Potent Transition-State Analogue Inhibitor of *Esherichia coli* Asparagine Synthetase A

Jun Hiratake, Mitsuteru Koizumi, Toru Nakatsu, Hiroaki Kato, and Jun'ichi Oda

A potent and slow-binding inhibitor of *E. coli* asparagine synthetase A (AS-A) was synthesized, and its inhibition behavior was characterized. The enzyme complexed with the inhibitor was analyzed by X-ray diffraction analysis to identify several key amino acid residues responsible for catalysis as well as for substrate recognition. AS-A catalyzes the formation of L-Asn from L-Asp and ammonia coupled with the hydrolysis of ATP to AMP and pyrophosphate. The reaction catalyzed by this enzyme is prototypic of mammalian asparagine synthetase (AS-B) which utilizes glutamine as a nitrogen source. In addition, asparagine synthetase is a potential target for chemotherapy to treat certain leukemias. We therefore designed and synthesized a transition-state analogue, *N*-adenylated *S*-methyl-L-cysteine sulfoximine **1**, based on the proposed reaction mechanisms of AS-A. The compound **1** strongly inhibited the *E. coli* AS-A in a time-dependent manner with an overall inhibition constant (K_i*) of 67 nM and with an onset rate of inactivation of 3.27 s^{-1} mM^{-1}. The inhibition was almost irreversible and no regain of enzyme activity was observed in 10 days after gel filtration. The inhibitor **1** was also used as a ligand for X-ray diffraction analysis of AS-A. The X-ray crystal structure of AS-A complexed with **1** revealed several key amino acid residues such as Arg 100, Gln 116 and Asp 46 responsible for catalysis as well as those for substrate recognition. An attempt to inhibit AS-A by each diastereomer of *S*-methyl-L-cysteine sulfoximine and ATP is also described. Since AS-A is prototypic of asparagine synthetases in terms of the chemistry in substrate activation, compound **1** should formulate a basis for future inhibitor design of asparagine synthetase B.

Keywords: Transition-state analogue inhibitor/ Asparagine synthetase A/ ATP-Dependent ligase/ Sulfoximine/ Slow-binding inhibition/ Irreversible inhibition/ X-Ray crystallography

L-Asparagine synthetase [L-aspartate: ammonia ligase (AMP-forming) EC 6.3.1.1] (AS-A) from *Escherichia coli* [1] is a typical member of ammonia-dependent asparagine synthetases and catalyzes the formation of L-Asn from L-Asp and ammonia with concomitant hydrolysis of ATP to AMP and pyrophosphate. The reaction catalyzed by AS-A is mechanistically prototypic of all the classes of ATP-utilizing C-N bond ligases in which the substrate carboxyl group is activated by adenylation followed by substitution by amine nucleophile. It is therefore highly desirable to obtain good transition-state analogue inhibitors of AS-A to probe the detailed reaction mechanisms and structure of asparagine synthetases and its mechanistically related ligases. We describe here the synthesis and characterization of a transition-state analogue, *N*-adenylated *S*-methyl-L-cysteine sulfoximine **1**.

The catalytic reaction of AS-A is thought to proceed by a two-step mechanism via an intermediate β-aspartyl adenylate; the first step is the formation of the intermediate, and the resulting adenylated β-carboxy is attacked

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Scope of research

Our research aims are to elucidate the chemistry-function relationships of various biocatalysts (enzymes) in combination with organic chemistry, molecular biology and X-ray crystallography. The biochemical and physiological roles of enzymes and hormone receptors are also studied from the chemical point of view. Main subjects are (1) Chemical, biochemical and molecular biological studies on β-primeverosidase, a major tea aroma-producing β-glycosidase concerned with tea-manufacturing process, and on its original physiological roles in tea plants, (2) Design and synthesis of transition-state analogue inhibitors of ATP-dependent ligases and glycosidases, (3) Development of a new method for functional cloning of plant hormone receptors and biochemical studies on plant hormone biosynthesis, (4) X-Ray crystallography of firefly luciferases and pyruvate phosphate dikinase from Maize, (5) Development of a new types of lipase by evolutionary molecular engineering.


Prof
SAKATA, Kanzo
(D Agr)


Assoc Prof
HIRATAKE, Jun
(D Agr)

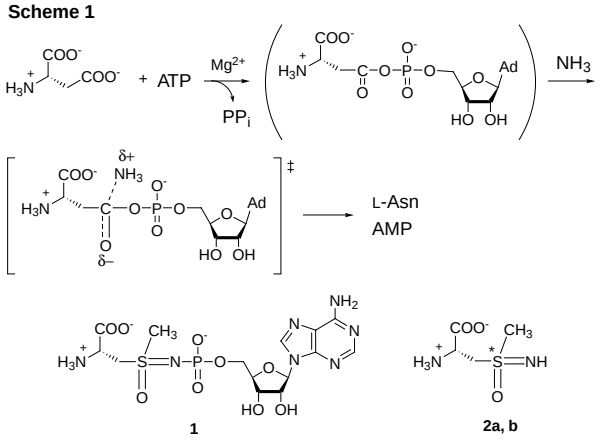

Instr
KATO, Hiroaki
(D Agr)


Instr
MIZUTANI, Masaharu
(D Agr)


Assoc Instr
NAKATSU, Toru
(D Agr)

INOUE, Makoto (DC), FUJII, Ryota (DC), MA, Seung-Jin (DC), FURUKAWA, Hiroshi (MC), TAKEGAWA, Mizuki (MC), EMA, Jun'ichi (MC), NAKANISHI, Tsugumi (MC), SATO, Tadashi (MC), GUO, Wenfei (PD), SHIMIZU, Tetsuya (MC, RS), KISHIMOTO, Masayuki (MC, RS), SHIMA, Masami (UG, RS), UEMURA, Miyuki (secretary)

Scheme 1



by ammonia in the second step to form L-Asn and AMP (Scheme 1). As a stable analogue of the transition state in the latter step, we designed *N*-adenylated *S*-methyl-L-cysteine sulfoximine **1** where the carbonyl to be attacked by ammonia is replaced by a tetrahedral sulfoximine sulfur atom with a methyl group mimicking ammonia. Compound **1** was synthesized in 9 steps from *S*-methyl-L-Cys via adenylation of sulfoximine nitrogen [2]. The resulting P-N bond was hydrolytically stable even under acidic conditions.

The *N*-adenylated sulfoximine **1** (1:1 mixture of diastereomers) was found to be an extremely potent slow-binding inhibitor that caused time-dependent inactivation of AS-A (Figure 1). The enzyme, for example, was totally inactivated in 15 min when 2.5 μM of **1** was present. The inhibition was virtually irreversible, and no regain of enzyme activity was observed after gel filtration. Since the inhibition was time-dependent, the onset rate of inactivation (k_{on}) was calculated from the progress curves and was found to be 3.27 s^{-1} mM^{-1}. The overall inhibition constant (K_i*) was calculated as 67 nM by measuring the inhibited enzyme activity after slow-binding inhibition equilibrium was reached. Considering that the kinetic constants for AS-A were K_m (Asp) = 1.69 mM, K_m (ATP) = 1.24 mM and k_0/K_m (Asp) = 28.5 $s^{-1}mM^{-1}$, the inhibitor **1** bound to the enzyme at a rate nine times as small as that of the enzymatic reaction, but with 25,000-fold higher affinity than its substrates.

We first attempted to inhibit AS-A with diastereomeric *S*-methyl-L-cysteine sulfoximine **2a**, **b** and ATP in the hope that a mechanism-based inactivation of AS-A might result by *in situ* adenylation of the sulfoximine by ATP within the enzyme active site. However, each diastereomer **2a** and **2b** was a poor and reversible inhibitor of AS-A with K_i = 3.27 and 0.76 mM, respectively, and no enzyme inactivation was observed after a prolonged incubation of the enzyme with **2a** or **2b** in the presence of ATP.

Since compound **1** was most likely to mimic the putative transition state, the enzyme complexed with **1** was crystallized and subjected to X-ray diffraction analysis [2]. The inhibitor **1** gave a very clear electron density in the enzyme active site, and several specific interactions were identified between the active site amino acid residues and the inhibitor **1** (Figure 2). Of particular interest is the interaction regarding the sulfoximine moiety. First, the sulfoximine oxygen (S=O) is hydrogen bonded to Arg 100 and Gln 116. Since S=O represents the oxyanion

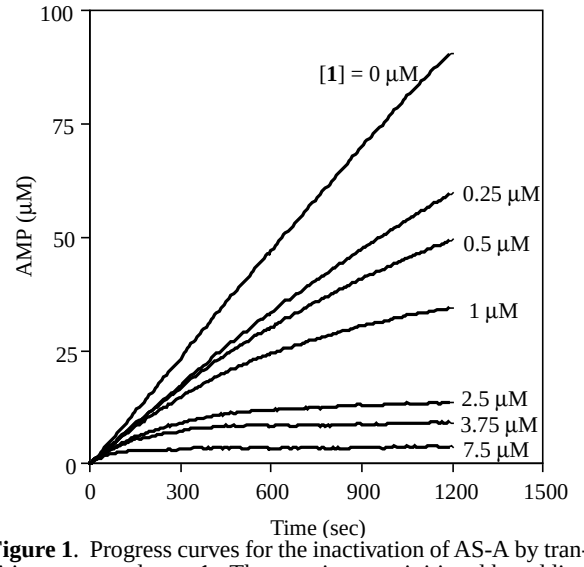


Figure 1. Progress curves for the inactivation of AS-A by transition-state analogue **1**. The reaction was initiated by adding the enzyme to an assay mixture containing L-Asp (1.5 mM), NH_4Cl (20 mM), ATP (3 mM) and the inhibitor **1** (0-7.5 μM) in 100 mM Tris-HCl (pH 7.8) at 37 °C.

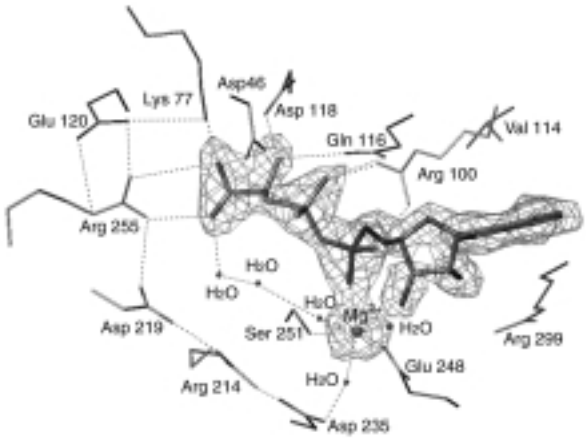


Figure 2. X-Ray crystal structure of *E. coli* AS-A active site complexed with the inhibitor **1**, showing the SIGMAA weighted F_O-F_c omit electron density map, superimposed with the refined structure of the inhibitor **1** and the active site amino acid residues. The contour level is 3.0 σ.

generated by nucleophilic attack of ammonia, these residues are most likely to be key catalytic residues responsible for stabilizing the tetrahedral oxyanion transition state or intermediate. Another point is the methyl attached to the sulfur atom. This methyl, which is supposed to mimic the -NH^{3+} moiety of the zwitterionic tetrahedral intermediate, was found to be 3.1 Å distant from Asp 46. This negatively charged residue probably plays a key role in the electrostatic stabilization of the -NH^{3+} part of the transition state or the intermediate and also acts as a base to abstract a proton from the tetrahedral intermediate completing the formation of the product asparagine.

References

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