Identification of Cysteine-523 in the Aspartate Binding Site of *Escherichia coli* Asparagine Synthetase B†

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ABSTRACT: The site-directed chemical modifier [*p*-(fluorosulfonyl)benzoyl]adenosine (5′-FSBA) inactivates *Escherichia coli* asparagine synthetase B activity following pseudo-first-order kinetics, with ATP providing specific protection, with a K_d of 12 μM. The 5′-FSBA modification appears to be covalent, even though a nonstoichiometric amount (less than 10%) of radiolabeled 5′-FSBA was associated with a totally inactivated enzyme. However, the inactivation by 5′-FSBA could be reversed upon the addition of dithiothreitol. These results are indicative of 5′-FSBA-induced disulfide bond formation, which requires the presence of at least two cysteine residues in the proximity of the ATP binding site. Identification of the critical cysteine residue was accomplished by sequential replacement of each cysteine in the protein by site-directed mutagenesis. Cys 523 was identified as the key residue involved in the formation of the 5′-FSBA-induced disulfide bond. Detailed kinetic analyses and comparison with similar enzymes, suggest that this cysteine residue, while in close proximity to the ATP binding site, is actually involved in aspartate binding in asparagine synthetase B.

Escherichia coli asparagine synthetase B (AS-B)[1] catalyses the ATP-dependent conversion of aspartate to asparagine using either glutamine or ammonia as a nitrogen source. In the absence of aspartate, the enzyme functions as a glutaminase, which is stimulated by the addition of ATP (Boehlein et al., 1994a). AS-B is a member of the *purF* subfamily of glutamine amidotransferases (GAT), characterized by an essential N-terminal cystiene residue (Boehlein et al., 1994b; Zalkin, 1993). Other members of this subfamily include glucosamine 6-phosphate synthetase and phosphoribosylpyrophosphate synthetase (PRPP), for which partial or whole crystal structures are now available (Smith et al., 1994; Isupov et al., 1996; Kim et al., 1996).

The reaction mechanism whereby the glutamine amide is transferred to an acceptor molecule has been under a great deal of investigation for both asparagine synthetase (Boehlein et al., 1994a,b, 1996; Stoker et al., 1996) and several other GAT enzymes (Zalkin, 1993). Even though there are several crystal structures available, there is still a controversy over the form of nitrogen which attacks the activated intermediate, with some suggesting protected ammonia (Mei & Zalkin, 1989), an activated glutamine amide (Richards & Schuster, 1992), or the tetrahedral intermediate formed en route to formation of the enzyme bound thioester of glutamic acid (Stoker et al., 1996). On the other hand, very little information has been obtained for the synthetase component of the AS-B reaction.

The mechanism by which aspartic acid is activated involves the formation of an unstable intermediate, β-aspartyl-AMP. The existence of this reaction intermediate has been validated through isotopic labeling studies with ^{18}O aspartate for both the bovine liver AS and *E. coli* AS-A (Leuhr & Schuster, 1985; Cedar & Schwartz, 1969). It is presumed that the carboxylate anion of aspartate attacks the α-phosphorus of ATP (I), forming a pentacovalent intermediate (II), to generate the mixed anhydride, β-aspartyl-AMP (III) (Scheme 1). Attack of the β-aspartyl-AMP by a nitrogen source then leads to the stable amide group of asparagine and AMP.

Although several amino acid residues have been implicated in having a role in the binding and catalysis in the GAT domain of AS-B (Boehlein et al., 1994a,b), to date, no information is available on the nucleotide or aspartate binding domain. Since active site affinity reagents have been very useful in obtaining information about amino acid residues located within the active site of enzymes, and one such affinity tag, [*p*-(fluorosulfonyl)benzoyl]adenosine (5′-FSBA), has been used with much success to probe the nucleotide binding sites of a wide variety of enzymes (Colman, 1983, 1990; Potter & Powers-Lee, 1992; Harlow & Switzer, 1990), the chemical modification of AS-B with 5′-FSBA was examined. In many of the previous reports, stable conjugates to 5′-FSBA were isolated, and the location of the residues in the primary amino acid sequence were used to identify the active site. Further, the extended structure of 5′-FSBA would suggest that the reactive sulfonylfluoride group could react in the vicinity where ATP and aspartate interact to form the β-aspartyl-AMP. Therefore, a combination of chemical modification with 5′-FSBA and site-specific mutagenesis was utilized to identify residues in the active site of AS-B.

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[1] 5′-FSBA, [*p*-(fluorosulfonyl)benzoyl]adenosine; DTT, DL-dithiothreitol; AS-B, *E. coli* asparagine synthetase B; GAT, glutamine amidotransferase; DMSO, dimethyl sulfoxide; PK, pyruvate kinase; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); CSA, cysteine sulfinic acid.

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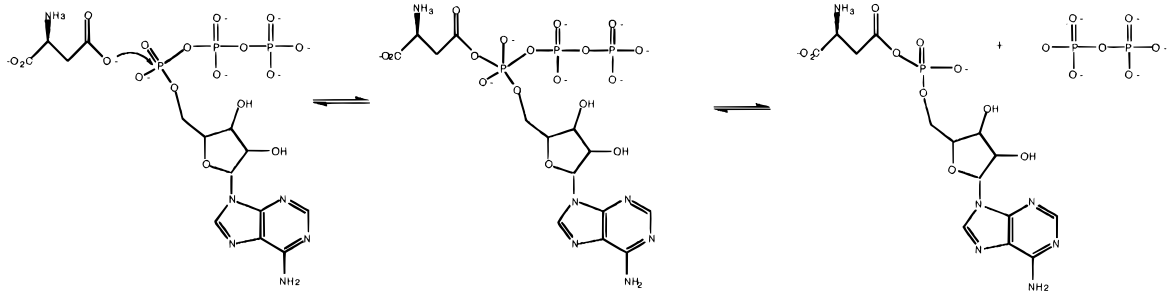
Scheme 1



(I) (II) (III)

MATERIALS AND METHODS

5′-FSBA Inactivation. A stock solution of 10 mM 5′-FSBA was made in ethanol:dimethyl sulfoxide (1:1, v:v). The concentration of the 5′-FSBA was determined spectrophotometrically using ϵ-259 nm = 1.35×10^4 M^{-1} cm^{-1}. Inactivation was performed at room temperature in reactions containing 100 mM Tris-HCl, pH 8, 8 mM $MgCl_2$, 5% ethanol, 5% dimethyl sulfoxide, 0.2 μg/μL asparagine synthetase B, and varying concentrations of 5′-FSBA. 5′-FSBA was omitted in control reactions. At given time points, samples were withdrawn and diluted 8-fold into reaction buffer (100 mM Tris-HCl, pH 8.0, 15 mM $MgCl_2$, 10 mM ATP, 10 mM glutamine, 10 mM aspartate, and PPi coupling reagent (Sigma Chemical Company, Technical Bulletin No. BI-100), which was prewarmed at 37 °C. Production of pyrophosphate was monitored through a continuous assay system, in which the amount of pyrophosphate produced is proportional to the decrease in [NADH]. In control reactions where 5′-FSBA was omitted, the asparagine synthetase B retained over 95% of its original activity over the entire incubation period.

ATP Protection from 5′-FSBA Inactivation. A stock solution of 10 mM 5′-FSBA was prepared as described above. Inactivation was performed at room temperature in reactions containing 100 mM Tris-HCl, pH 8, 8 mM $MgCl_2$, 5% ethanol, 5% dimethyl sulfoxide, 0.2 μg/μL asparagine synthetase B, 1 mM 5′-FSBA, and varying amounts of ATP. 5′-FSBA was omitted in control reactions. At given time points, samples were withdrawn and diluted 8-fold into reaction buffer, and the amount of pyrophosphate released was measured as described above.

Irreversibility of 5′-FSBA. In order to determine if covalent modification of the enzyme had occurred, 200 μg of wild-type AS-B was incubated with 2 mM 5′-FSBA (the final concentration of DMSO/ETOH was 10%, in order to keep the 5′-FSBA in solution) for a given time and then desalted by the method of Penefsky (1977). Control reactions were performed in which enzyme was incubated with 10% DMSO/ETOH 1:1. Initial velocity studies of the modified and unmodified enzyme were then employed to determine if the 5′-FSBA modification was covalent.

Reversibility of 5′-FSBA Inactivation by DTT. In order to determine if covalent modification of cysteines was occurring, a reaction containing 4 μg of enzyme was treated with 0.1 mM 5′-FSBA in the presence of 100 mM Tris-HCl, pH 8, 8 mM $MgCl_2$, 5% ethanol, 5% dimethyl sulfoxide for varying amounts of time, and asparagine synthetase activity was measured. At 31 min, a 20 mM final concentration of DTT was added to $^1/_2$ of the sample and to the other half water was added to keep the concentration of enzyme equal. Immediately following the addition of DTT, asparagine synthetase activity was measured by the amount of pyrophosphate released as described above.

Substrate and Substrate Analog Protection from 5′-FSBA Inactivation. A stock solution of 10 mM 5′-FSBA was prepared as described above. Inactivation was performed at room temperature in reactions containing 100 mM Tris-HCl, pH 8, 8 mM $MgCl_2$, 5% ethanol, 5% dimethyl sulfoxide, 0.4 μg/μL asparagine synthetase B, 2 mM 5′-FSBA, and varying amounts of substrates or substrate analogs. Control reactions were performed in each of the experimental conditions listed above, but 5′-FSBA was replaced with the same amount of DMSO/ETOH. At 20 min, samples were withdrawn and diluted 8-fold into reaction buffer, and the amount of pyrophosphate released was measured as described above.

Determination of the Stoichiometry of [^{14}C]5′-FSBA Labeling in Wild-Type AS-B Protein and Rabbit Muscle Pyruvate Kinase. Fluorosulfonylbenzoyladenosine 5′-P-[adenine-8-^{14}C]- (41.9 mCi/mmol) was obtained from NEN research products. AS-B protein was desalted using the method of Penefsky (1977), to remove the DTT which was added to the enzyme for storage. Approximately 13.6 nmol of AS-B was inactivated with 5′-FSBA by incubating the enzyme with 0.45 mM [^{14}C]5′-FSBA (2.13 mCi/mmol) at room temperature for approximately 1.5 h or until the AS-B activity was less than 10% of the control reaction (5′-FSBA). The 5′-FSBA treated enzyme was then separated from free analogs using G-50 sephadex spin column. The amount of radiolabeled protein and the concentration of protein in each sample was calculated. The protein concentration was determined with the Bio-Rad protein reagent kit using γ globulin as a standard. For pyruvate kinase, approximately 16 nmol of enzyme (Sigma) was inactivated with 5′-FSBA by incubating the enzyme with 2.0 mM [^{14}C]5′-FSBA (0.33 mCi/mmol) at room temperature for approximately 4 h or until the PK activity was less than 20% of the control reaction (5′-FSBA). PK activity was determined spectrophotometrically by following the decrease in NADH, in a coupled assay with lactate dehydrogenase. The 5′-FSBA treated enzyme was then separated from free analogs using G-50 sephadex spin column and the amount of radiolabeled protein and the concentration of protein in each sample was calculated.

Preparation of Cysteine Mutants of Asparagine Synthetase B. Mutations in which each of the eight cysteine residues

Table 1: Summary of Oligonucleotides Used for the Construction of Mutations in *E. coli* AS-B

oligonucleotide	oligonucleotide sequence
SS59	5′ CCA TGG AAG CTT GAT TTC CCC GGG GCT GTT TCG CAT TTC TTA 3′
SS65	5′ GCT TCC CAT ATG TGT TCA ATT TTT GGC GTA TTC GAT 3′
SS148	5′ AAC CAT CTC GGT ACC GTG CAT CAC 3′
SS253	5′ GCA CTG TAC GAC AGC G 3′
SS347	5′ GTG GTC ACC GCA TAA GTT TC 3′
SS371	5′ GCT GAG GCC GTG CCG GGG GGC CCT TCC 3′
SS372	5′ CCA ACA AAG CGA TGT CAG CC 3′
SS373	5′ AAA ATG GAA AAA CAC ATC TTA AGG GAA GCT TTT GAA GC 3′
SS375	5′ CCG GGC GGG CCC TCC GTC GCT GCT TCT TCC 3′
SS376	5′ CCG TTA CCG GCC ATT TTA TC 3′
SS377	5′ GCT TTG TTG GCA CGC GCG GCG TCA TAC 3′
SS378	5′ ATC GTG CGC GCA ACT GGC AC 3′
SS379	5′ CAG ACC GGA TCC GAC GCT GAA GT 3′

were replaced by alanine were constructed by PCR megaprimer mutagenesis. All mutagenic oligonucleotides are shown in Table 1. Briefly, two primers, one mutagenic primer and one 3′ or 5′ of the target region, were used to create a megaprimer, which was subsequently used with a third primer for production of the megaproduct (Satar & Sommers, 1990). This product was then digested with the appropriate restriction endonucleases and cloned into the native vector digested with the same enzymes. All inserts were sequenced using the USB reagent kit with Sequenase 2.0, and purified according to standard procedures.

Determination of $K_{m(app)}$ for Wild-Type AS-B and AS-B Mutants. Apparent affinity constants $K_{m(app)}$ for AS-B substrates were determined by incubating purified wild-type AS-B or mutant AS-B in reaction mixtures in which all but one of the substrates were at saturating conditions. The concentration of the substrates for wild-type and AS-B mutants when held constant were approximately 10 times their K_m value, unless otherwise noted. The highest concentration of aspartate used in these assays was 150 mM, so mutant enzymes with aspartate K_ms over 15 mM were not truly saturating. All assays contained 100 mM Tris-HCl, pH 8.0, 8 mM $MgCl_2$, and 3−15 μg of the appropriate purified enzyme. A 10-fold variation in substrate, with the K_m falling within these limits, was used to determine the K_m for both wild-type and mutant enzymes. The initial velocity of each reaction was determined spectrophotometrically by following the production of pyrophosphate during asparagine synthesis, using pyrophosphate reagent (Sigma Chemical Company, Technical Bulletin No. BI-100). Each assay was run two to four times and the averages are presented. The rate and concentration data were fitted to the Michaelis−Menton equation using the software program Graph Pad Prism.

Preparation and Purification of Chromium Nucleotides. The bidentate chromium nucleotides were prepared and purified according to the procedure of Cleland (Cleland, 1982).

RESULTS

Fluorosulfonylbenzoyl analogs of nucleotides have been used as site specific labels of nucleotide binding sites for many proteins (Colman, 1983, 1990). The prototype of these compounds, 5′-FSBA, retains the purine and ribose structures, while substituting a reactive sulfonyl fluoride for the γ-phosphoryl group of ATP, when the analog is arranged in an extended conformation. A similar extended geometry for AMPPcP in yeast aspartyl tRNA synthetase has been shown to adopt a conformation where, by analogy, the 5′-FSBA electrophilic reactive center would be predicted to be in the region of the active site where aspartate is bound during formation of β-aspartyl-AMP (Cavarelli et al. 1994). The reagent is very electrophilic and has been shown to react covalently with several amino acid residues, including tyrosine, lysine, serine, histidine, and cystiene (see Coleman, 1983, 1990 and references therein). In an attempt to gain insight about the particular amino acid residues involved in formation of β-aspartyl-AMP, an investigation was undertaken into the interaction of the affinity analog 5′-FSBA with AS-B.

The reaction of 5′-FSBA with *E. coli* AS-B follows several general characteristics of a site-specific affinity label. First, the incubation of purified AS-B with 5′-FSBA resulted in a time-dependent inactivation of the enzyme (Figure 1A). This inactivation was consistent with the following reaction:

$$\mathrm{E} + \mathrm{I} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{E{\cdot}I} \xrightarrow{k_3} \mathrm{EX} \qquad \text{where } K_i = k_2/k_1 \qquad (1)$$

where K_i is the dissociation constant, k_3 is the rate of inactivation at saturating inactivator (I), and EX is the inactive enzyme. The inactivation of the enzyme was first order with respect to 5′-FSBA concentration for approximately 25 min as seen from the plot of the ln% activity vs time (Figure 1A). The kinetic constants were determined from a replot of the reciprocal first-order rates (k_{obs}) versus the reciprocal inactivator concentration according to eq 2:

$$1/k_{obs} = K_i/k_3[\mathrm{I}] + 1/k_3 \qquad (2)$$

The dissociation constant K_i was estimated to be 1.2 mM, and k_3, the maximal rate of inactivation at saturating 5′-FSBA, was calculated to be 0.49 min^{-1} (Figure 1B).

Another general characteristic of a site-specific affinity reagent is that the presence of the natural substrate, which binds to the same site on the enzyme, should yield some protection from inactivation by the reagent. Therefore, if 5′-FSBA inhibition requires base and sugar binding to the ATP binding site of AS-B, then the presence of ATP in the reaction should provide specific protection against 5′-FSBA inactivation. ATP was found to protect AS-B from the 5′-FSBA inactivation of asparagine synthetase activity in a concentration-dependent manner. The natural logarithm of

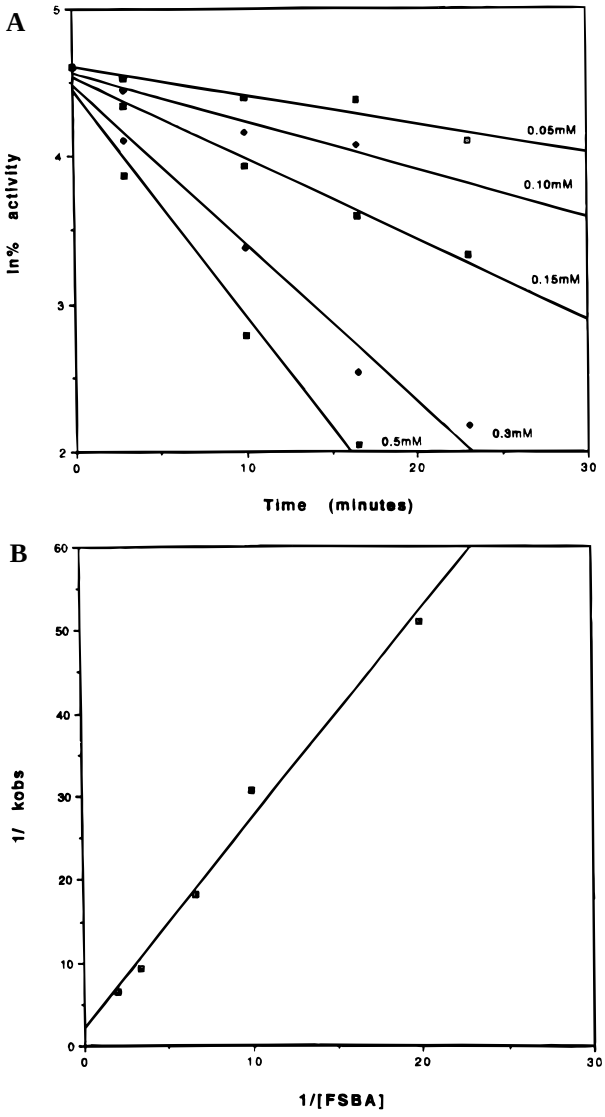


FIGURE 1: (A) Inactivation of *E. coli* AS-B by 5′-FSBA. *E. coli* AS-B (0.2 μg/μL) was incubated with varying concentrations of 5′-FSBA (0.05, 0.10, 0.15, 0.30, and 0.50 mM) as indicated on the graph, at room temperature in 100 mM Tris-HCl, pH 8, 8 mM $MgCl_2$, 5% ethanol, 5% dimethyl sulfoxide. At the times indicated, aliquots were withdrawn and assayed for asparagine synthetase activity. Control reactions were performed in which no 5′-FSBA was added. (B) Dependence of pseudo-first-order rate constant of inactivation of *E. coli* AS-B activity on 5′-FSBA concentration. The inactivation constants, k_{obs}, were calculated from the curves shown in Figure 1A.

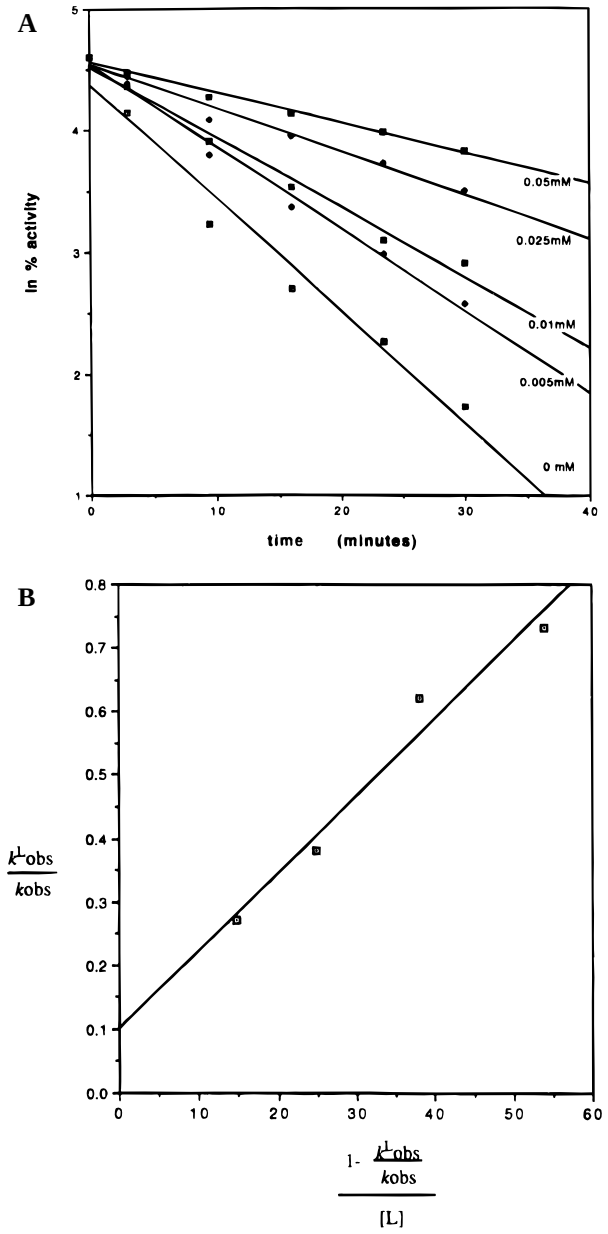


FIGURE 2: (A) Protection from 5′-FSBA inactivation of *E. coli* AS-B activity by Mg·ATP. *E. coli* AS-B (0.2 μg/μL) was incubated with 1 mM 5′-FSBA and protected with ATP at the concentrations indicated on the graph. The *E. coli* AS-B activity in the absence of 5′-FSBA was taken as the control. (B) Determination of the ATP K_d for *E. coli* AS-B. The inactivation constants, k_{obs}, in the presence of varying concentrations of ATP were calculated from Figure 2A.

the percent activity remaining vs time, at various concentrations of ATP, were plotted to obtain k_{obs} at various ATP concentrations (Figure 2A). This data clearly shows that protection by ATP is occurring. An ATP K_d of 12 μM was extrapolated from a replot using eq 3 [from Caremil (1987)], where L is the ligand, ATP.

$$\frac{k^{L}_{obs}}{k_{obs}} = \frac{k_2}{k_1} + \left[\frac{1 - \frac{k^{L}_{obs}}{k_{obs}}}{[L]}\right] \quad (3)$$

Since ATP provides specific protection against 5′-FSBA inactivation (Figure 2A), and the asparagine synthetase reaction is believed to go through a β-aspartyl-AMP intermediate (Leuhr & Schuster, 1985; Cedar & Schwartz, 1969), it may be proposed that ATP, in the presence of aspartate, should offer enhanced protection against 5′-FSBA inactivation. It is clear from the data presented in Table 2 that this is the case. For example, when the enzyme is incubated with 1.0 mM 5′-FSBA for approximately 20 min in the absence of substrates, only 8.8% of the activity remained. At approximately an equimolar ratio of ATP to enzyme (0.005 mM), 14.5% activity remained, whereas when 0.005 mM ATP and 5 mM aspartate were present, 79.1% activity remained. The presence of aspartate alone offered no protection against 5′-FSBA. The presence of cysteine sulfinic acid, an analog which is competitive with aspartate (Parr et al., 1996), ammonium chloride, or glutamine alone or in combination with ATP, offered very little or no protection from 5′-FSBA inactivation (see Table 2).

Table 2: Protection of the Glutamine-Dependent Activity of AS-B against 5′-FSBA Inactivation by Various Substrates and Substrate Analogs[a]

added compound	% activity remaining
	8.8
0.005 mM ATP	14.5
5 mM CSA	9.0
5 mM aspartate	11.2
5 mM glutamine	15.5
100 mM ammonium chloride	8.5
0.005 mM ATP + 5 mM CSA	13.2
0.005 mM ATP + 5 mM aspartate	79.1
0.005 mM ATP + 5 mM glutamine	18.4

[a] Inactivation was performed at room temperature in reactions containing 100 mM Tris-HCl, pH 8, 8 mM $MgCl_2$, 5% ethanol, 5% dimethyl sulfoxide, 5 μg of wt AS-B, and 1.0 mM 5′-FSBA. 5′-FSBA was omitted in control reactions. At 20 min, reactions were diluted 8-fold into reaction buffer (100 mM Tris-HCl, pH 8.0, 15 mM $MgCl_2$, 10 mM ATP, 10 mM glutamine, 10 mM aspartate, and PPi coupling reagent, which was prewarmed at 37 °C). Production of pyrophosphate was monitored through a continuous assay system, as mentioned above. All standard errors are less that 10%.

In order to determine the stoichiometry of the affinity label, it was necessary to ascertain if the inhibition by 5′-FSBA was irreversible. This was accomplished in the following two ways. First, AS-B was incubated in the presence or absence of 2 mM 5′-FSBA for a given time and then excess 5′-FSBA removed by gel filtration. The kinetic constants (apparent K_m and k_{cat} for aspartate) for the modified and unmodified enzymes were determined by measuring the rate of formation of product at varying concentrations of aspartate. The apparent K_m and k_{cat} for the glutamine-dependent aspartate reaction for the modified enzyme were 1.1 mM and 0.76 s^{-1}, respectively, whereas the kinetic constants for the unmodified enzyme were 1.1 mM and 0.94 s^{-1}. The change in the k_{cat} for the reaction and not in the apparent K_m is consistent with the formation of an irreversible inactive enzyme inhibitor complex. Also, AS-B was incubated with 5′-FSBA until no further enzyme activity was observed. Excess reagent was then removed through a gel filtration column and the activity assay was performed again. The 5′-FSBA-treated enzyme remained completely inactive, indicating that the 5′-FSBA was bound extremely tight if it was not covalent.

The covalent incorporation of 5′-FSBA was measured by incubating approximately 13.6 nmol of AS-B with [^{14}C]5′-FSBA (2.13 mCi/mmol). The enzymatic activity was measured at various times, until the activity remaining was less than 10% of the original activity. Unincorporated label was then removed from the enzyme by gel filtration, the protein concentration determined, and the radioactivity measured. Even though the enzymatic activity was decreased by greater than 90% of its original activity, less than 10% of the enzyme was labeled with [^{14}C]5′-FSBA. In control experiments, whereby 5′ FSBA was used to inactivate rabbit muscle pyruvate kinase, a stoichiometry of approximately 0.7:1 nmol of 5′-FSBA to nmol of enzyme was seen. Similar results with 5′-FSBA have been reported by several other investigators (Rutherford et al., 1991; Takata & Fujioka, 1984). In each of these cases, a cysteine residue initially reacted with the 5′-FSBA to yield a thiolsulfonate derivative, followed by rapid displacement of the sulfinic acid derivative by an adjacent cysteine, to generate a disulfide bond. This reaction sequence led not only to the lack of correlation between label and enzyme, but the concurrent loss of free sulfhydral groups and the reversibility of this inhibition by dithiothreitol.

Table 3: Reactivation of Asparagine Synthetase Activity with Dithiothreitol, Following Inactivation by Incubation with 5′-FSBA[a]

treatment	specific activity (nmol/min/mg)
−FSBA −DTT	635
+FSBA −DTT	420
−FSBA +DTT	630
+FSBA +DTT	702

[a] Asparagine synthetase activity was assayed in the presence or absence of 0.1 mM 5′-FSBA for 31 min as described in the Materials and Methods. Aliquots were then assayed for asparagine synthetase activity in the presence or absence of 20 mM DTT. All standard errors are less than 10%.

Table 4: Titration of Free Cysteines with DTNB[a]

experimental conditions	Cysteine residues detected/ mole of monomer
wild-type AS-B (+FSBA)	2.0
wild-type AS-B (-FSBA)	3.2
difference	1.2
C1A (+FSBA)	1.4
C1A (−FSBA)	2.8
difference	1.4

[a] Two reactions were performed in which approximately 10 nmol of wt AS-B was either incubated with DMSO/ETOH or completely inactivated with 5′-FSBA. Excess 5′-FSBA was removed by a spin column and the eluate added to a solution containing 1% SDS, 0.1 M Tris-HCl, pH 8, 0.01 mM EDTA. DTNB, 0.01 M in 0.05 M sodium phosphate buffer, pH 7, was then added to the previous mixture, and the absorbance at 412 nm was monitored. The reaction was allowed to proceed until no further increase in absorbance was seen over a blank. The amount of free cysteines were calculated using 13 600 M^{-1} as an extinction coefficient. The same procedure was then repeated with C1A, a mutant with cysteine 1, changed to an alanine.

In order to examine if a disulfide bond was forming between two adjacent cysteine residues, the reversibility of the 5′-FSBA inhibition by DTT was investigated. AS-B was inactivated with 5′-FSBA to approximately 60% of wt AS-B activity, followed by the addition of 20 mM DTT, incubation for 1 min, and determination of AS enzymatic activity. As can be seen in Table 3, the addition of DTT to the enzyme treated with 5′-FSBA resulted in a complete restoration of AS-B activity. These results, in accord with the very low level labeling of the enzyme by [^{14}C]5′-FSBA, strongly support the hypothesis that a thiolsulfonate derivative of 5′-FSBA was formed at the active site, which was subsequently attacked by a second cysteine, forming a disulfide bond which was then reduced by DTT.

Attempts were next made to quantify the loss of free cysteine residues in the wild-type and 5′-FSBA inactivated protein. The free sulfhydryls were titrated with 5′5′-dithiobis(2-nitrobenzoic acid) (DTNB) and quantified by following the absorbance of the 3-carboxalato-4-nitrothiophenolate using 13 600 M^{-1} as the extinction coefficient. The modification with DTNB was performed on both 5′-FSBA-treated and -untreated enzyme. As a comparison, DTNB titration was also performed on C1A, a mutant enzyme in which cysteine 1 is changed to an alanine (Table 4). Similar results were obtained with the native wild-type enzyme or with the enzyme that had been reduced and denatured (data not shown). Although the exact number of free cysteines were not obtained in these experiments, several conclusions

Table 5: Effect of 5′-FSBA on the Glutaminase Activity of *E. coli* AS-B[a]

	glutaminase reaction		synthetase reaction	
reaction mix	+5′-FSBA (nmol/min/mg)	−5′-FSBA (nmol/min/mg)	+5′-FSBA (nmol/min/mg)	−5′-FSBA (nmol/min/mg)
Gln	720	720		
Gln + ATP	1190	1250		
Gln + ATP + Asp	1245	1570	125	1057
Gln + ATP + CSA	1240	1655		
Gln + Asp	825	785		
Gln + CSA	790	745		

[a] Inactivation of the glutaminase activity of AS-B was performed at room temperature in reactions containing 100 mM Tris-HCl, pH 8, 8 mM $MgCl_2$, 5% ethanol, 5% dimethyl sulfoxide, 0.2 μg/μL asparagine synthetase B, and 1 mM 5′-FSBA. 5′-FSBA was omitted in control reactions. At 12 min, 10 μL of sample was withdrawn and diluted 10-fold into one of six different reaction mixes. Concentrations of each of the substrates when added were glutamine, 10 mM; ATP, 10 mM; aspartate, 10 mM; and cysteine sulfinic acid, 10 mM. Each reaction was incubated for 10 min at 37 °C and then terminated by boiling for 3 min. Production of pyrophosphate was monitored through an endpoint assay, in which the amount of pyrophosphate produced is proportional to the decrease in [NADH]. In control reactions where 5′-FSBA was omitted, the asparagine synthetase B still retained over 95% of its original activity over the entire incubation period. Production of glutamine was monitored by an end point assay in which the amount of glutamate formed was proportional to the increase in [NADH].

can be made. First, there was a decrease of approximately 0.4 cysteines/mol when comparing wt AS-B and C1A mutant enzyme. This trend was consistent in the 5′-FSBA modified wt AS-B and C1A, where there was a decrease of 0.6 cysteine. Moreover, the decrease in the number of cysteines between untreated enzyme versus 5′-FSBA treated enzyme for wild type AS-B and C1A was 1.2 and 1.4, respectively. While exact quantitation was not obtained, these experiments support the hypothesis that at least one, if not more cysteines are rendered unreactive to DTNB due to 5′-FSBA modification.

Since all the data presented thus far supported the hypothesis that 5′-FSBA inactivation caused the formation of an intramolecular disulfide bond, two critical questions remained to be addressed. First, was the sulfonyl fluoride reacting directly in the ATP binding site or in the aspartate binding site, and second, which cysteine residue was critical in binding or catalysis. To address the first question, several experiments utilizing unique properties of the glutaminase reaction were examined in the presence and absence of 5′-FSBA.

Although the glutaminase reaction does not consume a nucleotide, Mg·ATP has recently been shown to stimulate the reaction (Boehlein et al., 1994a). If 5′-FSBA was initially bound to the enzyme in a conformation similar to that of Mg·ATP, the covalent modification of the enzyme resulting from the inactivation would produce an enzyme no longer capable of ATP stimulation, since the reactive sulfonyl fluoride group would be in the proximity of the γ-phosphate of ATP. To test this hypothesis, AS-B was first incubated in the presence or absence of 5′-FSBA and the glutaminase activity of both treated and untreated enzymes was measured in the presence of saturating glutamine (10 mM). Both treated and untreated enzymes had similar specific activities, indicating that the 5′-FSBA had no effect on this reaction (Table 5). Next, AS-B was incubated in the presence or absence of 5′-FSBA, and then the glutaminase activity was measured in the presence of 10 mM glutamine and 10 mM Mg·ATP. These results are interesting in that the presence of Mg·ATP still stimulated the glutaminase activity, approximately 1.7-fold, for both the 5′-FSBA-treated and -untreated enzyme. One explanation for this result is that the 5′-FSBA modification results in disulfide bridge formation outside of the ATP binding site. As shown in Table 5, the presence of Mg·ATP and cysteine sulfinic acid (CSA), an aspartate analog, further stimulates the glutaminase activity of untreated enzyme, approximately 1.3-fold more than glutamine and Mg·ATP, and 2.3-fold more than glutamine alone. As support for the hypothesis that 5′-FSBA is modifying the aspartate binding site, it is noteworthy that the additional stimulation due to the CSA is lost in the 5′-FSBA-treated enzyme. The presence of CSA alone does not effect the glutaminase activity. In the presence of all three substrates, aspartate, Mg·ATP, and glutamine, the production of both glutamate and PPi were followed. In the treated enzyme, the rate of glutamate formation is similar to that of enzyme with glutamine and ATP, whereas in the untreated enzyme, a stimulation due to the presence of aspartate is seen. When looking at the formation of PPi, it can be seen that the 5′-FSBA-treated enzyme has approximately $^1/_{10}$ of the glutamine-dependent AS activity as the untreated enzyme (Table 5).

While it is clear that 5′-FSBA modifies a cysteine residue in the aspartate binding site of AS-B, the ribose and base of 5′-FSBA are initially bound to the ATP binding site of the enzyme. This opens the opportunity to perform experiments utilizing the glutaminase activity of AS-B that will provide insight into the bound conformation of ATP at the active site. From the crystal structure of the active site of yeast aspartyl tRNA synthetase, it has been shown that Mg·ATP adopts a bent conformation which is stabilized by a magnesium ion which is coordinated to the pyrophosphate group. On the other hand, AMPPcP, an ATP analog in which the oxygen linking the β- and γ-phosphate groups is replaced by a methylene group, is perturbed in such a way that the interaction with Mg^{2+} is changed and this may be the reason why the analog adopts a different conformation in the enzyme. In both cases, the adenosine moiety and the α-phosphate occupy the same position (Cavarelli et al., 1994). Several experiments comparing the activities of the enzymes with the addition of substrates and substrate analogs were examined (see Tables 6 and 7). First it was shown that ATP in the presence of Mg^{2+} stimulated the glutaminase reaction ~1.7-fold, whereas in the absence of Mg^{2+}, ATP became a slight inhibitor of the glutaminase activity. Next, it was shown that AMPPcP + Mg^{2+} could not stimulate the glutaminase activity of wt AS-B alone or in the presence of aspartate, albeit a slight stimulation was seen in the presence of PPi (Table 6). Even though the adenosine moiety and α-phosphate of AMPPcP should be in a similar conformation to that of ATP, it seems that the Mg^{2+} ion must be bound properly for activation to occur. Furthermore, AMPPcP was not found to be an inhibitor of the glutamine-dependent AS reaction at concentrations up to 3 mM. This may imply that a large majority of the binding energy associated with ATP is due to interactions between the enzyme and the Mg^{2+} ion. On the other hand, bidentate Cr·ATP, which is known to adopt a bent conformation (Cleland, 1982), did stimulate the glutaminase reaction. These reactions were performed in Bis-Tris at pH 6.5 to avoid breakdown of the Cr·ATP. As

Table 6: Effect of Different Substrates and Substrate Analogs on the Production of Glutamate at pH 8.0[a]

added substrate	% activity
$MgCl_2$	100
ATP	86
ATP + $MgCl_2$	166
ATP + Asp + $MgCl_2$	221
AMPPcP + $MgCl_2$	96
AMPPcP+ Asp + $MgCl_2$	95
AMPPcP + PPi + $MgCl_2$	114
AMP + $MgCl_2$	130
AMP + PPi + $MgCl_2$	209
AMP + PPi + Asp + $MgCl_2$	198

[a] Wt AS-B, 2 μg, was incubated at 37 °C for 15 min in 100 mM Tris, pH 8.0, containing 10 mM glutamine, and the amount of glutamate produced was measured. All other substrate and substrate analog concentrations were held at 5 mM with the exception of $MgCl_2$, which was held at 8 mM. Percent activity was calculated in relation to enzymatic activity determined in buffer with 8 mM $MgCl_2$. All standard errors are less that 10%.

Table 7: Effect of ATP and Chromium ATP on the Production of Glutamate at pH 6.5[a]

added substrate	% activity
	100
$MgCl_2$	97
ATP	90
ATP + $MgCl_2$	128
Cr·ATP	84
Cr·ATP + $MgCl_2$	114

[a] Wt AS-B, 2 μg, was incubated at 37 °C for 15 min in Bis-Tris/Tris, pH 6.5, (100 mM each) containing 20 mM glutamine, and the amount of glutamate produced was measured. Added substrate concentrations are as follows: $MgCl_2$, 16 mM; ATP, 4.0 mM; Cr·ATP, 4.0 mM. Percent activity was calculated in relation to enzymatic activity in buffer alone. All standard errors are less that 10%.

a control, reactions in the presence of ATP or ATP with $MgCl_2$ were also performed. It is clear that the stimulation by Mg·ATP is greatly reduced at this pH (see Table 7). Even so, it is obvious from these results that Cr·ATP only in the presence of $MgCl_2$ stimulates the glutaminase reaction. It is possible that the Cr·ATP used in these experiments, a combination of several isomers, may be obscuring the stimulatory effects and that the proper isomer of Cr·ATP may cause some stimulation of the glutaminase activity in the absence of Mg ion. However, it is clear that Cr·ATP in the presence of Mg^{2+} ion does enhance the glutaminase activity equally similar to that of the ATP-dependent stimulation. This may imply that a second Mg^{2+} ion is required for the stimulation of the glutaminase activity.

The second question to be addressed was which of the cysteine residues were being modified by 5′-FSBA. Therefore, a series of mutations were constructed whereby each of the eight cysteine residues in wt AS-B were individually replaced by an alanine. The N-terminal cysteine, C1, which is critical for both the glutaminase and glutamine-dependent AS activity, was previously constructed (Boehlein et al., 1994b). The seven additional enzymes containing individual cysteine replacements were purified, and the kinetic constants for the glutamine-dependent AS activity were determined and compared to wt AS-B. As can be seen in Table 8, five of the seven mutations constructed, C168A, C386A, C423A, C436A, and C514A, had very similar kinetic constants with respect to glutamine. In fact, the specificity constant, k_{cat}/K_m for glutamine was changed less than 3-fold in all of these cases. As mentioned previously, mutation of C1 to an alanine resulted in an enzyme which had an undetectable glutamine-dependent AS activity (Boehlein et al., 1994b). Replacement of C99 with an alanine produced an enzyme with a 7.5-fold increase in the glutamine K_m but an unaltered k_{cat}. It is very reasonable that the mutation of this residue may have caused an alteration in glutamine binding since the cognate residue D98, in both PRPP amidotransferase and glucosamine-6-phosphate synthetase, is proposed to be involved directly in glutamine binding. It is therefore likely that replacement of C99 with alanine caused a local perturbation of the enzyme so that D98 was repositioned in such a way that optimum binding of glutamine could not occur. With the exception of C1A, replacement of C523 with an alanine residue caused the most dramatic effect on the enzymatic activity. This replacement had very little effect on the K_m for either glutamine or ATP, but had a tremendous effect on the K_m for aspartate, increasing it over 80-fold vs wt AS-B. The k_{cat} for this mutant was approximately $^1/_2$ that of wt AS-B.

The rate of inactivation by 5′-FSBA was then determined for this series of mutations. The loss of the glutamine-dependent AS activity of each of the cysteine mutants was followed for a total of 30 min, in the presence of 0.1 mM 5′-FSBA. The first-order rates of inactivation (k_{obs}) were then determined at this 5′-FSBA concentration (see Table 9). For the C1A mutant, a similar experiment was performed, except the ammonia-dependent activity was followed instead of glutamine-dependent activity. With the exception of C523A, the k_{obs} for 5′-FSBA inactivation of each of the mutant enzymes was comparable to the k_{obs} of wt AS-B. Following 30 min of incubation, each of the mutant enzymes had less than 40% activity remaining, whereas C523A retained 100% of the activity during the same incubation period. The dissociation constant K_i and the maximal rate of inactivation was then estimated for this mutant in a similar manner to that described for wt AS-B. The dissociation constant of 5′-FSBA for Cys 523 was 3.7 mM, and the maximal rate of inactivation at saturating 5′-FSBA was calculated to be 0.033 min^{-1}. Since the K_i was 3-fold larger and the k_3 was almost 15-fold slower, it is apparent that Cys 523 is somehow involved in the 5′-FSBA inactivation of wt AS-B.

If Cys 523 is the residue which is primarily attacked by 5′-FSBA, replacement of this residue by an alanine should produce an enzyme which is still inhibited by 5′-FSBA, but this inhibition would no longer be reversed by the addition of DTT. Therefore, the reversibility of 5′-FSBA inactivation by DTT was examined for C523A. The 5′-FSBA, at a final concentration of 2 mM, was added to 9 μg of C523A and incubated at room temperature for an hour. Following the incubation, 64% of the activity remained. DTT was then added to the assay mixture at a final concentration of 20 mM and incubated for an additional 2 min, and the activity was re-examined. No reactivation occurred. A sample was then allowed to incubate with 20 mM DTT for 30 min and then examined. Again, no increase in activity was seen. It is clear from this data that the 5′-FSBA inhibition is not reversible upon the addition of DTT for C523A, as it was for each of the other mutants (data not shown). An attempt was also made to label this mutant with ^{14}[C]5′-FSBA, but due to the increase in the dissociation constant, and decrease in the rate of inactivation, a high

Table 8: Apparent Kinetic Constants for the Glutamine-Dependent Activity of Wild-Type AS-B, C99A, C168A, C386A, C423A, C436A, C514A, C523A[a]

	glutamine			aspartate			atp		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
wild-type AS-B	0.69 ± 0.07	1.01 ± 0.05	1463	0.68 ± 0.07	1.05 ± 0.04	1544	0.18 ± 0.01	1.10 ± 0.03	6111
C99A	5.24 ± 0.46	1.31 ± 0.04	250	0.96 ± 0.08	1.37 ± 0.04	1427	0.21 ± 0.02	1.17 ± 0.04	5571
C168A	0.83 ± 0.08	0.86 ± 0.04	1036	1.68 ± 0.08	1.23 ± 0.04	732	0.39 ± 0.03	1.15 ± 0.05	2951
C386A	0.51 ± 0.07	0.69 ± 0.04	1169	0.88 ± 0.07	0.77 ± 0.03	875	0.20 ± 0.02	0.87 ± 0.04	4350
C423A	0.77 ± 0.93	1.09 ± 0.06	1415	1.91 ± 0.27	1.35 ± 0.12	706	0.44 ± 0.05	1.46 ± 0.08	3319
C436A	0.74 ± 0.07	0.73 ± 0.02	986	0.85 ± 0.06	0.74 ± 0.02	871	0.29 ± 0.045	0.87 ± 0.06	3000
C514A	0.92 ± 0.03	0.74 ± 0.02	804	1.70 ± 0.06	0.79 ± 0.01	465	0.29 ± 0.04	0.78 ± 0.03	2701
C523A	0.37 ± 0.02	0.40 ± 0.006	1081	55.7 ± 3.11	0.52 ± 0.01	9.3	0.24 ± 0.22	0.53 ± 0.02	2208

[a] C1A has no detectable glutamine-dependent activity.

Table 9: Determination of k_{obs} at 0.1 mM 5′-FSBA for wt AS-B and Each of the Cysteine Mutants[a]

enzyme	k_{obs} (1/min)
wt AS-B	4.83×10^{-2}
C1A	2.92×10^{-2}
C99A	4.39×10^{-2}
C168A	4.33×10^{-2}
C386A	3.31×10^{-2}
C423A	4.69×10^{-2}
C436A	4.12×10^{-2}
C514A	5.22×10^{-2}
C523A	0

[a] Inactivation was performed at room temperature in reactions containing 100 mM Tris-HCl, pH 8, 8 mM $MgCl_2$, 5% ethanol, 5% dimethyl sulfoxide, 2.0−3.0 μg of the appropriate mutant enzyme, and 0.1 mM 5′-FSBA. 5′-FSBA was omitted in control reactions. At given time points, samples were withdrawn, and diluted 8-fold into reaction buffer (100 mM Tris-HCl, pH 8.0, 8 mM $MgCl_2$, 5 mM ATP, 10 mM glutamine, 10 mM aspartate, and PPi coupling reagent, which was prewarmed at 37 °C). The only exceptions were for C99A, where the glutamine concentration was 20 mM, for C523A, where the aspartate concentration was 150 mM, and for C1A, where glutamine was replaced by 100 mM NH_4Cl.

enough specific activity of the radiolabeled 5′-FSBA could not be obtained experimentally.

DISCUSSION

The goal of this investigation was to learn more about the amino acid residues involved in the synthetase domain of the *E. coli* AS-B. Since a crystal structure is not yet available for any asparagine synthetase, 5′-FSBA, a reactive derivative and analog of ATP, was used to gain insight into the amino acid residues in or in close proximity to the ATP binding site. It was determined that 5′-FSBA was a site specific affinity label of AS-B, whereby several criteria held true. 5′-FSBA was capable of providing concentration-dependent, saturable, enzyme inactivation. The first-order rate constant, k_3, 0.49 min^{-1} and dissociation constant, K_i of 1.2 mM were determined. It was also shown, that the true substrate Mg•ATP provided specific protection against the 5′-FSBA inactivation, with a K_d of 12 μM. On the basis of these results, it is highly likely that the 5′-FSBA interacts in the active site of AS-B.

5′-FSBA Inactivates AS-B by Formation of a Disulfide Bridge between two Spatially Constrained Cysteine Residues. Several experiments provided overwhelming evidence that the 5′-FSBA does not remain covalently attached to AS-B, yet still irreversibly inactivates the enzyme. The strongest evidence for this is the lack of any correlation between the loss of enzymatic activity and the incorporation of radiolabeled 5′-FSBA. The lack of incorporation of label along with the fact that DTT fully reverses the inactivation provides strong evidence that 5′-FSBA catalyzes the formation of a disulfide bond, which is responsible for the loss of asparagine synthetase activity. 5′-FSBA-induced inactivation of enzymatic activity by disulfide bridge formation has been suggested for several enzymes including bovine adrenal 3β-hydroxysteriod dehydrogenase/steroid isomerase, (Rutherford et al., 1991), rabbit muscle pyruvate kinase (Annamalai & Colman, 1981), myosin subfragment 1 (Togashi & Reisler, 1982), and rat liver *S*-adensylhomocysteinase (Takata & Fujioka, 1984; Gomi et al., 1986). The proposed mechanism for the 5′-FSBA disulfide bridge formation is as follows: first, a cysteine residue reacts with 5′-FSBA to form a sulfonylbenzoyl-adenosylated cysteine; next, a contiguous cysteine residue attacks the intermediate, displacing the sulfonylbenzoyl adenosine and forming an intramolecular disulfide bond.

In order to determine the first cysteine residue inactivated by 5′-FSBA, a series of site-directed mutants were constructed. Each of the eight mutant enzymes, in which cysteine was replaced by alanine, was inactivated with 5′-FSBA, and the k_{obs} at one fixed 5′-FSBA concentration was determined. In each case, the k_{obs} at 0.1 mM 5′-FSBA was similar to that of wild-type, except in the mutant where cysteine 523 was replaced by alanine. In this case, using 0.1 mM 5′-FSBA, no decrease in the enzymatic activity was seen for the entire time studied. In order to determine if the dramatic change in the 5′-FSBA inactivation was primarily due to the binding of the reagent or the rate of inactivation, both the K_i and k_3 were determined for this mutant enzyme. It was found that the K_i for 5′-FSBA in the C523A mutant was approximately 3-fold larger, and k_3 was approximately 15-fold slower than the inactivation of wild-type AS-B. This is the reason why no inactivation was seen in the initial screening, with 0.1 mM 5′-FSBA. It was also found that DTT did not reverse the inhibition, as it did for wild-type AS-B (see Table 9) and each of the other cysteine mutants (data not shown). On the basis of this evidence, it is proposed that Cys 523 is the first residue which is modified by 5′-FSBA. Since removal of this residue results in C523A whereby the inactivation by 5′-FSBA is an order of magnitude slower compared to wild-type AS-B and the inactivation is no longer reversible by DTT, this indicates that a cysteine residue is no longer the primary target of the reagent in C523A.

While the proposed mechanism for the 5′-FSBA formation of a disulfide bond requires two spatially adjacent cysteines, either in a native state or following a conformational change brought upon by the binding of 5′-FSBA, it is curious that in no cases was radiolabeled enzyme detected upon the addition of [^{14}C]5′-FSBA. If Cys 523 was the primary target of [^{14}C]5′-FSBA, then removal of the spatially adjacent cysteine by site-directed mutagenesis should have resulted in an enzyme whereby Cys 523 was labeled by the reagent, but whose inactivation was still reversible by DTT. As shown in the Results, this was not the case. None of the mutant enzymes were found to have an appreciable amount of incorporated label. The only enzyme in which labeling may have occurred was the mutant enzyme C523A, but experimental limitations excluded the determination of radiolabled product. Some evidence which supports 5′-FSBA covalent modification of C523A is that DTT no longer reverses the inactivation, and the K_i and k_3 for the inactivation are significantly altered. These changes in the inactivation and dissociation constants are probably due to modification of an amino acid residue in close proximity to Ala 523. It has been noted for both rat liver *S*-adensylhomocysteine hydrolase (Aksamit et al., 1994) and guanidoacetate methyltransferase (Takata et al., 1991) that three spatially adjacent cysteines may be involved in the disulfide bond formation. In the case of *S*-adensylhomocysteine hydrolase, the two cysteine residues involved in the 5′-FSBA disulfide bond formation were located by chemical modification. Mutagenesis was then performed on one of the cysteine residues, and 5′-FSBA modification produced a disulfide bond with a third cysteine residue. This third cysteine residue was located by chemical modification. It is possible to explain the lack of incorporation of label seen in experiments with the AS-B cysteine mutants if three spatially adjacent cysteine residues are involved in a manner similar to that of *S*-adensylhomocysteine hydrolase. We propose that Cys 523 is the first residue inactivated by 5′-FSBA, followed by release of the reagent and formation of a disulfide by either of two spacially constrained cysteine residues.

Modified Cysteine Residue Is in the Aspartate Binding Pocket, Rather Than the ATP Binding Domain of AS-B. In order to determine if there was a correlation between the 5′-FSBA inactivation of the asparagine synthetase and glutaminase activities of AS-B, several experiments in the presence or absence of the reagent were performed. The glutaminase activity of modified or unmodified wt AS-B, in the presence or absence of ATP, aspartate, and CSA, was determined. Although the glutaminase activity and the ATP-dependent stimulation of the glutaminase activity of the 5′-FSBA-modified enzyme was not effected by the presence of the reagent generated disulfide bridge (see Table 5), the CSA and aspartate stimulations of the glutaminase activity were abolished. This interesting observation indicated strongly that the 5′-FSBA generated intramolecular disulfide bond was not being formed in the ATP binding site, but rather in the aspartate binding site. Additional evidence for this hypothesis was gained from the kinetic analyses of the cysteine mutants, especially that of C523A (see Table 8). Since C523A has already been implicated as the primary residue responsible for the attack on 5′-FSBA, resulting in formation of the disulfide bond, it is noteworthy that mutation of this residue caused an 82-fold increase in the K_m for aspartate, with little change in the kinetic constants for ATP or glutamine. While we cannot disregard the possibility of a subtle conformational change in C523A, the striking consistency of the apparent kinetic constants for ATP and glutamine argue strongly for a specific role for Cys 523 in aspartate binding. The mutation of each of the other cysteine residues had very little effect on the kinetic constants. These results are consistent with the hypothesis that 5′-FSBA generated chemical modification occurs in the aspartate binding site.

Given the hypothesis that Cys 523 is located in the aspartate binding domain, this would suggest that the 5′-FSBA was initially bound to the enzyme so that the adenosine moiety was in a similar position to that of ATP, since ATP confers specific protection from the reagent. It would also suggest that 5′-FSBA would be in an elongated conformation such that the reactive sulfonyl fluoride extended into the aspartate binding site. Since a crystal structure is not available for AS-B, the proximity of these two sites is not certain. However, there are several crystal structures for an enzyme which catalyzes an extremely similar reaction, aspartyl tRNA synthase (Cavarelli et al., 1994). The electron density for ATP or AMPPcP, an ATP analog, in the active site of aspartyl tRNA synthase shows that ATP and AMPPcP adopt different conformations. ATP adopts a bent conformation which is stabilized by Mg^{2+} ions, whereas the AMPPcP adopts an elongated conformation which extends into the pocket which the amino acid substrate aspartate will occupy (Cavarelli et al., 1994). Since 5′-FSBA also does not bind Mg^{2+} ions, it is likely that it also adopts an elongated conformation, extending into the aspartate binding domain since the bent conformation, which is less favorable than the elongated conformation, requires Mg2+ for stabilization. In order to test this hypothesis, several experiments with ATP and ATP analogs in the presence and absence of $MgCl_2$ were performed. It was shown that AMPPcP does not stimulate the glutaminase activity in the presence or absence of $MgCl_2$ (see Table 6). It was also shown that ATP only in the presence of $MgCl_2$ stimulated activity. These data suggest that the addition of $MgCl_2$ is required for the binding of ATP in the bent conformation, and this bent confirmation is required for the stimulation of the glutaminase activity. This could then be the reason why AMPPcP does not stimulate the glutaminase activity. Further, bidentate chromium ATP, a substitution inert complex, was tested as a stimulator of the glutaminase activity. Surprisingly, Cr·ATP stimulated the glutaminase activity only in the presence of added $MgCl_2$. These results may suggest that two metal ions are required for the stimulation of the glutaminase activity.

It is of great interest that even though cysteine 523 appears to be involved in aspartate binding (the K_m for aspartate is raised greater that 80-fold in the C523A mutant), addition of 5 mM aspartate to wt AS-B offers no protection against inactivation by 5′-FSBA even though this concentration is almost 10-fold greater than the aspartate K_m. This data implies that AS-B requires a preferred order of binding, whereby aspartate cannot bind to the enzyme prior to the addition of ATP. Although the data in this case does not prove that the ATP, which protects against inactivation, is binding in a catalytically competent manner, it does provide some evidence for a preferred binding order. It is clear that ATP, at slightly more than stoichiometric amounts, in the presence of aspartate, provides exceptional protection from

5′-FSBA inactivation (see Table 2) and CSA stimulates the glutaminase reaction similarly to that of ATP and aspartate (see Table 5) but cannot protect the enzyme from 5′-FSBA inactivation at an equivalent amount of ATP. This may be an indication that upon addition of ATP and aspartate, a tightly bound β-aspartyl-AMP intermediate may be formed, which is not readily turned over. In contrast, CSA cannot form the activated intermediate with ATP, and although it can stimulate the glutaminase activity, it is not tightly bound, and thus 5′-FSBA inactivation readily occurs.

The overall goal of these experiments was to locate critical amino acid residues in the active site of AS-B, and Cys 523 has been shown to be involved in the aspartate binding domain. Structurally, this is interesting in light of the recent paper which suggests amino acid residues 317−322 of AS-B are part of both the aspartate and ATP binding domains (Boehlein et al., 1997). This would imply that the C-terminal amino acid residues must loop back and be in the proximity of amino acid residues 317−322. The presence of cysteine 523 in the aspartate binding domain may also be a reason why mutant enzymes, in which the C-terminal domain was truncated by site-directed mutagenesis, were both insoluble and inactive (data not shown). Another interesting finding in this study is that there appear to be no critical disulfide bonds in AS-B. Each mutant enzyme, with the exception of C1A, had a substantial glutamine-dependent asparagine synthetase activity, suggesting that none of the cysteine residues were critical for folding or overall structure of the enzyme. Further, although most chemical modification studies produce a covalently linked and modified peptide fragment that can be used to identify active site residues, these residues then need to be mutated to test the specificity of the modification. In an alternate approach, we were able to combine site-directed mutagenesis directly after chemical modification in order to identify a residue in the active site of AS-B.

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