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

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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*<sub>i</sub>) of 67 nM and with an onset rate of inactivation of 3.27 s<sup>-1</sup> mM<sup>-1</sup>. 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...
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 Kcat (Asp) = 1.69 mM, Km (ATP) = 1.24 mM and kcat/Km(Asp) = 28.5 s⁻¹mM⁻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 sulfoximines 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 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 -NH₃⁺ 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 -NH₃⁺ 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