[17] or uncompetitive inhibition[18] have also been reported. In some case, these diverse mechanisms for the inhibition of sulfonylurea may be a misleading experimental artifact arisen from the differences in the enzyme assay method or the analysis of the results. Despite the availability of the sulfonylurea and imidazolinone bound AHAS crystal structures,[10,19,20] the mechanism of inhibition by these herbicides is still not understood. Inhibition of the AHAS reaction was known as a time-dependent and biphasic process with an initial weak inhibition followed by a slow transition into a final steady-state where the inhibition is more potent.[9,21] These reasons would also make it more difficult to analyze the mechanism of inhibition.

Chang *et al.* revealed that AHAS from *Arabidopsis thaliana* was strongly inhibited by the sulfonylurea herbicide, chlorsulphuron, and the imidazolinone herbicide, imazapyr.[9] Moreover, the crystal structures of these herbicides in complex with *A.thaliana* AHAS have been determined.[10] Both classes of herbicide were bound within the channel leading to the active site and blocked the substrate access. A interesting thing of these reports is that two herbicides of sulfonylurea and imidazolinone are sharing the binding sites.[10,22] Recently, inhibition of the mycobacteria by these herbicides has been showed that their influences were something different from that of *A. thaliana* AHAS.[11,23] The sulfonylurea herbicide strongly inhibited the mycobacteria AHAS. However the imidazolinone herbicide, which is useful herbicides on the *A.thaliana* AHAS, hardly inhibited the mycobacteria enzyme. Although the sulfonylurea on the *A. thaliana* AHAS followed the non-competitive inhibition mechanism, we don't know whether or not that the same herbicide on the mycobacteria AHAS will act as the non-competitive inhibition. In this study, we observed that inhibition of AHAS from *Mycobacterium tuberculosis* by sulfonylurea in non-competitive. So we can, at least, estimate that the sulfonylurea can bind to either free enzyme or substrate binding enzyme in the active site. Previous report

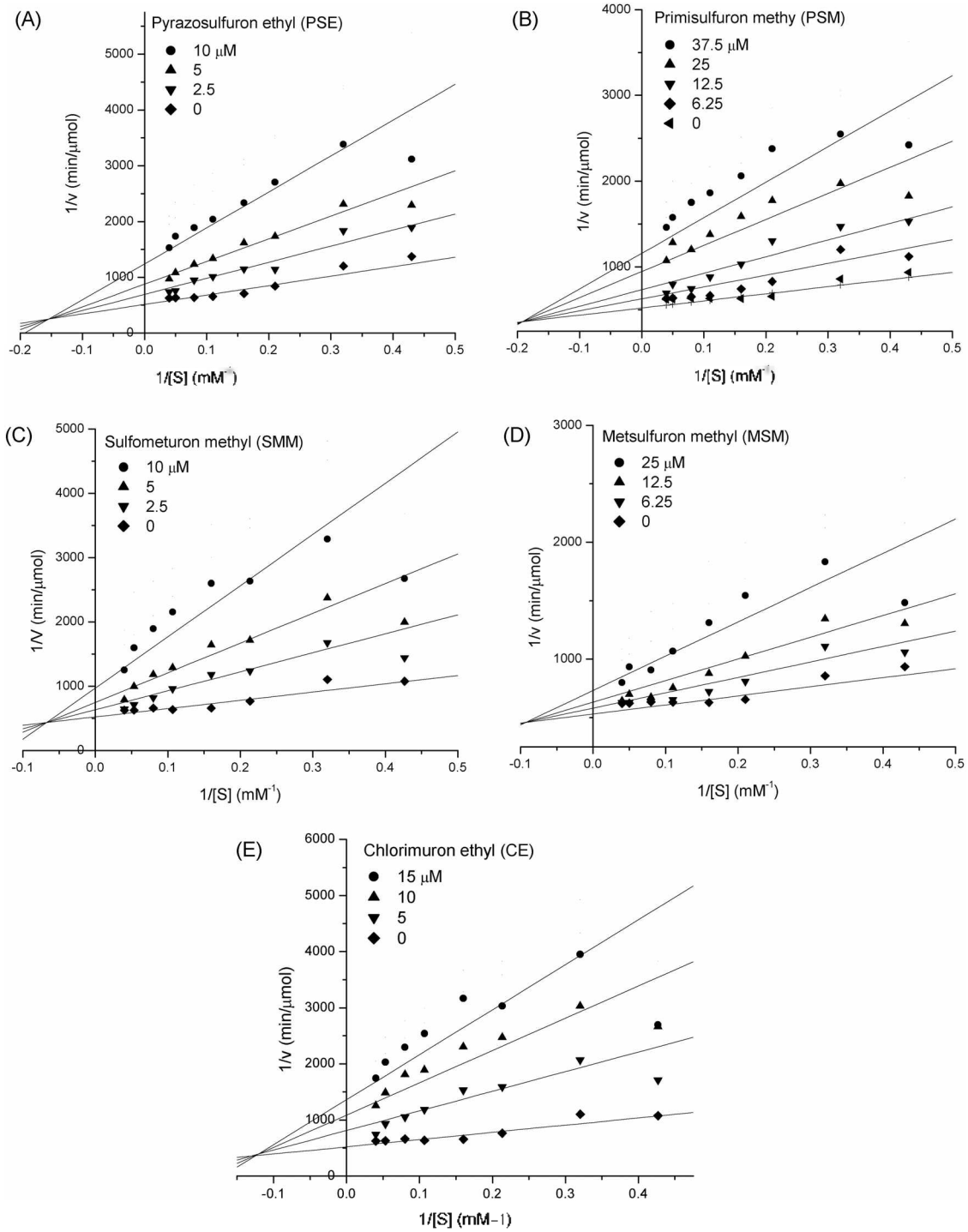

Figure 3. Inhibition of *M. tuberculosis* AHAS by sulfonylureas. Initial rates were measured as a function of the concentration of pyruvate at fixed inhibitor concentrations. The data were fitted to Eq. (1) and is shown in the form of a double-reciprocal plot. The inhibition constants are: (A) PSE (K_{is} = 3.6 ± 0.8 μM, K_{ii} = 7.1 ± 0.9 μM); (B) PSM (K_{is} = 9.4 ± 3.3 μM, K_{ii} = 30.7 ± 5.2 μM); (C) SMM (K_{is} = 1.9 ± 0.5 μM, K_{ii} = 11.6 ± 3.1 μM); (D) MSM (K_{is} = 9.0 ± 2.9 μM, K_{ii} = 66.7 ± 17.7 μM); (E) CE (K_{is} = 2.7 ± 0.7 μM, K_{ii} = 9.8 ± 1.7 μM).

for the mechanism of inhibition showed that herbicide-induced inactivation may correspond to a change in enzyme conformation disfavoring the V conformation of ThDP cofactor.[24] However, we did not show in this study that ThDP itself makes any influence for the inhibition of mycobacteria AHAS by herbicide. Although inhibition mechanism for the sulfonylurea is identical with that from plant AHAS, further studies will be required to determine how strong binding of ThDP to mycobacteria AHAS in the presence of the sulfonylurea. We guess that dissociation constant of ThDP in existence and nonexistence of the sulfonylurea will be determined by monitoring the quenching of the intrinsic

fluorescence of the enzyme after addition of increasing concentrations of ThDP.[25] This approach for understanding the mechanism of inhibition will extend the possibilities for the design of new inhibitors and the development of new anti-TB drugs.

Experimental Section

Enzyme purification and assay. The catalytic subunit of acetohydroxyacid synthase from *M. tuberculosis* (mAHAS) was purified by Ni^{2+}-charged His tag affinity chromatography as described previously.[11] The AHAS activity was also measured according to the cited reference.[11] Briefly, initial rates were determined at 37 °C for 1 hr in a reaction mixture containing 100 mM potassium phosphate, pH 7.5, 100 mM sodium pyruvate, 10 mM $MgCl_2$, 1 mM ThDP, and 50 μM FAD. The mAHAS and reaction mixture were pre-incubated for 15 min, respectively. The reaction was initiated by adding a mAHAS (0.5 μg) to the reaction mixture (final 200 μL) and was stopped after 1 hr by addition of 30 μL of 4N-H_2SO_4, and further incubated at 65 °C for 15 min. Then 100 μL of reaction product was mixed with 90 μL of 0.5% (w/v) creatine and 90 μL of 5% (w/v) α-naphtol (in 2.5 M NaOH, freshly prepared) individually batched, and incubated at 65 °C for 15 min. The absorbance of the red-colored complex ($\varepsilon_{525nm} = 20{,}000\ M^{-1}cm^{-1}$) was measured at 525 nm using a Lambda25 UV/VIS Spectrometer (Perkin-Elmer, Wellesley, USA). One unit (U) of activity is defined as that producing 1 μmol of acetolactate per minute under the standard conditions.

Determination of inhibition constant. Initial rates of mAHAS in the presence of herbicides were determined by the standard assay condition as described above. The reaction mixture consisted of 100 mM potassium phosphate, pH 7.5, 1 mM ThDP, 10 mM $MgCl_2$, 50 μM FAD, sodium pyruvate (2.3-25 mM), and several concentrations of herbicides (5 μL). All of herbicides are dissolved in DMSO, and the final percent concentration of DMSO in the reaction mixture is 2.5%. The inhibition constants of the 5 potent herbicides were determined using the BASIC program designed by the algorithms of Cleland.[12] All five potent herbicides followed the non-competitive inhibition mechanism, and the K_{is} and K_{ii} values of these herbicides were calculated by fitting Eq. (1) to the data. In this equation, [S] and [I] are the concentrations of pyruvate and sulfonylurea inhibitor, respectively, and K_{is} is a slope inhibition constants, whereas K_{ii} is an intercepts inhibition constant.

$$\frac{1}{V} = \frac{K_m}{V_{max}}\left(1 + \frac{[I]}{K_{is}}\right)\frac{1}{[S]} + \frac{1}{V_{max}}\left(1 + \frac{[I]}{K_{ii}}\right) \quad (1)$$

Aknowledgement. This work was supported in part by research grants from Taegu University (2005).

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