Probing the Mechanism of Nitrogen Transfer in *Escherichia coli* Asparagine Synthetase by Using Heavy Atom Isotope Effects†

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ABSTRACT: In experiments aimed at determining the mechanism of nitrogen transfer in *purF* amidotransferase enzymes, ^{13}C and ^{15}N kinetic isotope effects have been measured for both of the glutamine-dependent activities of *Escherichia coli* asparagine synthetase B (AS-B). For the glutaminase reaction catalyzed by AS-B at pH 8.0, substitution of heavy atom labels in the side chain amide of the substrate yields observed values of 1.0245 and 1.0095 for the amide carbon and amide nitrogen isotope effects, respectively. In the glutamine-dependent synthesis of asparagine at pH 8.0, the amide carbon and amide nitrogen isotope effects have values of 1.0231 and 1.0222, respectively. We interpret these results to mean that nitrogen transfer does not proceed by the formation of free ammonia in the active site of the enzyme and probably involves a series of intermediates in which glutamine becomes covalently attached to aspartate. While a number of mechanisms are consistent with the observed isotope effects, a likely reaction pathway involves reaction of an oxyanion with β-aspartyl-AMP. This yields an intermediate in which C−N bond cleavage gives an acylthioenzyme and a second tetrahedral intermediate. Loss of AMP from the latter gives asparagine. An alternate reaction mechanism in which asparagine is generated from an imide intermediate also appears consistent with the observed kinetic isotope effects.

Asparagine synthetase (AS)[1] catalyzes the synthesis of asparagine from aspartic acid and ATP using either ammonia or glutamine as a nitrogen source. This enzyme also hydrolyzes glutamine to glutamate in the absence of aspartic acid (Figure 1). On the basis of its primary structure, AS-B is a member of the *purF* family of glutamine-dependent amidotransferases (Buchanan, 1973; Zalkin, 1993), which are characterized by an N-terminal cysteine that is essential for both glutamine-dependent enzyme activities (Badet-Denisot et al., 1993; Mei & Zalkin, 1989; Sheng et al., 1993; Van Heeke & Schuster, 1989). The *purF* family also includes glutamine phosphoribosylpyrophosphate amidotransferase (GPA) (Makaroff et al., 1983; Tso et al., 1982a,b; Zalkin & Truitt, 1977) and glutamine:fructose-6-phosphate amidotransferase (GFAT) (Badet et al., 1987, 1988; Golinelli-Pimpaneau et al., 1989; Winterburn & Phelps, 1971). Selective inhibitors of AS have potential clinical application since leukemia cells appear to be sensitive to chemotherapeutic agents which decrease levels of circulating asparagine (Andrulis & Barrett, 1989; Broome, 1963, 1968; Broome & Schwartz, 1967; Burchenal, 1970; Kiriyama et al., 1989). To date, however, extensive screening studies, employing hundreds of aspartate, glutamine, and ATP analogs, have failed to identify any high-affinity AS inhibitors (Cooney et al., 1976, 1980; Mokotoff et al., 1975a,b). We have therefore undertaken detailed mechanistic studies of asparagine synthetases in an effort to pursue rational approaches to discovering potent and selective AS inhibitors (Boehlein et al., 1994a,b; Sheng et al., 1993).

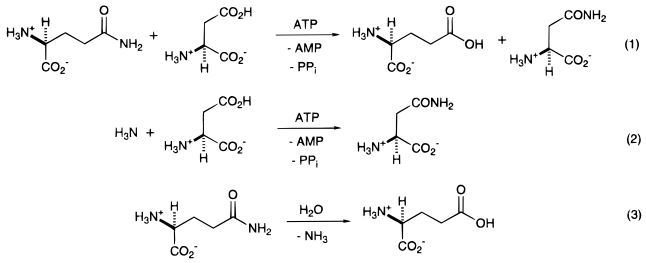

FIGURE 1: Reactions catalyzed by *Escherichia coli* asparagine synthetase B. (1) Glutamine-dependent asparagine synthesis in the presence of ATP and aspartic acid. (2) Asparagine synthesis using ammonia as a nitrogen source. (3) Hydrolysis of glutamine to yield glutamate and ammonia. This reaction proceeds in both the absence and presence of ATP.

The central mechanistic issue in all glutamine-dependent amidotransferase chemistry concerns the reaction by which nitrogen is transferred from the primary amide of glutamine

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[1] Abbreviations: AS-B, asparagine synthetase B; GAT, glutamine-dependent amidotransferase; GFAT, glutamine:fructose-6-phosphate amidotransferase; GPA, glutamine 5′-phosphoribosylpyrophosphate amidotransferase; HEA, Hereus elemental analyzer; IMP, inosine monophosphate; IRMS, isotope ratio mass spectroscopy; KIE, kinetic isotope effect; PEP, phosphoenolpyruvate.

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to a specific electrophilic acceptor. In the case of asparagine synthetase, the nitrogen acceptor is β-aspartyl-AMP, an intermediate that is formed by reaction of aspartate with ATP (Cedar & Schwartz, 1969; Leuhr & Schuster, 1985). On the other hand, despite the finding that Cys-1, present in all *purF* amidotransferases, is critical for glutamine-dependent activity (Badet et al., 1987; Mei & Zalkin, 1989; Sheng et al., 1993; Van Heeke & Schuster, 1989), the molecular mechanism of nitrogen transfer remains poorly defined (Boehlein et al., 1994a; Richards & Schuster, 1992). A highly plausible hypothesis is that the N-terminal, glutamine-utilizing (GAT) domain acts as thiol protease, liberating ammonia within the enzyme active site that mediates subsequent nitrogen transfer. This idea is also consistent with the observation that all *purF* amidotransferases catalyze glutamine hydrolysis to yield glutamate and ammonia in the absence of other substrates (Mei & Zalkin, 1989). Recent studies on GFAT employing substrate analogs as inhibitors have also suggested that nitrogen transfer may involve enzyme-bound ammonia (Badet-Denisot et al., 1995). On the other hand, there are no conserved histidine residues within the GAT domain of known *purF* amidotransferases, and no Cys-His catalytic dyad, analogous to that observed in papain (Arad et al., 1990; Drenth et al., 1968; Kamphuis et al., 1984), was evident in a recent crystal structure of *Bacillus subtilis* GPA complexed to AMP (Smith et al., 1994). Ammonia-mediated nitrogen transfer, for which little direct evidence has been obtained in studies on *purF* amidotransferases, has also been questioned for a number of chemical reasons (Richards & Schuster, 1992).

In addition to cloning the asparagine synthetase encoded by the *asnB* gene of *Escherichia coli* (AS-B) (Scofield et al., 1990), we have developed expression and purification protocols that allow the production of large quantities of AS-B (Boehlein et al., 1994a). In common with other asparagine synthetases (Ray et al., 1984; Sheng et al., 1993; Van Heeke & Schuster, 1989), AS-B can utilize either ammonia or glutamine as a nitrogen source (Boehlein et al., 1994a). As yet, recent site-directed mutagenesis experiments have failed to identify catalytically important histidines in the AS-B GAT domain (Boehlein et al., 1994a). Indeed, only Asn-74 in the AS-B GAT domain has been shown to play a catalytic role in both glutamine hydrolysis and nitrogen transfer (Boehlein et al., 1994b). This residue is completely conserved in all *purF* amidotransferases (Zalkin, 1993). In light of these observations, we have probed the nitrogen transfer mechanism using a series of isotopically labeled glutamines as AS-B substrates.

Heavy atom isotope effects have been widely used in studying enzyme reaction mechanisms as they provide information about the rate-determining step as well as prior moderately slow steps (O'Leary, 1980). Despite the small magnitude of such isotope effects, they can be measured sufficiently accurately using the remote label method and isotope ratio mass spectroscopy (IRMS) (O'Leary & Marlier, 1979). Using multiple isotope methods (O'Leary, 1989), we have determined, for both glutamine-dependent AS-B activities, the kinetic isotope effects (KIEs) associated with placing either ^{13}C or ^{15}N in the primary amide of glutamine. We now report that, while $^{13}(V/K)$ remains essentially unchanged, the amide ^{15}N KIEs are different for the AS-B-catalyzed glutamine-dependent synthetase and glutaminase reactions. These results may represent the first direct evidence against the mediation of nitrogen transfer by enzyme-bound ammonia in *purF* amidotransferases.

MATERIALS AND METHODS

Recombinant AS-B was obtained by overexpression in *E. coli* and purified as described previously (Boehlein et al., 1994a). Purified *E. coli* adenylosuccinate synthetase was generously provided by Dr. Herbert Fromm, Department of Biochemistry and Biophysics, Iowa State University (Bass et al., 1987). Phosphoenolpyruvate (PEP), ATP, GTP, IMP, NADH, α-ketoglutarate, L-aspartic acid, and L-glutamine were supplied by Sigma. Type VI pyruvate kinase (rabbit muscle), type II glutamate dehydrogenase (bovine liver), *E. coli* glutaminase, yeast adenylosuccinate lyase, and 5′-adenylic acid deaminase (rabbit muscle) were also purchased from Sigma. Prior to each experiment, all enzymes, with the exception of AS-B, were dialyzed exhaustively against 50 mM imidazole, (pH 7.4), 10 mM $MgCl_2$, and 50 mM KCl. For stability reasons, AS-B was dialyzed against 50 mM imidazole in 40% glycerol. Control experiments confirmed that these procedures eliminated any extraneous nitrogen sources. [2,5-$^{15}N_2$, 5-^{13}C]-L-Glutamine and [2-^{15}N, 5-^{13}C]-L-glutamine were synthesized using standard procedures.[2] All other chemicals were obtained commercially and were of the highest available purity. A Cary 2200 UV−visible spectrophotometer, equipped with a Brinkman MGW Lauda Model RM-20 temperature-controlled water bath, was used for all spectrophotometric enzyme assays. Solid samples (5 mg) were combusted in a Heraeus elemental analyzer (HEA) system, over CuO at 1000 °C. The resulting nitric oxides were then quantitatively reduced to N_2 over copper metal at 600 °C. The N_2 was adsorbed onto silica gel at −198 °C in a trapping box. The isotopic composition of the N_2, after desorption from the silica gel, was determined using a Finnegan Delta-S isotope ratio mass spectrometer.

Isotope Effect Nomenclature. The nomenclature used throughout this manuscript is modified from that of Northrop (1977). Here the leading superscript denotes the isotope responsible for the effect on the kinetic parameter. For example, a ^{15}N isotope effect on V/K is written as $^{15}(V/K)$ and would represent $[(V/K)^{14}N/(V/K)^{15}N]$, i.e. (V/K) for the ^{14}N-containing species relative to that of the ^{15}N-containing species. $^{13}(V/K)$ is defined in a similar manner except that ^{12}C is replaced by ^{13}C in the substrate of interest. To designate the position of the ^{15}N isotopic label for the multiply labeled compounds used in these experiments, we employ a subscript notation. Thus $^{15}(V/K)_5$ indicates the isotope effect arising from a ^{15}N attached to C-5 in the glutamine substrate. Multiple isotope effects are written as a combination of superscripts and subscripts. For example, $^{13,15,15}(V/K)_{2,5}$ is the isotope effect arising from having ^{13}C at C-5 and ^{15}N labels connected to carbons C-2 and C-5.

Determining the Extent of the AS-B-Catalyzed Glutaminase and Synthetase Reactions. The extent of AS-B-catalyzed glutamine hydrolysis was assayed, at 25 °C, by measuring liberated ammonia using glutamate dehydrogenase. Assay solutions were composed of 10 mM α-ketoglutarate, 10 mM NADPH, 10 mM $MgCl_2$, 50 mM imidazole buffer (pH 7.4),

[2] Stoker, P. W. (1994) Ph.D. Thesis, University of Nebraska−Lincoln, Lincoln, NE. Full details of this synthesis will be published elsewhere. All compounds exhibited satisfactory spectroscopic and microanalytical properties.

and glutamate dehydrogenase (1.4 u). A known volume of the reaction solution was added to the assay mixture, and the amount of NADPH was consumed by formation of glutamate determined by monitoring at 340 nm. In AS-B-catalyzed asparagine synthesis, the extent of reaction was assayed by measuring the amount of pyrophosphate produced. This assay employed a commercially available reagent kit purchased from Sigma (technical bulletin number B1-100).

Analytical Procedures for the Isolation and Isotopic Analysis of Glutamate. Amberlyst A-26 resin was treated with 100 mM HCl and then rinsed with distilled water until the washes were neutral. This resin (65 mL) was then packed into a column (o.d., 2.4 cm) and equilibrated with 10 mM imidazole buffer (pH 6.5) (300 mL). In the isotope effect measurements, solutions containing glutamate were adjusted to pH 6.5−7.0 before being passed through an Amberlyst A-26 column (flow rate, 2 mL/min). Upon application of the reaction solution, the column was initially rinsed with 10 mM imidazole buffer (pH 6.5), (100 mL) followed by distilled water (300 mL). Finally, 0.1 M AcOH (145 mL) was passed through the column. This fraction was collected and the solvent removed under reduced pressure until the total volume was less than 15 mL. Lyophilization then gave a solid residue. A portion of the solid (5 mg) was then combusted and the isotopic composition of the glutamate analyzed using IRMS/HEA.

AS-B-Catalyzed Hydrolysis of Glutamine. AS-B (4−6 u) was added to a reaction solution (100 mL total volume) containing 12 mM natural abundance glutamine, 3 mM ATP, 10 mM $MgCl_2$, and 50 mM imidazole buffer (pH 8), that had been equilibrated at 35 °C for 30 min. The concentration of ammonia in aliquots of the reaction solution was measured using the glutamate dehydrogenase assay. After approximately 20% of the glutamine substrate had been hydrolyzed in the enzymatic reaction (60−105 min), the reaction solution was filtered through an Amicon YM10 filter to remove AS-B. The filtrate was then diluted with an equal volume of distilled water and the solution pH adjusted to 6.5, before the addition of activated charcoal (1 g) for 5 min to remove nucleotides. After further filtration, separation and isotopic analysis of the glutamate product was carried out as described above. Identical conditions were employed for experiments employing both other isotopically labeled glutamine substrates.

AS-B-Catalyzed Hydrolysis of Glutamine in the Presence of ATP. AS-B (5−6 u) was added to a reaction solution (100 mL total volume) containing 12 mM natural abundance glutamine, 3 mM ATP, 10 mM $MgCl_2$, and 50 mM imidazole at pH 8. The hydrolysis reaction was allowed to proceed for 45 min before the mixture was filtered through an Amicon YM10 filter to remove AS-B. The concentration of ammonia in the reaction solution was assayed using glutamate dehydrogenase. The reaction solution was then treated with activated charcoal (1 g) for 5 min and filtered to remove nucleotides. After adjustment of the filtrate pH to 6.5−7.0, the separation and isotopic analysis of the glutamate product was carried out as described above. Identical conditions were employed for both other isotopically labeled glutamine substrates.

Glutamine-Dependent Synthesis of Asparagine. A reaction solution (150 mL total volume) containing 12 mM natural abundance glutamine, 3 mM aspartate, 2 mM ATP, 10 mM $MgCl_2$, and 50 mM imidazole at pH 8 was equilibrated for 30 min at 35 °C before the addition of AS-B (4−6 u). Enzyme-catalyzed asparagine formation was terminated by filtration through an Amicon YM10 filter to remove AS-B. An aliquot of the filtrate was assayed for pyrophosphate. The filtrate was then treated with activated charcoal (1 g) for 5 min and filtered to remove nucleotides. The pH was then adjusted to 7.4, and then nucleotides were added until the reaction solution contained 8 mM IMP, 2 mM GTP, and 35 mM PEP. An aliquot of the solution was then taken and kept to assay for possible noncatalyzed substrate hyrolysis. After the addition of adenylosuccinate synthetase (3 u), adenylosuccinate lyase (10 u), pyruvate kinase (100 u), and adenylic acid deaminase (10 u), the solution was incubated at 35 °C overnight (Figure 2). To ensure that no aspartate remained in solution, an aliquot was assayed for pyruvate. The aliquot taken from the reaction solution before addition of the coupled enzyme system was also assayed at this time. Pyruvate concentrations were measured using lactate dehydrogenase. Assay solutions were composed of 15 mM NADPH and 50 mM imidazole buffer (pH 7.4). Aliquots of reaction solutions containing pyruvate were added to the assay mixture so that the final concentration of pyruvate was between 0.1 and 0.2 mM. After mixing, lactate dehydrogenase (1−2 u) was added and the decrease in NADPH monitored at 340 nm. On determining that all aspartate had been consumed, filtration of the reaction mixture through an Amicon YM10 filter removed the four enzymes. As usual, nucleotides were removed from the filtrate by stirring with activated charcoal (1.25 g) for 5 min. After filtration, the solution was diluted with doubly distilled, deionized water (100 mL) and its pH adjusted to 6.5−7.0. Separation and determination of the isotopic composition of the glutamate product was carried out following the usual procedure. Identical conditions were employed for the isotopically labeled glutamine substrates.

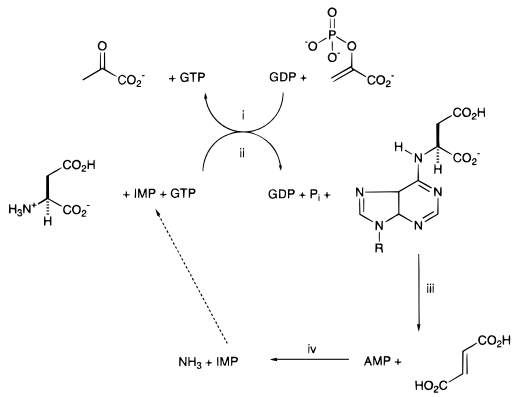

FIGURE 2: Four-enzyme-coupled system to remove aspartic acid in the presence of glutamic acid. The overall conversion is aspartate + PEP → fumarate + NH_3 + pyruvate. Enzymes: (i) pyruvate kinase, (ii) adenylosuccinate synthetase, (iii) adenylosuccinate lyase, and (iv) adenylic acid deaminase.

Data Analysis. The ^{13}C and ^{15}N isotope effects on the AS-B-catalyzed hydrolysis of glutamine were measured using the method of internal competition in which changes in the isotopic composition of the product are determined over the course of the reaction (O'Leary, 1980). The ^{15}N isotope effect for isotopic substitution in the α-amino group, $^{15}(V/K)_2$, was computed from the following equation:

$$^{15}(V/K)_2 = \log(1 - f)/\log[1 - (f\, R_s/R_o)]$$

Table 1: Heavy Atom Kinetic Isotope Effects in the Glutamine-Dependent Activities of AS-B[a]

	glutaminase (−ATP)	glutaminase (+ATP)	asparagine synthesis
$^{15}(V/K)_2$	1.0005 ± 0.0001	0.9984 ± 0.0005	1.0062 ± 0.0007
$^{13}(V/K)$	1.0245 ± 0.0038	1.0256 ± 0.0019	1.0231 ± 0.0020
$^{15}(V/K)_5$	1.0095 ± 0.0044	1.0065 ± 0.0032	1.0222 ± 0.0023

[a] $^{15}(V/K)_2$ is the KIE associated with placing ^{15}N in the α-amino group of glutamine; $^{13}(V/K)$ and $^{15}(V/K)_5$ are the KIEs associated with placing either ^{13}C or ^{15}N in the primary amide of glutamine, respectively.

where R_s is the ^{15}N/^{14}N ratio at this position of glutamate after the reaction has proceeded to a fractional extent expressed by f, and R_o is the initial ^{15}N/^{14}N ratio in the substrate. Complete hydrolysis of natural abundance glutamine, using glutaminase, yielded the ^{15}N content in the α-amino group. R_o was determined to be $-7.825 \pm 0.12\%$.[3]

When [2-^{15}N, 5-^{13}C]glutamine was used as the substrate, the ^{13}C isotope effect at C-5, $^{13}(V/K)$, could be calculated from the observed isotope effect $^{13,15}(V/K)$ using the expression

$$^{13}(V/K) = {}^{13,15}(V/K)/{}^{15}(V/K)_2$$

$^{13,15}(V/K)$ was again determined by observation of R_s as a function of the reaction extent f. For [2-^{15}N, 5-^{13}C]glutamine, complete hydrolysis using glutaminase gave $R_o = 205.1 \pm 6.9\%$. In a similar manner, the isotope effect when ^{15}N was present in the primary amide of glutamine was determined using the expression

$$^{15}(V/K)_5 = {}^{13,15,15}(V/K)_{2,5}/{}^{15}(V/K)_2\,{}^{13}(V/K)$$

The value of $^{13,15,15}(V/K)_{2,5}$ was measured using triply labeled [2,5-$^{15}N_2$, 5-^{13}C]glutamine. For this substrate, complete hydrolysis using glutaminase gave $R_o = 246.1 \pm 15.9\%$.

RESULTS

Isotope Effects Observed in AS-B-Catalyzed Glutamine Hydrolysis. $15(V/K)_2$ was determined for the AS-B-catalyzed hydrolysis of glutamine, in the absence of other AS-B substrates at pH 8.0 and 35 °C, by running the reaction until 26% of the substrate had reacted. The ^{15}N content of the α-amino group of the glutamate product was analyzed, giving an average value for $^{15}(V/K)_2$ of 1.0005 ± 0.0001 (three determinations) (Table 1). To measure $^{13}(V/K)$, an isotopically enriched substrate, [2-^{15}N, 5-^{13}C]glutamine, was subjected to AS-B-catalyzed hydrolysis. Two experiments were carried out, at pH 8.0 and 35 °C, in which reaction proceeded to an extent of either 12 or 27%. Once again, the ^{15}N content of the α-amino group of the glutamate product was analyzed so as to determine $^{13,15}(V/K)_2$. The use of this value and the observed $^{15}(V/K)_2$ then yielded an average value for $^{13}(V/K)$ of 1.0245 ± 0.0038 (three determinations) (Table 1). In a similar manner, the determination of $^{15}(V/K)_5$ was accomplished using the isotopically enriched substrate, [2,5-$^{15}N_2$, 5-^{13}C]glutamine. A solution of this substrate was hydrolyzed by AS-B to an extent of 20% at pH 8.0 and 35 °C, before the ^{15}N content of the α-amino group of the glutamate product was analyzed and $^{13,15,15}(V/K)_{2,5}$ measured. $^{13,15,15}(V/K)_{2,5}$ in combination with $^{15}(V/K)_2$ and $^{13}(V/K)$ then yielded an average value for $^{15}(V/K)_5$ of 1.0095 ± 0.0044 (three determinations) (Table 1). $^{15}(V/K)_2$, $^{13,15}(V/K)_2$, and $^{13,15,15}(V/K)_{2,5}$ were then determined by identical procedures for the AS-B-catalyzed glutamine hydrolysis in the presence of 5 mM ATP. Under these conditions, the rate of glutamine hydrolysis was enhanced 5-fold relative to that observed when ATP was absent. ATP did not appear to be consumed by the enzyme under the conditions for glutamine hydrolysis. While $^{15}(V/K)_2$ was determined to be 0.9984 ± 0.0005 for the ^{15}N label in the α-amino group of glutamine, both the $^{13}(V/K)$ and $^{15}(V/K)_5$ KIEs were of similar magnitude to those measured when ATP was not present in the reaction solution (Table 1).

Isotope Effects Observed in AS-B-Catalyzed Asparagine Synthesis. All studies were conducted such that the extent of reaction never exceeded 8% so as to avoid AS-B inhibition by asparagine formed by the synthetase. As in the hydrolysis studies, heavy atom KIEs were measured by analysis of the ^{15}N content of the α-amino group of glutamate produced in the reaction. However, as asparagine synthesis required the presence of 3 mM aspartic acid and 2 mM ATP, several experimental modifications were required for these determinations. First, the reaction volume was increased to 150 mL to offset problems arising from the limited extent of reaction due to asparagine inhibition of AS-B. Second, ATP was removed from the reaction mixture by adsorption on activated charcoal before analysis of the isotopic composition of glutamate. Given difficulties in the separation of aspartate from glutamate by ion-exchange chromatography, a coupled four-enzyme system was used to deaminate aspartate to fumarate (Figure 2). These enzymes were removed from the reaction mixture by filtration through an Amicon YM10 filter, and any nucleotides added as substrates for the four-enzyme system were adsorbed onto activated charcoal in a second step before isolation of glutamate. The $^{15}(V/K)_2$ value for asparagine synthesis, determined using natural abundance glutamine, was determined to be 1.0062 ± 0.0007 (Table 1). In similar experiments employing either [2-^{15}N, 5-^{13}C]-glutamine or [2,5-$^{15}N_2$, 5-^{13}C]glutamine, $^{13}(V/K)$ and $^{15}(V/K)_5$ were determined to be 1.0231 ± 0.0020 and 1.0222 ± 0.0023, respectively.

DISCUSSION

The chemical mechanism of amide bond hydrolysis by thiol proteases has been the subject of extensive investigation (Brocklehurst et al., 1987; Lowe & Yuthavong, 1971b; Ménard & Storer, 1992). It seems to be generally accepted that the thiolate anion of a cysteine residue attacks the amide to give a tetrahedral intermediate **1** (Figure 3A), although recent calculations have suggested that prior protonation of the carbonyl group may be required for sulfur attack (Arad et al., 1990). In the case of papain and other cysteine proteases, the first step is probably fast and reversible. Amide resonance is lost in tetrahedral intermediate **1**, increasing the basicity of the nitrogen atom. Subsequent proton transfer yields **2**, in which the leaving group ability of the nitrogen is enhanced, and loss of ammonia generates an acylthioenzyme **3** (Figure 3A). For papain-catalyzed hydrolysis of N-benzoylargininamide, $^{15}(V/K)$ was found to be 1.023 at pH 8.0 (O'Leary et al., 1974). In combination with other evidence (Lowe & Yuthavong, 1971a,b; Whitaker & Bender, 1965), this observation supports rate-limiting

[3] This value is determined relative to the isotopic composition of atmospheric nitrogen and is negative because the sample was depleted in ^{15}N (Kiick, 1991).

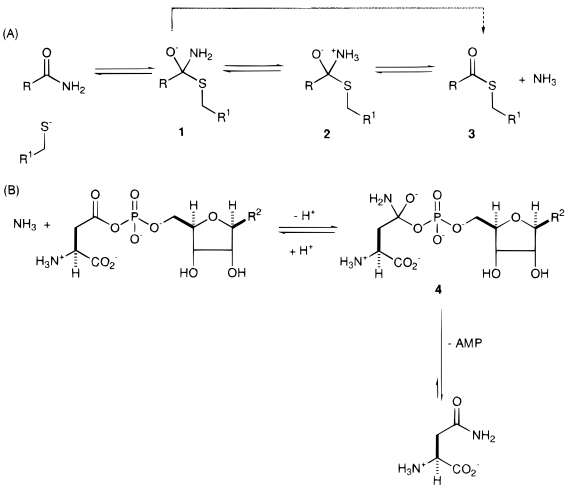

FIGURE 3: Mechanism of ammonia-mediated nitrogen transfer. (A) Initial attack of a thiolate anion upon the amide generates a tetrahedral intermediate **1** in which the basicity of the amide nitrogen is increased. Subsequent N-protonation then occurs to give **2**. In papain, His-159 is the source of this proton. However, experiments with site-directed AS-B mutants have failed, as yet, to identify a catalytically important histidine residue in the AS-B GAT domain. Cleavage of the C−N bond in **2** gives a thioacylenzyme **3** and ammonia. It is also possible that C−N bond breaking occurs simultaneously with N-protonation (dashed arrow). Intermediate **3** then undergoes hydrolysis to yield an acid, regenerating the catalytic thiolate. In papain, C−N cleavage is thought to represent the rate-limiting step. (B) In AS-B, the enzyme-bound ammonia would react with β-aspartyl-AMP to give **4**. Loss of AMP from **4** yields asparagine.

C−N bond cleavage, i.e. partitioning of the initial tetrahedral intermediate favors regeneration of the amide and the thiolate anion. While most mechanistic proposals assume that **2** is formed in the reaction, C−N bond cleavage may also occur simultaneously with N protonation.

As Cys-1 is essential for glutamine-dependent activity in AS-B (Boehlein et al., 1994a), it is likely that AS-B-catalyzed glutamine hydrolysis proceeds by a mechanism similar to that proposed for thiol proteases (Figure 3A). Further, the magnitude of $^{13}(V/K)$ measured for the glutaminase activity in our experiments is almost identical to $^{13}(V/K)$ reported for urease (Medina et al., 1981). In this enzyme, for which a crystal structure has recently been reported (Jabri et al., 1995), nickel atoms catalyze the attack of water on urea. The reported isotope effect probably reflects the rehybridization of the carbonyl carbon from a planar to a tetrahedral geometry intermediate, suggesting that chemistry is rate-limiting compared to conformational changes, substrate binding, or product release. Both formation of the tetrahedral intermediate **1** and subsequent C−N bond cleavage would, however, be expected to exhibit very similar KIEs given that the transition states for these two steps are almost identical in structure, only the roles of sulfur and nitrogen being reversed. The association of $^{13}(V/K)$ specifically with either of these steps is a general problem in studying amide hydrolysis using ^{13}C KIEs. Given that sulfides are usually better leaving groups than amines, C−N bond breaking is likely to be rate-determining in the AS-B glutaminase activity. That $^{15}(V/K)_5$ is only 1.0095 is therefore interesting since it is substantially smaller than $^{15}(V/K)$ for papain-catalyzed amide hydrolysis (O'Leary et al., 1974).

Two reasons may underly the observed $^{15}(V/K)_5$ value for the AS-B glutaminase activity. First, the assignment of the rate-determining step may be incorrect. Thus, formation of the tetrahedral intermediate **1** could be slow relative to C−N bond breaking. For example, it is possible that papain catalyzes formation of a tetrahedral adduct by distorting the amide bond of its secondary amide substrates away from planarity, in a manner similar to that of chymotrypsin (Hollands et al., 1969). This mechanism may not be available to AS-B, given that it must hydrolyze a primary amide, and the difference in $^{15}(V/K)_5$ values might therefore only reflect changes in commitment factors. Smaller $^{15}(V/K)_5$ values are therefore observed for serine proteases relative to thiol proteases as formation of the tetrahedal adduct is rate-limiting (O'Leary & Kluetz, 1970, 1972). On the other hand, questions regarding the similarity and relative importance of the oxyanion holes in serine and thiol proteases appear to argue against this explanation (Carter & Wells, 1990; Mackenzie et al., 1986; Ménard et al., 1991, 1995). A second plausible reason for the reduced magnitude of $^{15}(V/K)_5$ is that the nature of the N protonation step, required to transform nitrogen into a leaving group, differs in the papain- and AS-B-catalyzed hydrolysis reactions. In papain, all mechanistic studies support the idea that proton transfer proceeds in a concerted manner with C−N bond cleavage, i.e. that there is general acid catalysis of amine loss from the tetrahedral intermediate **1** (Figure 3A). Hence, His-159 of papain appears ideally located, relative to the active site thiol, to function as a general acid catalyst (Kamphuis et al., 1984). In contrast, there is presently no evidence either from site-directed mutagenesis experiments for a critical histidine residue in the AS-B GAT domain (Boehlein et al., 1994a) or in the crystal structure of *B.* subtilis GPA (Smith et al., 1994). N protonation might therefore proceed via specific acid catalysis, in which the proton is provided from the solvent, a proton transfer that might be facilitated by the α-amino group of Cys-1. The isotope effect associated with N protonation is likely to be large and inverse (Hermes et al., 1985), acting to reduce the magnitude of $^{15}(V/K)_5$ arising from C−N bond breaking. We presently favor this mechanistic proposal and therefore conclude that, during AS-B-catalyzed glutamine hydrolysis, N protonation precedes rate-limiting breakdown of tetrahedral intermediate **1** (Figure 3A).

In common with other amidotransferases (Buchanan, 1973; Zalkin, 1993), the glutaminase activity of AS-B is stimulated approximately 5-fold by the presence of ATP (Boehlein et al., 1994b). In the absence of aspartate, there is no detectable production of ADP, AMP, or pyrophosphate under these reaction conditions, indicating that ATP is not consumed.[4] Despite the increased rate of glutamine hydrolysis, $^{13}(V/K)$ and $^{15}(V/K)_5$ are almost unchanged relative to their values in the absence of ATP (Table 1). While $^{15}(V/K)_2$ is small and inverse, the magnitude of this effect appears to be negligible, and its interpretation in terms of enzyme/substrate interactions is presently unclear. For example, $^{15}(V/K)_2$ may represent a binding isotope effect or a perturbation of the pK_a of this amino group caused by the presence of ATP in the AS-B active site. Given the $^{13}(V/K)$ and $^{15}(V/K)_5$ values, it is clear that the hydrolysis mechanism is unchanged by the presence of ATP and that C−N bond breaking, combined with prior N protonation, remains rate-limiting.

In AS-B-catalyzed asparagine synthesis, the KIE associated with the remote label, $^{15}(V/K)_2$, was found to be higher than

[4] P. H.-Tari, unpublished results.

that seen in both studies of the glutaminase activity of the enzyme. This suggests that the α-amino group of the glutamine becomes tightly bound within the AS-B active site in the presence of the other two substrates, consistent with the view that AS-B undergoes conformational changes during asparagine synthesis. Ammonia-mediated nitrogen transfer has been widely assumed to occur in both *purF* and *trpG* amidotransferases (Chaparian & Evans, 1991; Mei & Zalkin, 1989) (Figure 3). If this assumption is correct, then similar $^{13}(V/K)$ and $^{15}(V/K)_5$ values would be expected for the glutamine-dependent synthetase activity of AS-B, unless attack of ammonia on β-aspartyl-AMP to give **4** were rate-limiting (Figure 3B). The latter possibility is ruled out, however, by $^{13}(V/K)$ observed for the synthetase reaction which is similar in magnitude to that seen in glutamine hydrolysis (Table 1). Once again, the magnitudes of $^{13}(V/K)$ and $^{15}(V/K)_5$ in AS-B-catalyzed asparagine synthesis indicate that substrate binding or product release is probably not rate-limiting for this amidotransferase. While $^{13}(V/K)$ is almost unchanged for both glutamine-dependent reactions catalyzed by AS-B, consistent with a common mechanism of glutamine breakdown, $^{15}(V/K)_5$ is markedly increased in the synthetase reaction. Given that $^{15}(V/K)_5$ represents changes in chemical bonding about the nitrogen atom as the reactant changes to the transition state, and therefore arises from localized structural effects, it is unlikely that the different $^{15}(V/K)_5$ values are associated with conformational dissimilarities in the AS-B active site during the two reactions. Moreover, if structural changes in the protein were significant, then it is likely that $^{13}(V/K)$ would also be different in the two AS-B-catalyzed reactions. Reconciling this difference in $^{15}(V/K)_5$ on the basis of a common mechanism therefore appears to demand that the first step in the hydrolysis reaction is rate-limiting, since $^{15}(V/K)_5$ for the synthetase reaction is only consistent with slow C−N bond breaking, as in papain (O'Leary et al., 1974).

On the basis of these data, we therefore conclude that the glutamine-dependent reactions catalyzed by AS-B are likely to proceed by different mechanisms. As the number of chemical mechanisms for amide hydrolysis appear limited, the simplest explanation of our observations is that nitrogen transfer from glutamine is *not* mediated by enzyme-bound ammonia, at least for the *purF* amidotransferase AS-B. There are several chemical rationales for using intermediates in which glutamine becomes covalently attached to aspartate with concomitant AMP release to mediate nitrogen transfer (Richards & Schuster, 1992). The formation of such intermediates, however, requires that the primary amide be activated so as to become nucleophilic. While several mechanisms of nitrogen transfer can be written that do not involve ammonia formation, it is clear from the observed $^{13}(V/K)$ and $^{15}(V/K)_5$ values that steps involving the carbonyl of the primary amide must still be rate-limiting given the magnitude of $^{13}(V/K)$. In addition, the transition state giving rise to $^{15}(V/K)_5$ must be different during nitrogen transfer and glutamine hydrolysis. One mechanistic possibility involves attack of a tetrahedral adduct on β-aspartyl-AMP (Figure 4). Thus, reaction of the thiolate anion derived from Cys-1 with glutamine would generate **5** in which the nitrogen reactivity would be enhanced. Although such an intermediate might be an oxyanion, recent calculations have suggested that protonation of the carbonyl oxygen might be essential

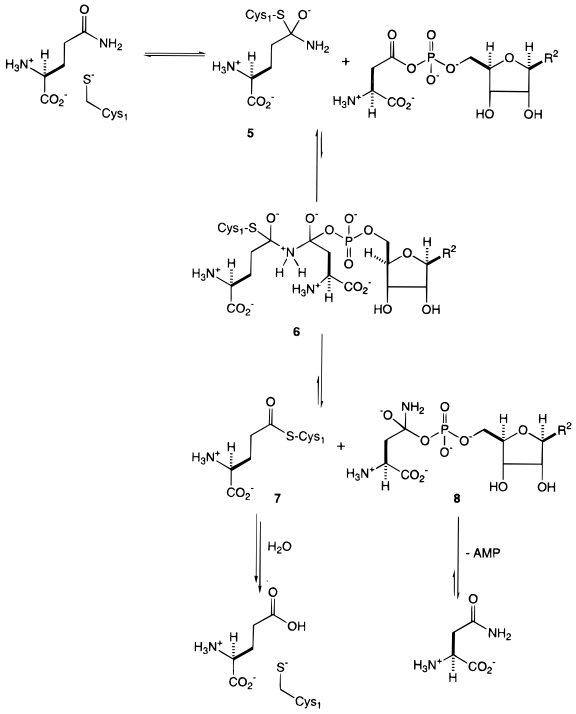

FIGURE 4: Alternative mechanism for glutamine-dependent nitrogen transfer in AS-B. Initial attack of the Cys-1 thiolate on glutamine generates tetrahedral intermediate **5**. Reaction of β-aspartyl-AMP with the nucleophilic nitrogen atom in **5** then yields **6**, in which glutamine is covalently linked to β-aspartyl-AMP. C−N bond cleavage then yields thioacylenzyme **7** and tetrahedral intermediate **8**. Loss of AMP from **8** gives asparagine without the need for general acid catalysis. As in papain-catalyzed amide hydrolysis, **7** reacts with water to give glutamate, regenerating the catalytic thiolate of Cys-1.

for promotion of nucleophilic attack of the sulfur atom (Arad et al., 1990). Attack of the nucleophilic nitrogen on β-aspartyl-AMP could then readily proceed to give the complex intermediate **6**. C−N bond cleavage would then yield the acylthio ester **7** and a new tetrahedral adduct **8** which would give asparagine by loss of AMP (Figure 4). In this mechanism, $^{15}(V/K)_5$ and $^{13}(V/K)$ would therefore arise from C−N bond breaking in **6**. We note, however, that the observed $^{15}(V/K)_5$ and $^{13}(V/K)$ values for glutamine-dependent asparagine synthesis do not definitively rule out a previous mechanistic proposal in which the primary amide of glutamine reacts directly with β-aspartyl-AMP to give an imide intermediate (Richards & Schuster, 1992; Sheng et al., 1993).

In summary, these experiments represent the first direct measurements of heavy atom kinetic isotope effects for any glutamine-dependent amidotransferase. Our data appear to raise further questions concerning the participation of enzyme-bound ammonia in the transfer of nitrogen from the primary amide of glutamine, at least for AS-B. The results of similar measurements on other *purF* and *trpG* amidotransferases are eagerly anticipated.

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