Characterization of Inhibitors Acting at the Synthetase Site of *Escherichia coli* Asparagine Synthetase B†

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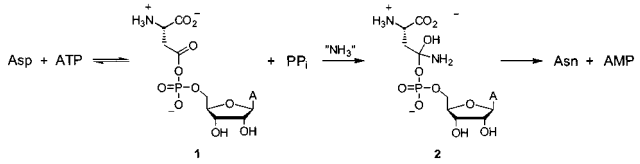
ABSTRACT: Asparagine synthetase catalyzes the ATP-dependent formation of L-asparagine from L-aspartate and L-glutamine, via a β-aspartyl-AMP intermediate. Since interfering with this enzyme activity might be useful for treating leukemia and solid tumors, we have sought small-molecule inhibitors of *Escherichia coli* asparagine synthetase B (AS-B) as a model system for the human enzyme. Prior work showed that L-cysteine sulfinic acid competitively inhibits this enzyme by interfering with L-aspartate binding. Here, we demonstrate that cysteine sulfinic acid is also a partial substrate for *E. coli* asparagine synthetase, acting as a nucleophile to form the sulfur analogue of β-aspartyl-AMP, which is subsequently hydrolyzed back to cysteine sulfinic acid and AMP in a futile cycle. While cysteine sulfinic acid did not itself constitute a clinically useful inhibitor of asparagine synthetase B, these results suggested that replacing this linkage by a more stable analogue might lead to a more potent inhibitor. A sulfoximine reported recently by Koizumi et al. as a competitive inhibitor of the ammonia-dependent *E. coli* asparagine synthetase A (AS-A) [Koizumi, M., Hiratake, J., Nakatsu, T., Kato, H., and Oda, J. (1999) *J. Am. Chem. Soc. 121*, 5799−5800] can be regarded as such a species. We found that this sulfoximine also inhibited AS-B, effectively irreversibly. Unlike either the cysteine sulfinic acid interaction with AS-B or the sulfoximine interaction with AS-A, only AS-B productively engaged in asparagine synthesis could be inactivated by the sulfoximine; free enzyme was unaffected even after extended incubation with the sulfoximine. Taken together, these results support the notion that sulfur-containing analogues of aspartate can serve as platforms for developing useful inhibitors of AS-B.

Asparagine synthetase B (AS-B),[1] encoded by the *asnB* gene in *Escherichia coli* (*1*), catalyzes the synthesis of L-asparagine from L-aspartic acid, in an ATP-dependent reaction for which the nitrogen source can be either L-glutamine or free ammonia (*2*). Aspartate activation is accomplished by formation of β-aspartyl-AMP **1** (β-AspAMP) in the C-terminal synthetase site of the enzyme (Scheme 1) (*3*). In a spatially distinct active site located in the AS-B N-terminal domain, glutamine is hydrolyzed to glutamate and a molecule of free ammonia that is believed to be transferred to the synthetase site along an intramolecular channel (*4*, *5*). Similar structural motifs for channeling enzyme-bound ammonia have been observed in crystal structures of three other glutamine-dependent amidotransferases (*6*−*12*). Nucleophilic attack of ammonia on the activated carbonyl group of β-AspAMP **1** then yields the tetrahedral intermediate **2** that subsequently collapses to give asparagine and AMP (Scheme 1). The kinetic and chemical mechanisms of ammonia-dependent AS-A are therefore similar to those observed for aminoacyl tRNA synthetase (aaRS) (*13*, *14*) in which a stable acyl-AMP intermediate is formed on the enzyme prior to nucleophilic attack by tRNA (*15*, *16*). Yet, while the ammonia-dependent asparagine synthetases (*17*, *18*) and aminoacyl-tRNA synthetases in prokaryotes are homologous proteins that share a common ancestor (*19*−*21*), glutamine-dependent dependent asparagine synthetases appear to be evolutionarily unrelated to either of these enzyme families (*4*, *22*).

Scheme 1



There appears to be an inverse correlation between the susceptibility of leukemia cells to drug therapy and their

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[1] AS-B, *Escherichia coli* asparagine synthetase B; βAspAMP, β-aspartyl-AMP; aaRS, aminoacyl tRNA synthetase; GPATase, glutamine 5′phosphoribosylpyrophosphate amidotransferase; GFAT, glutamine fructose-6-phosphate amidotransferase; CSA, L-cysteine sulfinic acid; WT, wild type; NMR, nuclear magnetic resonance; TCA, trichloroacetic acid; PP_i, inorganic pyrophosphate.

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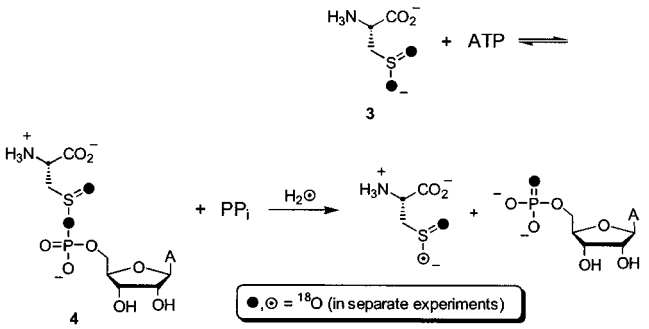
Scheme 2



capacity for intracellular asparagine biosynthesis. As a consequence, the enzyme L-asparaginase, which catalyzes the hydrolysis of asparagine to aspartate and ammonia (*23*, *24*), is widely used in chemotherapeutic protocols for treating acute lymphoblastic leukemia (*25*, *26*). Unfortunately, the clinical utility of L-asparaginase in cancer therapy is often limited by the appearance of tumors that are resistant to further therapy (*27*). Potent, specific inhibitors of human AS may therefore have significant potential both as agents for treating leukemia and as tools for investigating the cellular basis of resistance to L-asparaginase treatment. Although the high-throughput screening of small molecule libraries (*28*−*30*) offers an approach to obtaining potent AS inhibitors, our current strategy is to exploit information on (i) the interactions that catalyze the formation and breakdown of the β-AspAMP intermediate **1** and (ii) the molecular mechanisms underlying channel formation in the discovery of bioactive compounds with high selectivity. Inhibitors that are targeted to the N-terminal active site are likely to be nonselective because this catalytic domain is structurally and mechanistically similar in all Ntn amidotransferases (*4*, *8*, *22*, *31*, *32*), a family that includes important cellular enzymes such as glutamine 5′phosphoribosylpyrophosphate amidotransferase (GPATase) (*33*) and glutamine fructose-6-phosphate amidotransferase (GFAT) (*34*). We now report investigations into the mechanisms of two inhibitors that specifically interact with the AS-B synthetase site. Our experiments suggest that L-cysteine sulfinic acid (CSA) **3** (*35*, *36*), a weak competitive inhibitor with respect to aspartate (*37*, *38*), exerts its effects by accelerating the ordinarily negligible rate of futile ATP hydrolysis by forming the novel, unstable β-AspAMP analogue **4** (Scheme 2). We also demonstrate that the *N*-adenylated sulfoximine **5** (Figure 1), which is a tight-binding inhibitor of ammonia-dependent asparagine synthetase (AS-A) (*39*), is one of the most potent AS-B inhibitors reported to date. In contrast to its behavior with AS-A, however, sulfoximine derivative **5** appears able to form a high-affinity complex with AS-B only when the enzyme is undergoing catalytic turnover. The unexpected difference in the kinetic mechanism by which the sulfoximine derivative inhibits AS-A and AS-B is most likely a consequence of conformational changes that are unique to glutamine-dependent asparagine synthetases due to constraints imposed by the structural elements required to channel ammonia between two independent active sites.

MATERIALS AND METHODS

Enzyme Preparation and Purification. Wild type (wt) AS-B was expressed in *Escherichia coli* and purified by

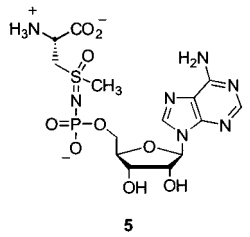


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FIGURE 1: Structure of sulfoximine **5** as prepared and described in ref *39*.

anion exchange chromatography, as described elsewhere (*40*). Protein concentrations were determined using the BioRad (Hercules, CA) assay kit based on a standard curve constructed using known quantities of AS-B.

*AS-B Catalyzed ATP Hydrolysis in the Presence of (S)-Aspartic Acid, (R)-CSA **3**, or (2S,3S)-β-Methylaspartate.* Wild type AS-B (0.39−0.58 nmol) was incubated with 0.1 mM [2,8-$^3H_2$]-ATP (SA: 1×10^6 cpm/mmol), 8 mM $MgCl_2$, and either 5 mM aspartate, 5 mM CSA **3** or 5 mM (2*S*,3*R*)-β-methylaspartate in 100 mM Bis-Tris HCl, pH 6.5 (75 μL total volume). The reaction was quenched by the addition of TCA (4% final concentration) after a variable time (10 s−5.5 min). The pH of the solution was then raised to pH 8 with Tris HCl, and the nucleotides were separated by chromatography on DE81 paper (eluant: 1/50 sat. NH_4OAc, pH 2.75) in a closed system for 3 h. The radioactivity associated with [2,8-$^3H_2$]AMP was then determined using a Beckman LS60001C scintillation counter. This assay reports the total of [AMP] plus [β-AspAMP]. To verify that all of the latter species hydrolyzed prior to paper chromatography analysis, we varied the time between quenching of the reaction and determination of the AMP from 2 to 105 min. No effect on the time dependence of $[AMP]/[E]_o$ was observed in these control experiments, validating our assumption concerning the hydrolytic stability of free β-AspAMP **1** under the conditions used to quench the reaction.

^{31}P NMR Spectroscopy. ^{31}P NMR spectra were recorded at 25 °C, with broadband ^1H decoupling, using a Varian VXR-300 spectrometer (121 MHz for ^{31}P). ^{31}P chemical shifts were determined relative to an external reference of 90 mM NaH_2PO_4 ($\delta = 0.0$). In general, reaction samples contained a small amount of unreacted ATP. The concentration of each species was calculated by comparison of peak area to that of the external standard. Assignment of the ^{31}P resonance in AMP was confirmed by the addition of an authentic sample.

*AS-B Catalyzed ATP Hydrolysis in the Presence of (R)-CSA **3** in 40% $H_2{}^{18}O/H_2{}^{16}O$.* Wild type AS-B (11.8 nmol) was incubated with 10 mM CSA, 10 mM ATP, and 15 mM $MgCl_2$ in 50 mM Tris HCl, pH 8.0, dissolved in 40% $H_2{}^{18}O/H_2{}^{16}O$ (600 μL total volume), for 3 h at 37 °C. Control reactions were performed using identical conditions, except that either enzyme or CSA was omitted. After this time, both samples were divided into two portions for analysis using ^{31}P NMR and electrospray mass spectroscopy. For the NMR experiments, glycine (4.5 mg), EDTA (32.2 mg), and 99.9% D_2O (50 μL) were added to the reaction sample (500 μL). The solution pH was then adjusted to 9 (final volume 600 μL). Samples for mass spectroscopy were separated and analyzed by reverse-phase (C_{18}) HPLC/ESI-MS and MS/MS. AMP, CSA, and ATP were detected in positive ionization mode, while detection of phosphate and inorganic pyrophos-

phate was performed using negative ionization mode. Peaks corresponding to parent, or fragment, ions in which ^{18}O had been incorporated or was absent were then identified and integrated. The peak ratios were then used to measure the extent of ^{18}O incorporation.

*Preparation of ^{18}O-Labeled (R)-CSA **3**.* Wild type AS-B (33.6 nmol) was incubated with 5 mM CSA **3**, 50 mM ATP, 55 mM $MgCl_2$ in 50 mM Tris HCl, dissolved in 50% $H_2{}^{18}O$/$H_2{}^{16}O$ (1 mL total volume), for 24 h at 37 °C. After this time, 1.6 μmol of ATP remained in solution. Analysis of recovered **3** using electrospray mass spectrometry, as described above, showed that all of the ^{18}O-label present was found in the sulfinic acid moiety. Doubly, singly, and unlabeled CSA were present in the sample at levels of 53, 28, and 19%, respectively. Activated charcoal was used to remove nucleotides from the sample before use of the labeled CSA in subsequent experiments.

*AS-B Catalyzed ^{18}O-Transfer from ^{18}O-Labeled (R)-CSA **3** to AMP wt AS-B.* Twenty-four nanomoles was added to 4 mM ^{18}O-labeled CSA **3** (prepared as described above), 10 mM ATP, 15 mM $MgCl_2$ in 50 mM Tris HCl, pH 8 (500 μL total volume). After incubation at 37 °C for 3 h, the solution was centrifuged, and the sample was prepared for ^{31}P NMR spectroscopic assay, as outlined above.

*Kinetics of Inhibition of AS-B by Sulfoximine **5**.* Progress curves were generated by incubating purified AS-B (1.14 μg) in reaction mixtures containing 100 mM Tris HCl, pH 8.0, 100 mM ammonium chloride, 0.5 mM ATP, 10 mM aspartate, 1 mM DTT, and 8.0 mM $MgCl_2$. The velocity of each reaction was monitored spectrophotometrically by following the production of pyrophosphate during asparagine synthesis using pyrophosphate reagent (Sigma Chemical Company, Technical Bulletin No. BI-100). Kinetic constants were obtained by nonlinear regression using equations derived from the full kinetic expression using the software program Prism (Graph Pad, San Diego, CA).

The possibility that wt AS-B catalyzed the breakdown of sulfoxime **5** to yield AMP was explored by incubating wt AS-B (10 μg) with either glutamine (5 mM) or ammonium chloride (100 mM) in the presence of sulfoximine **5** (25 μM). The amount of AMP formed was monitored using a coupling assay whereby the amount of AMP formation is proportional to the decrease in NADH absorbance. The coupling mixture contained 5 mM ATP, 10 mM $MgCl_2$, 100 mM Tris, pH 8.0, 1 mM phosphenolpyruvate, 0.24 mM NADH, 100 mM KCl, 1 mM DTT, 0.32 U myokinase, 4.0 U lactate dehydrogenase, and 1.6 U pyruvate kinase. The decrease in absorbance of NADH was observed at 340 nm over a period of 5 min, and the slope of the absorbance vs time used to calculate the velocity of the reaction.

To determine the form of AS-B inhibited by sulfoximine **5**, the enzyme was treated with inhibitor and various combinations of substrates. In these studies, wt AS-B (2 μg) was incubated either in the presence or absence of sulfoximine **5** for 10 min at 37 °C. The mixtures were then applied to a 1-mL G-50 Sephadex gel filtration spin column to remove excess substrate and inhibitor molecules (*41*). One-half of the eluant was used to determine the protein concentration (BioRad protein assay kit) and the other half was used to determine the enzymatic activity. Activity was determined by adding the eluant to a mixture containing saturating substrates for the AS-B reaction, (10 mM glutamine, 10 mM aspartate, and 5 mM ATP) along with the coupling mixture which contained 10 mM $MgCl_2$, 100 mM Tris, pH 8.0, 1 mM phosphoenolpyruvate, 0.24 mM NADH, 100 mM KCl, 1 mM DTT, 0.32 U myokinase, 4.0 U lactate dehydrogenase, and 1.6 U pyruvate kinase. The decrease in absorbance of NADH was observed at 340 nm over a period of 5 min and the slope of the absorbance vs time used to calculate the velocity of the reaction.

The kinetics of the partially inhibited wt AS-B were determined by incubating wt AS-B (32 μg) with a saturating substrate mixture (10 mM aspartate, 5 mM ATP, 10 mM glutamine, 100 mM Tris HCl, pH 8.0, and 8 mM $MgCl_2$) in the presence or absence of sulfoximine **5** for 5 min. Then the entire mixture was applied to a 1 mL G-50 sephadex gel filtration spin column to remove excess substrate and inhibitor molecules. A portion of the eluant was used to determine the protein concentration and another portion to determine the K_M for aspartate. Reaction mixtures contained varying amounts of aspartate (0−1.5 mM) along with 5 mM glutamine, 5 mM ATP, 10 mM $MgCl_2$, and 100 mM Tris, pH 8.0, and the amount of AMP produced was determined as outlined above. The kinetic constants were obtained by linear regression of double reciprocal plots using the software program Prism (Graph Pad, San Diego CA).

RESULTS

AS-B Catalyzed ATP Hydrolysis in the Absence of a Nitrogen Source. In AS-B catalyzed asparagine synthesis, ATP can bind to free enzyme, giving a catalytically significant E·ATP complex to which aspartate subsequently binds productively (*42*). An important consequence of this kinetic mechanism is that breakdown of either bound ATP or β-AspAMP **1** may occur prior to glutamine binding and nitrogen transfer. The ability of AS-B to catalyze futile ATP hydrolysis in the absence of a nitrogen source was therefore examined by incubation of the enzyme with radiolabeled [2,8-$^3H_2$]ATP, aspartate, and Mg^{2+} at pH 6.5. This pH was chosen to ensure that any adventitious ammonia present in solution would be unreactive. The reaction was quenched using TCA. Acyl-AMP intermediate **2** was hydrolyzed quantitatively under the conditions of our analysis so the final AMP concentration reported reflects the sum of β-AspAMP and free AMP present in the reaction mixture. After an initial "burst" (0.6 ± 0.2 $[AMP]/[E]_o$), the $[AMP]/[E]_o$ ratio remained almost constant over the time of the experiment (Figure 2). Fitting the data gave an estimated half-life of 23 min for the enzyme-bound β-AspAMP intermediate.

The rate of futile ATP hydrolysis in the *absence* of a nitrogen source was then probed using CSA **3** (*35*, *36*) and (2*S*,3*R*)-β-methyl aspartate (*43*, *44*) in place of aspartate. Both of these compounds are weak competitive inhibitors of AS-B (*37*). No AS-B-catalyzed ATP breakdown was observed when (2*S*,3*R*)-β-methyl aspartate was incubated with the enzyme and [2,8-$^3H_2$]ATP under identical conditions to those described above (data not shown). On the other hand, replacement of aspartate by CSA **3** enhanced the steady-state rate of AS-B catalyzed ATP hydrolysis to 0.87 ± 0.04 min^{-1} (Figure 2), approximately 30-fold higher than the breakdown rate observed in the presence of aspartate. The ATP breakdown reaction exhibited "burst" kinetics, the magnitude of which ($[AMP]/[E]_o = 0.7 \pm 0.1$), was identical,

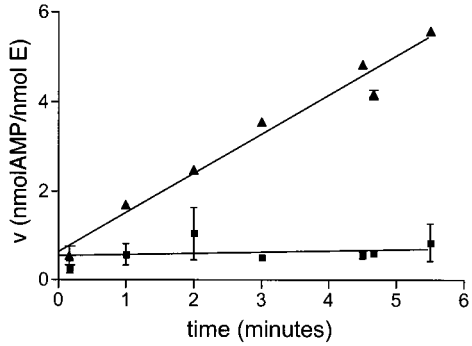

FIGURE 2: ATP hydrolysis in the presence of aspartate (■) or CSA (▲) in the absence of a nitrogen source. Wild type AS-B (0.39−0.58 nmol) was incubated with 0.1 mM [2,8-$^3H_2$]ATP (SA: 1×10^6 cpm/mmol), 8 mM $MgCl_2$, and either 5 mM aspartate or 5 mM CSA, in 100 mM Bis-Tris HCl, pH 6.5. The reaction was quenched by the addition of TCA (4% final concentration) after a variable time (10 s−5.5 min). The nucleotides were separated by chromatography on DE81 paper, and the radioactivity associated with [2,8-$^3H_2$]AMP was then determined. Error bars represent the standard deviation of triplicate determinations.

within experimental error, to that observed for the aspartate-dependent reaction. In the absence of the enzyme, **3** did not stimulate ATP breakdown under these reaction conditions. These data raised the possibility that **3** was forming the novel, sulfur-containing CSA-AMP analogue **4** in the AS-B synthetase site (Scheme 2).

^{31}P NMR and Electrospray Mass Spectroscopy Studies of CSA-Dependent Stimulation of AS-B Catalyzed ATP Hydrolysis in the Absence of a Nitrogen Source. CSA **3** and ATP were incubated with AS-B in 40% $H_2{}^{18}O/H_2{}^{16}O$, in the absence of glutamine. After standard workup procedures (*45*), the phosphorus-containing compounds present in the reaction mixture were examined using ^{31}P NMR spectroscopy (*46*). The ^{31}P signals observed for the nuclei associated with AMP and inorganic pyrophosphate (PP_i) were singlets, confirming that the extent of ^{18}O incorporation into these compounds under the reaction conditions did not exceed 5%. Assignment of the AMP resonance was confirmed by addition of an authentic, unlabeled sample to the system, and the concentration of each ^{31}P-containing species in the product mixture was quantitated by comparison of the area of its resonance peak to that of the external phosphate standard. Some conversion of PP_i to phosphate in a non-enzyme-catalyzed hydrolysis reaction took place during the preparation of the NMR sample, yielding a doublet for the ^{31}P-resonance of phosphate due to incorporation of ^{18}O from solvent.

To establish that ^{18}O became incorporated into CSA **3** under these reaction conditions, the sulfinic acid was recovered and examined using electrospray mass spectroscopy (*47*). Two molecular ions (M^+ and $(M+2)^+$), in a ratio of 1:2.9, were observed for recovered CSA **3**, confirming that ^{18}O had been incorporated into the aspartate analogue. This incorporation ratio is in excellent agreement with the theoretical value of 1:2.1 calculated on the basis of the $H_2{}^{18}O$ concentration in the reaction buffer. The location of the ^{18}O isotope in recovered **3** was also determined from the observation that both CSA **3** parent ions gave a single (M^+-88) fragment ion after loss of SO_2 in the mass spectrometer. This confirmed that incorporation only occurred in the sulfinate group of **3** under the reaction conditions. Control experiments showed that ^{18}O incorporation into recovered

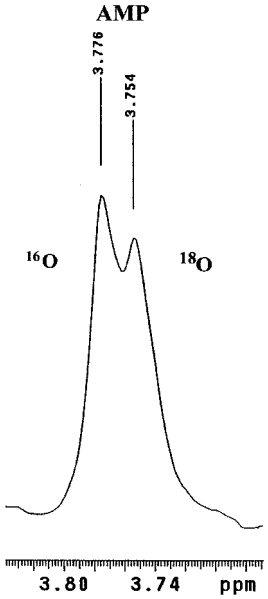

FIGURE 3: Expanded view of the ^{31}P resonance of AMP after incubation of ATP and ^{18}O-labeled CSA. Detailed experimental conditions are given in Materials and Methods. Nominal chemical shifts for AMP were determined by comparison to authentic materials. Peaks for ^{16}O- and ^{18}O-labeled AMP (separated by 0.022 ppm) are indicated.

CSA **3** did not take place when (i) ATP and CSA **3** were incubated without AS-B in 40% $H_2{}^{18}O/H_2{}^{16}O$ or (ii) CSA and AS-B were incubated in 40% $H_2{}^{18}O/H_2{}^{16}O$ in the absence of ATP (data not shown).

The direct transfer of ^{18}O from isotopically labeled CSA to AMP was then demonstrated. A sample of ^{18}O-CSA was prepared by extended incubation of unlabeled CSA **3** with ATP and AS-B in 50% $H_2{}^{18}O/H_2{}^{16}O$ for 24 h. The resulting solution was then passed through charcoal to remove nucleotides. The resulting sample of recovered CSA **3** was analyzed by reverse-phase (C_{18}) HPLC and electrospray MS/MS, the protonated form of the sulfinic acid being detected in positive ionization mode. The extent of ^{18}O-incorporation into CSA **3** was determined by integration of the peak heights for the M^+, $(M+2)^+$, and $(M+4)^+$ parent ions. Doubly, singly, and unlabeled forms of CSA were present in the sample to the extent of 53, 28, and 19%, respectively. Again, analysis of the ions produced by fragmentation clearly showed that all of the oxygen label was present in the side chain sulfinate moiety. After lyophilization, the labeled ^{18}O-CSA **3** was incubated with fresh AS-B and unlabeled ATP in buffer containing only $H_2{}^{16}O$. NMR spectroscopic analysis of the AMP formed under these incubation conditions showed that the ^{31}P resonance now gave two signals in a 1:1 ratio, separated in chemical shift values by 0.02 ppm (Figure 3). The upfield shift of the ^{31}P resonance was consistent with direct transfer of ^{18}O from specifically labeled CSA **3** into AMP (Scheme 2) (*48*). ^{18}O-transfer did not take place when the labeled CSA **3** was incubated with ATP in the absence of enzyme.

Electrospray Mass Spectroscopy Studies of AS-B Catalyzed Nitrogen Transfer to CSA 3. Sulfinic acid **3** was incubated with AS-B in the presence of $^{15}NH_3$, ATP, and Mg^{2+} to investigate whether enzyme-catalyzed nitrogen transfer could take place to generate the corresponding sulfinamide. In these experiments, the solution pH was maintained at 8.0. Control

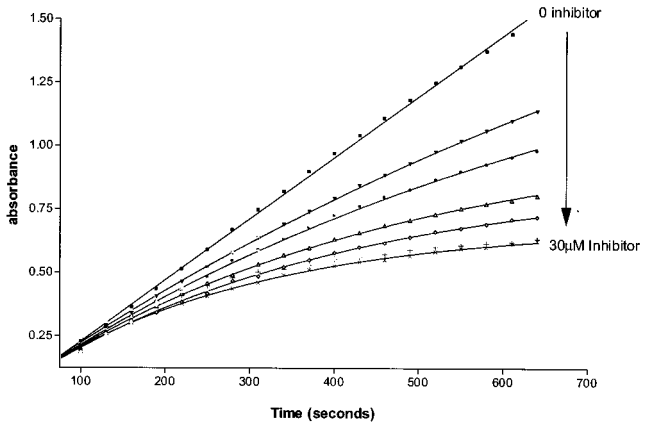

FIGURE 4: Progress curves for PP_i formation in the presence of sulfoximine **5**. Reaction mixtures contained AS-B (1.14 μg), 100 mM Tris HCl pH 8.0, 100 mM ammonium chloride, 0.5 mM ATP, 10 mM aspartate, 1 mM DTT, and 8.0 mM $MgCl_2$. Sulfoximine **5** concentration ranged from 0 to 30 μM. Asparagine formation and PPi production are always at a 1:1 ratio (*42*).

experiments were performed under identical conditions except that ATP was omitted from the reaction mixture. After 24 h, the reaction mixture was analyzed by reverse-phase (C_{18}) HPLC and electrospray MS/MS, the protonated forms of the amino acids present in solution being detected in positive ionization mode. Under these conditions, one mass unit would have separated the molecular ions for the acid **3** and the sulfinamide. Identical mass spectra were obtained from both samples, in which only the peak arising from the sulfinic acid **3** was present (data not shown). No evidence for nitrogen transfer was therefore obtained in these experiments.

Characterization of the Kinetics of AS-B Inhibition by Sulfoximine 5. Sulfoximine **5** has been shown previously to be a potent inhibitor of AS-A ($K_I = 67$ nM), the prokaryotic, ammonia-dependent asparagine synthetase (*39*). A series of kinetic studies have demonstrated that **5** binds to free AS-A and is presumably bound in the synthetase active site where it likely mimics the transition state for the attack of ammonia on β-AspAMP. Incubation of AS-A is effectively irreversible: no enzymatic activity was regained after 10 days. If sulfoximine **5** affected AS-B similarly, mixing free enzyme with inhibitor should reveal a time-dependent loss in activity. This was tested by incubating **5** (10 μM) with either AS-A or AS-B (1 μg) for various times, then determining the activity by end point assays. After 1 h of incubation with the sulfoximine **5**, AS-A was completely inactivated. By contrast, no loss of activity was seen for AS-B. These results suggested that the inhibitor was unable to bind free AS-B with high affinity, but it was possible that **5** would exhibit high affinity for other forms of the enzyme present during asparagine synthesis. We therefore assayed the ability of **5** to inhibit AS-B by observing the amount of product formed (PP_i) versus time when the enzyme was incubated with the inhibitor in the presence of aspartate, ATP, and ammonia. Enhanced curvature was observed with increasing concentrations of inhibitor (Figure 4). These observations could be interpreted to indicate either time dependence of inhibition, turnover dependence of inhibition or conversion of sulfoximine **5** into an active inhibitor produced by AS-B.

The possibility that AS-B was capable of catalyzing the breakdown of **5** was tested using an enzyme-based system

Table 1: Assay Conditions Leading to AS-B Inactivation by Adenylated Sulfoximine **5**[a]

enzyme	glutamine	aspartate	ATP	NH_4Cl	sulfoximine **5**	% activity remaining
AS-B	+	+	+	−	−	100
AS-B	+	+	+	−	+	35
AS-B	−	+	+	+	−	100
AS-B	−	+	+	+	+	47
AS-B	+	−	+	−	+	100
AS-B	−	−	+	+	+	98
AS-B	+	−	−	−	+	100
C1A	+	+	+	−	−	100
C1A	+	+	+	−	+	97
C1A	+	−	−	−	−	100
C1A	+	−	−	−	+	97
C1A	−	+	+	+	−	100
C1A	−	+	+	+	+	13

[a] Substrates when present were kept at approximately 10 times their K_m values: ATP, 5 mM, Asp 10 mM, Gln, 5 mM, NH_4Cl, 100 mM. The inhibitor concentration was 5 μM. The velocity of AS-B was normalized as 100%, and the velocities of the inhibitor data are expressed as a percentage of this rate. Each value represents the average of triplicate results.

in which AMP production could be monitored by coupling to NADH oxidation. After establishing that **5** did not inhibit the assay, it was determined that AS-B did not hydrolyze **5** to AMP in the absence of substrates for asparagine synthesis (data not shown).

To determine the form of the enzyme inhibited by sulfoximine **5**, various combinations of enzyme, substrates, and inhibitor were examined. Experiments were performed by incubating AS-B with various E + S + I combinations (Table 1). Unbound small molecules were removed by rapid gel filtration chromatography, and then the protein content and ammonia-dependent asparagine synthetase activity were determined under conditions where all substrates were saturating. The results are shown in Table 1. When all substrates necessary for glutamine-dependent asparagine synthesis were present in the incubation mixture along with sulfoximine **5** (5 μM), only 35% of the activity remained, as compared to a similar sample where no inhibitor was added. A comparable experiment was performed with ammonia as the AS-B nitrogen source, and a similar result was seen: 47% of the activity remained. On the other hand, when the incubation mixture contained glutamine plus ATP or ammonia plus ATP, but in the absence of aspartate, **5** caused no decrease in activity. These results demonstrate that the sulfoximine is an effective inhibitor of AS-B only when the enzyme is undergoing catalytic turnover to form asparagine. This contrasts sharply with its behavior with AS-A (39) and suggests that sulfoximine **5** may bind to a form of AS-B produced during the catalytic mechanism but not to the resting state of the free enzyme. To determine whether the presence of glutamine, or the γ-glutamyl thioester, in the N-terminal domain was necessary for binding of **5**, we investigated the ability of the adenylated sulfoximine to inhibit the ammonia-dependent synthetase activity of the C1A AS-B mutant. This enzyme (wherein cysteine 1 is replaced by alanine) has a functional ammonia-dependent asparagine synthesis activity and binds glutamine extremely tightly, but it cannot hydrolyze glutamine or perform the glutamine-dependent asparagine synthesis reaction (*40*). When C1A AS-B was performing ammonia-dependent asparagine synthesis, the presence of **5** eliminated all but 13% of the

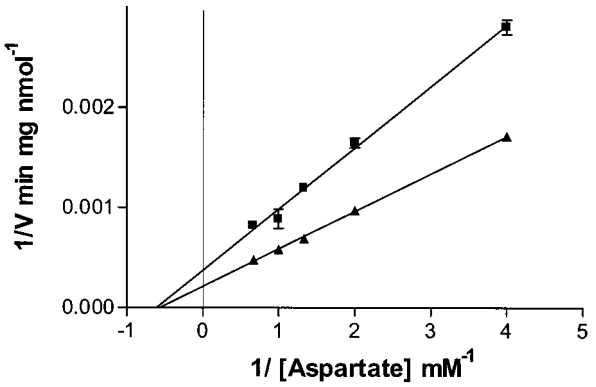

FIGURE 5: Steady-state kinetics of partially inhibited (■) and uninhibited AS-B (▲). Reaction mixtures contained partially inhibited or uninhibited AS-B (0.7 μg), varying amounts of aspartate (0−1.5 mM) along with 5 mM glutamine, 5 mM ATP, 10 mM $MgCl_2$, and 100 mM Tris, pH 8.0. The amount of AMP produced was determined as outlined above. Error bars represent the standard deviation of triplicate determinations.

catalytic activity. When the C1A AS-B mutant was incubated with all the substrates necessary for glutamine-dependent asparagine synthesis (but no catalysis was occurring), no inhibition was seen in the presence of **5**. These results suggest that **5** inhibits AS-B only when the enzyme is actively turning over.

To determine whether **5** is a dead-end inhibitor, the steady state kinetics of the partially inhibited enzyme were investigated. The partially inhibited AS-B was separated from substrates and inhibitors by gel filtration and then re-assayed to determine kinetic constants. When the remaining catalytic rate was approximately 40% of the uninhibited control, the rates were determined in the presence of varying concentrations of aspartic acid. As shown in Figure 5, the aspartate K_M was indistinguishable from that of the uninhibited enzyme (1.4 ± 0.1 and 1.3 ± 0.09 mM, respectively). This suggests that the inhibited enzyme is not capable of catalysis, and that **5** forms a dead-end complex with AS-B.

If the sulfoximine inhibits the glutamine-dependent activity of AS-B by binding to an enzyme form or conformation created at or near the site of aspartyl-AMP formation, it should not have any effect on glutamine hydrolysis, which from our crystal structure (*4*) occurs some 45 Å across the enzyme surface. To test this hypothesis, AS-B that had been completely inactivated with respect to ammonia-dependent synthesis of asparagine was separated from free **5** and substrates by gel filtration and assayed for glutaminase activity. No inhibition of glutaminase activity was observed as compared to that of uninhibited enzyme (data not shown).

DISCUSSION

The mechanistic sequence catalyzed by the C-terminal, synthetase site of AS-B proceeds in four steps (Scheme 1). First, the carboxylate group must react with the α-phosphate of ATP to yield a pentacoordinate intermediate from which the acyl-AMP derivative **1** is formed. AS-B must then deliver ammonia to the synthetase active site which reacts with **1** to give a tetrahedral intermediate **2**. In the final step, AMP release from **2** then yields asparagine. In many respects, this sequence of chemical transformations is similar to those observed in aminoacyl tRNA synthetases, although glutamine-dependent AS does not structurally resemble any of these enzymes, including aspartyl-tRNA synthetase (*49*). The observation that glutamine can bind productively to AS-B after the release of PP_i, however, implies that the reactive β-AspAMP intermediate **1** must be stabilized in the synthetase site so as to prevent futile ATP hydrolysis, presumably through interactions similar to those seen in the complex between tyrosyl-AMP and tyrosyl-tRNA synthetase (*50*). When AS-B was incubated with ATP alone, there was no detectable formation of AMP under our reaction conditions. ATP hydrolysis did occur, however, when aspartate and ATP were mixed with the enzyme, albeit with a small steady-state rate (Figure 2). Coincidentally, the steady-state rate of AS-B catalyzed ATP hydrolysis in the absence of a nitrogen source is similar to that reported for tyrosyl tRNA synthetase in the absence of $tRNA^{Tyr}$ (*51*). The specificity of this effect was then probed using substrate analogues. The observation that, in the absence of a nitrogen source, AS-B catalyzed ATP hydrolysis was stimulated by the presence of CSA **3**, but not by β-methylaspartate, was unexpected (Figure 2). Since sulfinates are generally observed to act as *S*-nucleophiles rather than *O*-nucleophiles (*52*−*54*), and the fact that CSA is a weak competitive inhibitor of AS-B with respect to L-aspartate (*37*), our initial hypothesis was that the binding of **3** caused structural changes in the enzyme that facilitated the direct attack of water on ATP. We were therefore surprised to find that a reaction containing enzyme and saturating levels of ATP and **3** exhibited a burst of AMP formation corresponding to 0.7 ± 0.2 enzyme equivalents. This instead suggested that CSA and ATP might be reacting to form an anhydride intermediate in the AS-B active site analogous to the normal β-AspAMP intermediate. A similar addition reaction is catalyzed by ATP sulfurylase during sulfate ion activation (*55*). Nucleophilic attack by water at sulfur or phosphorus would then yield AMP and regenerate CSA.

To test this hypothesis, ATP and **3** were incubated with AS-B in 40% $H_2{}^{18}O/H_2{}^{16}O$, and the extent of ^{18}O-incorporation into AMP and recovered **3** was determined using ^{31}P NMR and electrospray mass spectroscopy. These experiments showed that ^{18}O had been predominantly, and specifically, introduced into the sulfinate moiety of CSA and not into AMP or PP_i under the reaction conditions. Careful analysis of the electrospray mass spectrum of the reaction products, however, revealed that less than 2% of the recovered AMP contained ^{18}O. Although possibly arising from an alternate hydrolytic mechanism, we reasoned that the labeled AMP was actually due to CSA that had been labeled with ^{18}O in a previous turnover. This lead to an alternate approach to demonstrate participation of intermediate **4** in AS-B catalyzed, CSA-stimulated ATP hydrolysis. CSA and ATP were incubated with AS-B in 55% $H_2{}^{18}O/H_2{}^{16}O$. The resulting solution containing ^{18}O-labeled CSA was then passed through charcoal to remove nucleotides, and added to unlabeled ATP and AS-B. After further incubation, the ^{31}P resonance of the AMP isolated from the reaction was observed to be a 1:1 doublet, indicating that ^{18}O had been transferred from the labeled CSA into AMP via intermediate **4** (Scheme 2).

Of the four possible pathways by which ATP hydrolysis can occur when CSA and ATP are bound within the AS-B synthetase site (direct attack of water on the α-phosphorus of ATP, direct attack by water on the β-phosphorus of ATP,

attack of water on the phosphorus of **4,** and attack of water at the sulfur of **4**), the first three are ruled out by our observations. CSA-dependent ATP hydrolysis therefore proceeds in a completely analogous fashion to that of the enzyme-catalyzed reaction when aspartate is the substrate, i.e., the sulfinate anion attacks ATP to yield the adenylated intermediate **4** with subsequent nucleophilic attack of water at sulfur to yield AMP and regenerate CSA. This appears to be the first example of a sulfinate moiety exhibiting similar chemical reactivity to a carboxylate anion in an enzyme-catalyzed transformation. We note that in polar solvents, sulfinates react with many electrophiles to yield sulfones (*56*, *57*), the reactivity of the oxygen anion being decreased by hydrogen bonding interactions. Conversely, activated electrophiles react with oxygen to give sulfinate esters in nonpolar environments. While it is likely that oxygen attack on ATP occurs due to the proximity of the reacting atoms when the substrates are bound within the AS-B synthetase site, our observations may also support the hypothesis that the sulfinate anion, and by analogy the carboxylate anion of bound aspartate, is not stabilized by hydrogen bonding to the enzyme on binding. The ability of AS-B to catalyze the formation of intermediate **4** with the sulfur containing analogue of aspartate suggests that other sulfur analogues of the productive intermediate in asparagine biosynthesis might also be potent and specific inhibitors of asparagine synthesis. The effectiveness of **5** supports this notion. While **5** fails to inhibit AS-B unless the enzyme is undergoing catalysis, its inhibition appears to be essentially irreversible. The only time when **5** was observed to inhibit AS-B was when all three asparagine synthesis substrates were present in the reaction mixture. Moreover, utilizing a site-specific mutant of AS-B enabled us to demonstrate that it was in fact turnover and not simply substrate binding that was required for sulfoximine inhibition. When the C1A mutant of AS-B was incubated with all the glutamine-dependent asparagine synthetase substrates, no inhibition of the ammonia-dependent activity was observed, even though we have previously demonstrated that C1A AS-B binds glutamine tightly (*40*). While turnover of the enzyme with respect to asparagine synthesis is required for sulfoximine inhibition, glutaminase activity alone is not sufficient (Table 1). The irreversibility of inhibition by **5** was shown by gel filtration of the inactivated protein. Even after incubation in the absence of **5** for several hours, inhibited AS-B remained inactive. Finally, we note that inhibition by **5** is very efficient: concentrations of inhibitor that were less than 10-fold higher than that of AS-B afforded complete inactivation.

The work presented here establishes the potential of sulfur-containing analogues of β-AspAMP as potent inhibitors of asparagine biosynthesis. We have shown that CSA inhibits AS-B, by competing for binding with aspartate, as well as by undergoing chemical transformation to an analogue of the normal intermediate. Sulfoximine **5** can be considered to be a stable analogue of the intermediate formed by CSA on the AS-B active site, which inhibits by binding to a form of enzyme created during the catalytic turnover of AS-B. The inhibition by **5** was also shown to be not only turnover dependent, but effectively irreversible. We do not believe that **5** reacts covalently with AS-B, but the form of enzyme or the exact site of the inhibitor−enzyme complex are not yet defined. The fact that it is a potent and essentially irreversible inhibitor, however, encourages further examination of this class of compounds as potential new anticancer compounds.

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