Glutamic Acid γ-Monohydroxamate and Hydroxylamine Are Alternate Substrates for *Escherichia coli* Asparagine Synthetase B†

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ABSTRACT: *Escherichia coli* asparagine synthetase B (AS-B) catalyzes the synthesis of asparagine from aspartic acid and glutamine in an ATP-dependent reaction. The ability of this enzyme to employ hydroxylamine and L-glutamic acid γ-monohydroxamate (LGH) as alternative substrates in place of ammonia and L-glutamine, respectively, has been investigated. The enzyme is able to function as an amidohydrolase, liberating hydroxylamine from LGH with high catalytic efficiency, as measured by k_{cat}/K_M. In addition, the kinetic parameters determined for hydroxylamine in AS-B synthetase activity are very similar to those of ammonia. Nitrogen transfer from LGH to yield aspartic acid β-monohydroxamate is also catalyzed by AS-B. While such an observation has been made for a few members of the *trpG* amidotransferase family, our results appear to be the first demonstration that nitrogen transfer can occur from glutamine analogs in a *purF* amidotransferase. However, k_{cat}/K_M for the ATP-dependent transfer of hydroxylamine from LGH to aspartic acid is reduced 3-fold relative to that for glutamine-dependent asparagine synthesis. Further, the AS-B mutant in which asparagine is replaced by alanine (N74A) can also use hydroxylamine as an alternate substrate to ammonia and catalyze the hydrolysis of LGH. The catalytic efficiencies (k_{cat}/K_M) of nitrogen transfer from LGH and L-glutamine to β-aspartyl-AMP are almost identical for the N74A AS-B mutant. These observations support the proposal that Asn-74 plays a role in catalyzing glutamine-dependent nitrogen transfer. We interpret our kinetic data as further evidence against ammonia-mediated nitrogen transfer from glutamine in the *purF* amidotransferase AS-B. These results are consistent with two alternate chemical mechanisms that have been proposed for this reaction [Boehlein, S. K., Richards, N. G. J., Walworth, E. S., & Schuster, S. M. (1994) *J. Biol. Chem. 269,* 26789−26795].

Despite recent success in obtaining structural information for *Bacillus subtilis* glutamine 5′-phosphoribosylpyrophosphate (PRPP)[1] amidotransferase (Smith et al., 1994) and *Escherichia coli* GMP synthetase (Tesmer et al., 1994), the molecular mechanism by which nitrogen is transferred in the glutamine-dependent reactions catalyzed by GAT enzymes remains to be defined unambiguously (Buchanan, 1973; Zalkin, 1993). As is the case for other members of the *purF* family (Badet-Denisot et al., 1993; Mei & Zalkin, 1989; Sheng et al., 1993; Van Heeke & Schuster, 1989; Zalkin, 1993), the N-terminal cysteine residue (Cys-1) is essential for both the amidohydrolase and glutamine-dependent synthetase activities of AS-B (Boehlein et al., 1994a). Using site-directed mutagenesis experiments, our group has recently established that Asn-74 (AS-B numbering) plays a functional role in both glutamine-dependent activities of *E. coli* asparagine synthetase B (AS-B) (reactions I and II) (Boehlein et al., 1994b). Both Cys-1 and Asn-74 are totally conserved in all members of the *purF* amidotransferase family (Zalkin, 1993). In a third activity, AS-B can utilize ammonia as a nitrogen source in asparagine synthesis (reaction III). While this is also the case for other asparagine synthetases and glutamine PRPP amidotransferases, we note that glucosamine-6-phosphate synthases do not seem to exhibit any ammonia-dependent activity (Badet et al., 1987; Winterburn & Phelps, 1971).

$$\text{L-Asp} + \text{L-Gln} + \text{ATP} \rightarrow \text{L-Asn} + \text{L-Glu} + \text{AMP} + \text{PP}_i \quad \text{(I)}$$

$$\text{L-Gln} + \text{H}_2\text{O} \rightarrow \text{L-Glu} + \text{NH}_3 \quad \text{(II)}$$

$$\text{L-Asp} + \text{NH}_3 + \text{ATP} \rightarrow \text{L-Asn} + \text{AMP} + \text{PP}_i \quad \text{(III)}$$

Given the general unreactivity of primary amides (Challis & Challis, 1970), activation of the amide nitrogen in glutamine has generally been assumed to involve "nascent" ammonia that is generated in a thiolate-catalyzed hydrolysis

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[1] Abbreviations: AS-B, *E. coli* asparagine synthetase B; AS, asparagine synthetase; ES, enzyme/substrate; GAT, glutamine-dependent amidotransferase; GMP, guanosine 5′-monophosphate; LAH, L-aspartic acid β-monohydroxamate; LGH, L-glutamic acid γ-monohydroxamate; PCR, polymerase chain reaction; PRPP, 5′-phosphoribosylpyrophosphate; TCA, trichloroacetic acid; TLC, thin-layer chromatography.

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reaction (Bearne & Wolfenden, 1995; Chaparian & Evans, 1991; Mei & Zalkin, 1989). While a Cys-His dyad has been observed in the crystal structure of the *trpG* amidotransferase, GMP synthetase,[2] direct evidence for ammonia-mediated nitrogen transfer remains elusive for *purF* enzymes (Badet-Denisot et al., 1995). Indeed, no similar catalytic dyad appears to be present in *B. subtilis* glutamine PRPP amidotransferase (Smith et al., 1994). Chemical arguments have also been presented which question the need for the intermediacy of ammonia in glutamine-dependent nitrogen transfer (Richards & Schuster, 1992). Kinetic evidence for the glutamine-dependent formation of an intermediate in asparagine synthesis has been obtained using site-specific mutants of human AS (Sheng et al., 1993), and recent kinetic isotope effect (KIE) measurements also appear to be inconsistent with ammonia-mediated nitrogen transfer (Stoker et al., 1996).

Experiments employing alternate substrates have been utilized previously to establish the mechanistic details of a variety of enzyme-catalyzed transformations (Huang, 1979). Given the chemical similarity between primary amides and acylhydroxamates, and ammonia and hydroxylamine, we have therefore probed the mechanism of glutamine-dependent nitrogen transfer using L-glutamic acid γ-hydroxamate (LGH) and hydroxylamine as alternate substrates for AS-B. We now report (1) that LGH is efficiently hydrolyzed by AS-B (reaction II) and (2) that hydroxylamine is an effective substitute for ammonia in the synthetase activity of AS-B (reaction III). However, although LGH can be employed as a nitrogen source by the enzyme in the synthetase reaction (reaction I), forming L-aspartic acid β-monohydroxamate, the rate of this nitrogen transfer reaction is reduced 10-fold relative to the case when glutamine is used as the nitrogen source. These results suggest that nitrogen transfer proceeds via direct attack of the acylhydroxamate on β-aspartyl-AMP.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and construction of the AS-B N74A Mutant. All strains were derivatives of *E. coli* K-12; BL21DE3pLys S (F^-, ompT, rb^-, mb^-) was obtained from Studier (Studier & Moffatt, 1986), while NM522 [sup E, thi, (lac-proAB), hsd5, (r^- m^-)/F′ pro AB, lac I^q Z M15] was supplied by Stratagene, as was plasmid pBluescript. The plasmid pETB, prepared in our laboratory, has been described elsewhere (Boehlein et al., 1994a). An AS-B mutant in which Cys-1 is replaced by alanine (C1A) was constructed by PCR cassette mutagenesis, and subcloned into pETB, as previously described (Boehlein et al., 1994a). The AS-B mutant in which Asn-74 is replaced by alanine (N74A) was constructed by PCR cassette mutagenesis, and subcloned into pETB, as previously outlined (Boehlein et al., 1994b). Wild-type AS-B and N74A were expressed, harvested, and purified using standard procedures (Boehlein et al., 1994a,b).

Determination of Kinetic Constants for the AS-B-Catalyzed Hydrolysis of LGH. The assay used to obtain the kinetic constants for the AS-B-catalyzed hydrolysis of LGH employed a modified procedure for obtaining the glutamate concentration using glutamate dehydrogenase in the presence of NAD^+ (Brent & Bergmeyer, 1974). Reaction mixtures with total volume of 100 μL contained 100 mM Tris-HCl (pH 8) and 8 mM $MgCl_2$. The concentration of LGH was varied between 0.1 and 50 mM. Reactions were initiated by the addition of wild-type AS-B or the N74A mutant (1−7 μg) and the mixtures incubated at 37 °C for 10 min. Addition of 20% trichloroacetic acid (TCA) (15 μL) was used to terminate the reaction. The resulting solution was added to 385 μL of a coupling reagent [300 mM glycine, 250 mM hydrazine (pH 9.1), 1 mM ADP, 1.6 mM NAD^+, and 2.2 units of glutamate dehydrogenase] and incubated for 10 min. The solution absorbance was then measured at 340 nm and the amount of glutamate produced in the reaction determined by comparison to a standard curve. The kinetic parameters for the glutaminase activity of AS-B were obtained using a similar procedure (Boehlein et al., 1994a,b) and are included in the tables herein for comparison. All values are averages derived from twot of four separate assays. Kinetic constants [K_M(app) and k_{cat}] were obtained by nonlinear regression analysis of Michaelis−Menten plots using the Prism software package, supplied by Graphpad, Inc. (San Diego, CA).

Assay of the Hydroxylamine-Dependent Synthetase Activity of AS-B and the AS-B N74A Mutant. The ability of hydroxylamine to act as an alternative nitrogen source to ammonia or L-glutamine was assayed by monitoring the amount of L-aspartic acid β-monohydroxamate (LAH) synthesized using spectrophotometric measurement of the colored complex formed by its reaction with ferric chloride (Anderson & Meister, 1966). Reaction mixtures were comprised of 10 mM aspartic acid, 5 mM ATP, 100 mM Tris-HCl (pH 8), and 8 mM $MgCl_2$ in a total volume of 100 μL. The hydroxylamine concentration was varied over a 5−50 mM range, and synthetase reactions were initiated by the addition of 6−10 μg of either wild-type AS-B or the AS-B N74A mutant. After incubation at 37 °C for 20 min, reactions were terminated by addition of 400 μL of a solution of 10% $FeCl_3 \cdot 6H_2O$ in 0.7 M HCl containing 3% TCA. The absorbance of the final mixture was then determined at 535 nm to yield the total amount of LAH after reference to a standard curve.

Chromatographic Separation of LAH from L-Aspartate. Two separate chromatography systems were used to resolve LAH from L-aspartic acid. First, the mixture of the two amino acids was applied to Whatman DE81 ion-exchange chromatography paper (3 cm × 21 cm) after mixing with $FeCl_3$ reagent. In this procedure, the acylhydroxamate reacted with ferric ions to form a colored complex. The chromatogram was then developed using 0.1 M NaCl as the mobile phase for approximately 3 h. The amino acids were visualized by spraying the paper with 0.1% ninhydrin in 95% ethanol followed by heating. Aspartate was found to migrate with the solvent front while the aspartic acid β-monohydroxamate, as its iron complex, remained at the elution origin. In the second system, PEI cellulose TLC plates (1.5 cm × 7 cm) were predeveloped in water and then allowed to dry at room temperature. After application of the sample, the plates were developed in water for approximately 45 min and dried. The amino acids were visualized by spraying with 0.1% ninhydrin in 95% ethanol followed by heating. In this system, aspartic acid β-monohydroxamate migrated with the solvent front whereas aspartate remained at the elution origin.

Chromatographic Assays for LAH Production by Wild-Type AS-B and the AS-B N74A Mutant. Either wild-type AS-B or the N74A AS-B mutant was incubated with a

[2] V. J. Davisson, personal communication.

Table 1: Kinetic Constants for the Amidohydrolase Activity of Wild-Type AS-B and N74A for L-Glutamine and LGH with or without ATP[a]

	Glutamine (ATP Absent)			Glutamine (5.0 mM ATP Present)		
	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})
wt AS-B	1.67 ± 0.01	1.25 ± 0.03	750 ± 13	1.30 ± 0.06	2.19 ± 0.03	1680 ± 55
N74A	0.36 ± 0.05	0.14 ± 0.01	390 ± 26	0.20 ± 0.03	0.16 ± 0.01	800 ± 70
	LGH (ATP Absent)			LGH (5.0 mM ATP Present)		
	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})
wt AS-B	0.80 ± 0.10	1.90 ± 0.10	2400 ± 172	0.74 ± 0.09	2.60 ± 0.09	3500 ± 306
N74A	0.43 ± 0.07	0.15 ± 0.01	350 ± 33	0.14 ± 0.04	0.12 ± 0.01	900 ± 173

[a] Data for the reactions involving wild-type (wt) AS-B or the N74A AS-B mutant and glutamine as substrate have been published previously (Boehlein et al., 1994b) and are included here for comparison.

mixture of 0.5 μCi ^{14}C uniformly labeled aspartic acid, $MgCl_2$, LGH, and ATP in a reaction volume such that the final concentrations of substrates were 2.44 mM aspartic acid, 5 mM LGH, 10 mM ATP, 100 mM Tris-HCl (pH 8), and 15 mM $MgCl_2$. Control experiments employed identical amounts and concentrations of all reagents except that ATP was omitted from the reaction mixture. Reactions were initiated by addition of the enzyme and terminated after 0, 15, and 30 min by boiling for 3 min. After cooling, each sample was centrifuged for 5 min to remove protein, and two 5 μL samples of the supernatant were spotted onto a strip of Whatman DE81 ion-exchange chromatography paper. One of these samples was applied directly to the paper, while the other was mixed with $FeCl_3$ reagent before its application to the paper. The paper was developed using water as eluant over a period of 3 h. After drying, each chromatogram was cut into 2 cm strips, which were placed into vials with ScintiVers II* fluid and analyzed directly in a Beckman LS 60001C scintillation counter. Alternatively, a 1 μl sample of the reaction mixture was applied to a PEI cellulose TLC plate, developed in water for 45 min, and then counted. The number of nanomoles of ^{14}C-labeled aspartic acid β-monohydroxamate in each sample was determined by subtraction of the counts in each strip obtained from reaction mixtures without ATP from those containing ATP. All determinations were carried out in duplicate.

Determination of Steady State Kinetic Constants for LGH and Glutamine for the Synthesis of LAH by Wild-Type AS-B and the AS-B N74A Mutant. Given that both chromatographic methods gave similar results, we determined the steady state kinetic constants, K_M(app) and k_{cat}, for the LGH-dependent synthesis of aspartic acid β-monohydroxamate for both wild-type AS-B and the N74A AS-B mutant using TLC on PEI cellulose plates. In these experiments, all reactions employed saturating amounts of ATP and aspartate containing 1 μCi ^{14}C uniformly labeled aspartic acid. The concentrations of substrates when saturating were 5 mM ATP, 5 mM aspartic acid, and 8 mM $MgCl_2$ in 100 mM Tris-HCl (pH 8). The variable concentrations of LGH in experiments using wild-type AS-B and the AS-B N74A mutant were 0.05−0.9 mM. Reactions were performed at several time points and were terminated by boiling for 3 min. Two 1 μL aliquots of each reaction mixture were applied to the PEI plate which was then developed for 45 min in water and allowed to dry before the amount of aspartic β-monohydroxamate was determined, as outlined above. All measurements were carried out in duplicate at each time point.

RESULTS

Our initial experiments determined the ability of LGH to act as a substrate in the amidohydrolase reaction catalyzed by AS-B (reaction II). The formation of L-glutamate from LGH was measured by following NADH production in a coupled assay system (Brent & Bergmeyer, 1974). In the absence of both aspartate and ATP, AS-B catalyzed the hydrolysis of LGH with a k_{cat}/K_M of 2400 ± 422 M^{-1} s^{-1} (Table 1). For comparison, k_{cat}/K_M for the hydrolysis of glutamine by AS-B was 3-fold lower [Table 1 and Boehlein et al. (1994b)]. The addition of ATP to the reaction mixture stimulated the hydrolysis of LGH by AS-B, albeit to a smaller extent than that observed for the glutaminase activity (Boehlein et al., 1994b). However, differences between the K_M and k_{cat} values for LGH and glutamine were significantly diminished in the amidohydrolase activity of N74A (Table 1), an AS-B mutant in which Asn-74 is replaced by alanine (Boehlein et al., 1994b).

The ability of hydroxylamine to replace ammonia as a nitrogen source in the synthetase activity of AS-B was also studied. In these experiments, the production of L-aspartic acid β-monohydroxamate was determined directly by observation of the formation of the colored compound produced by complexation of the acylhydroxamate product with ferric ions (Anderson & Meister, 1966). Ammonia could be used by AS-B in the synthesis of asparagine with a catalytic efficiency (k_{cat}/K_M) of 37 ± 0.3 M^{-1} s^{-1} [Table 2 and Boehlein et al. (1994b)]. The kinetic behavior of hydroxylamine with AS-B in the presence of ATP and aspartate was similar to that of ammonia, the 2-fold difference in k_{cat}/K_M arising from a 2-fold increase in the turnover number (k_{cat}) associated with hydroxylamine (Table 2). The increased turnover number for hydroxylamine-dependent synthetase activity might reflect the higher nucleophilicity of hydroxylamine relative to that of ammonia. Similar experiments with ammonia and hydroxylamine using N74A indicated that Asn-74 in the wild-type enzyme did not have a significant function in the ammonia-dependent synthetase activity of AS-B. Hence, only minor perturbations to either the apparent K_M or k_{cat} values for ammonia and hydroxylamine in the N74A-catalyzed synthetase reaction were observed relative to those determined for wild-type AS-B (Table 2).

Thus, AS-B not only catalyzed the hydrolysis of LGH but also utilized hydroxylamine as an alternate substrate to ammonia (reaction III). LGH could replace glutamine in the synthetase reaction (reaction I). Given that it is possible for the oxygen atom of the hydroxylamine moiety of LGH to attack β-aspartyl-AMP (Robinson & Jencks, 1967), we

Table 2: Kinetic Constants for the AS-B Synthetase Activity for L-Glutamine and LGH of Wild-Type AS-B and N74A[a]

		kinetic parameters		
enzyme	substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)
wt AS-B	ammonia	15.7 ± 0.4	0.59 ± 0.01	37 ± 0.3
N74A	ammonia	13.0 ± 0.4	0.65 ± 0.01	50 ± 0.8
wt AS-B	hydroxylamine	11.5 ± 2.0	1.03 ± 0.08	90 ± 8.6
N74A	hydroxylamine	17.1 ± 1.9	1.17 ± 0.07	68 ± 3.5
wt AS-B	L-glutamine	0.69 ± 0.07	1.01 ± 0.05	1460 ± 76
N74A	L-glutamine	0.040 ± 0.004	0.050 ± 0.002	1250 ± 75
wt AS-B	LGH	0.26 ± 0.02	0.150 ± 0.004	577 ± 29
N74A	LGH	0.09 ± 0.01	0.100 ± 0.004	1100 ± 321

[a] Data for the reactions involving wild-type (wt) AS-B or the N74A AS-B mutant and glutamine or ammonia as substrates have been published previously (Boehlein et al., 1994b) and are included here for comparison.

employed a direct chromatographic assay to measure the production of aspartic acid β-monohydroxamate. Incubation of ^{14}C-radiolabeled aspartic acid with ATP and LGH in the presence of AS-B gave a radioactive product which was separable from labeled aspartic acid in the reaction mixture by TLC on PEI cellulose. This material exhibited chromatographic behavior identical to that of a reference sample of authentic L-aspartic acid β-monohydroxamate in two separation systems. The high amidohydrolase activity of AS-B, however, precluded the observation of a 1:1 stoichiometry between glutamate and L-aspartic acid β-monohydroxamate when LGH was the nitrogen source. The observed K_M(app) for hydroxylamine of 11.5 mM (Table 2) in the synthetase reaction (reaction III) was also inconsistent with formation of aspartic acid β-monohydroxamate being due to the presence of free hydroxylamine produced by AS-B-catalyzed LGH hydrolysis. Further, the rate of β-monohydroxamate production did not increase as a function of time due to the presence of increasing levels of hydroxylamine released from the LGH substrate (data not shown). By monitoring the formation of L-aspartic acid β-monohydroxamate in the AS-B synthetase reaction, K_M(app) and K_{cat} for LGH were found to be decreased 3- and 10-fold, respectively, relative to the kinetic parameters for glutamine in glutamine-dependent asparagine synthesis (Table 2). As previous mutagenesis experiments had established that Asn-74 plays a functional role in nitrogen transfer (Boehlein et al., 1994b), the kinetic parameters associated with LGH in the synthesis of L-aspartic acid β-monohydroxamate by the N74A AS-B mutant were determined and compared to those of glutamine in N74A-catalyzed asparagine synthesis (reaction I) [Table 2 and Boehlein et al. (1994b)]. In contrast to wild-type AS-B, only minor differences in the turnover numbers for LGH and glutamine were observed in the synthetase activity of N74A.

The ability of LGH and L-glutamine to inhibit the ammonia-dependent activity of an AS-B mutant in which Cys-1 had been substituted by alanine (C1A) was also determined (Boehlein et al., 1994a). Although kinetic characterization of the ammonia-dependent synthetase activity of C1A suggests that this AS-B mutant is correctly folded, C1A possesses no glutamine- or LGH-dependent amidohydrolase or synthetase activities. Both LGH and glutamine inhibit the ammonia-dependent C1A synthetase activity, presumably by interfering with the positioning of ammonia within the active site. A full inhibition study was carried out for C1A in which, at various fixed concentrations of LGH, the amount of ammonia was varied while the levels of aspartic acid and ATP were kept constant. While the double reciprocal plot showed a complicated pattern, the simplest interpretation is that LGH is a partial competitive inhibitor with respect to ammonia (Figure 1). Similar kinetic behavior was observed in identical experiments employing L-glutamine as an inhibitor of C1A-catalyzed ammonia-dependent asparagine synthesis (Boehlein et al., 1994a). Using the linear portion of the slope replot, standard extrapolation methods gave a K_I value for LGH of approximately 1.9 μM, similar to the value of 4 μM previously determined for glutamine (Boehlein et al., 1994a). These

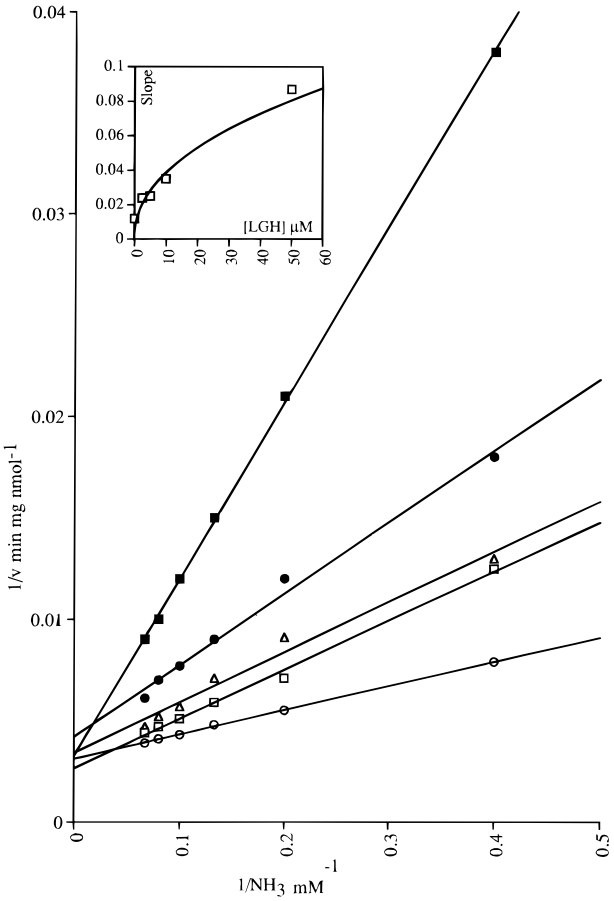

FIGURE 1: Inhibition of the ammonia-dependent synthetase activity of the C1A AS-B mutant by LGH. Each initial velocity is the average result of two parallel experiments. Double reciprocal plot of initial velocities of C1A with NH_4Cl (2.5, 5, 7.5, 10, 12.5, and 15 mM) at various fixed concentrations of LGH: (○) 0 mM, (□) 2.5 μM, (△) 5 μM, (●) 10 μM, and (■) 50 μM. The concentrations of aspartate, ATP, and $MgCl_2$ were maintained at 10, 5, and 8 mM, respectively. Reaction velocities were determined by monitoring pyrophosphate release, as described in Materials and Methods. The inset shows the replot of the slopes versus LGH concentration (micromolar).

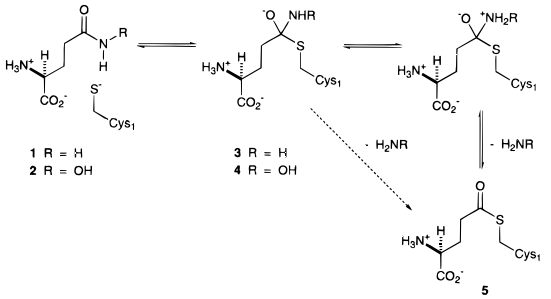

FIGURE 2: Mechanism of hydrolysis of LGH via an oxyanion intermediate. Attack of the thiolate of Cys-1 upon bound glutamine **1** (R = H) or LGH **2** (R = OH) occurs to yield an oxyanion **3** (R = H) or **4** (R = OH) which must then be protonated before loss of the leaving group to yield the acylenzyme **5**. The dotted arrow indicates that protonation may occur at the same time as C−N bond cleavage.

values support the idea that glutamine and LGH bind to AS-B in a similar manner under these reaction conditions.

DISCUSSION

Amidohydrolase Activity of Wild-Type AS-B and the N74A AS-B Mutant. LGH is an excellent substrate in AS-B-catalyzed hydrolysis, giving glutamate and hydroxylamine as the products. Furthermore, in the amidohydrolase reaction, breakdown of LGH by AS-B is stimulated by ATP to a similar extent as observed when glutamine is the substrate. If amide and acylhydroxamate hydrolysis is catalyzed by the GAT domain of *purF* amidotransferases using a mechanism analogous to that of thiol proteases (Brocklehurst et al., 1987), the tetrahedral intermediate **4** must be formed by reaction of the Cys-1 thiolate with the substrate (Figure 2). Subsequent C−N bond cleavage then yields the acylenzyme **5**, a step that is rate-limiting in the hydrolysis of amides by papain and other thiol proteases (O'Leary et al., 1974; Whitaker & Bender, 1965). Although C−N cleavage requires N protonation of the leaving group, acylhydroxamates are usually hydrolyzed faster than amides given that hydroxylamine ($pK_a \approx 8.2$) is a better leaving group than ammonia ($pK_a \approx 9.2$). A similar difference in the rates of hydrolysis of glutamine and LGH by AS-B has been reported for *E. coli* carbamoyl-phosphate synthetase (Lusty & Liao, 1993). This suggests that the side chain functional groups of LGH and glutamine are located in the same binding pocket of the AS-B GAT domain relative to the thiolate of Cys-1. Given our previous observations that Asn-74 plays a functional role in the catalysis of glutamine-dependent nitrogen transfer (Boehlein et al., 1994b), it is significant that the kinetic parameters associated with N74A-catalyzed LGH hydrolysis resulted in a reduced k_{cat}/K_M compared to the same reaction catalyzed by wild-type AS-B. In addition, k_{cat}/K_M values for LGH and glutamine were almost identical in the respective N74A-catalyzed hydrolysis reactions. ATP-dependent stimulation of amidohydrolase activity, however, was retained in the N74A AS-B mutant. These results support the participation of Asn-74 in the catalysis of LGH breakdown. Hence, noncovalent interactions between the Asn-74 side chain and the amide, or hydroxamate, moieties in glutamine LGH, respectively, give rise to the differences in kinetic behavior between glutamine and LGH observed in wild-type AS-B-catalyzed hydrolysis.

Probing the Interaction of Asn-74 with Substrate in AS-B Amidohydrolase Activity. Assuming that the mechanism of

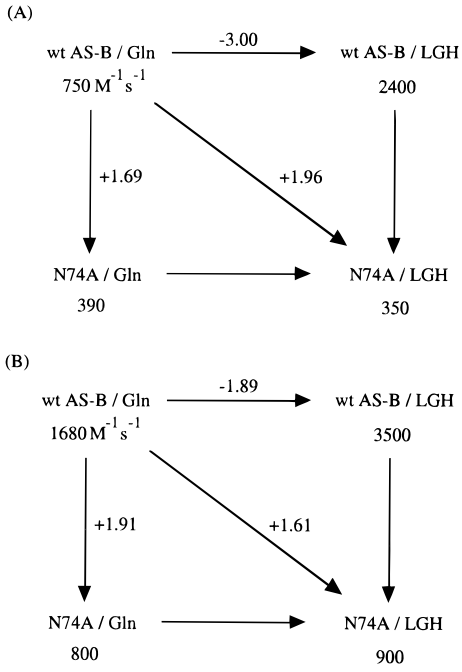

FIGURE 3: Thermodynamic cycles for the amidohydrolase activity of wild-type AS-B and the N74A AS-B mutant with glutamine and LGH. (A) Comparisons of k_{cat}/K_M for the amidohydrolase activity of wild-type AS-B and the variant N74A obtained by site-directed mutagenesis with glutamine and LGH, in the absence of ATP. Values of k_{cat}/K_M in $M^{-1}\ s^{-1}$ appear beneath each enzyme/substrate system. (B) Comparisons of k_{cat}/K_M for the amidohydrolase activity of wild-type AS-B and the variant N74A obtained by site-directed mutagenesis with glutamine and LGH, in the presence of ATP. Values of k_{cat}/K_M in $M^{-1}\ s^{-1}$ appear beneath each enzyme/substrate system. The energy values adjacent to the arrows are in kilooules per mole and were calculated using $\Delta G = -RT \ln[(k_{cat}/K_M)_B/(k_{cat}/K_M)_A]$, where A and B are the appropriate enzyme/substrate combinations. In all cases, $T = 310.15$ K.

LGH and glutamine hydrolysis was identical in both wild-type AS-B and the N74A AS-B mutant, we constructed thermodynamic cycles for the reaction in the absence and presence of ATP (Figure 3A,B). This approach has been widely used in probing side chain−side chain interactions upon binding and catalysis (Mildvan et al., 1992; Shortle, 1992; Wells, 1990), although site-specific mutations in previous studies have generally involved only amino acids within the protein. The evaluation of specific enzyme−substrate interactions using substrate modification combined with site-specific mutation of protein residues appears to be less well-known. Both free energy cycles that computed k_{cat}/K_M indicated that the effects of modifying glutamine to LGH and asparagine to alanine in the protein were synergistic (Mildvan et al., 1992). Although synergy may be observed due to extensive protein unfolding, the unchanged ability of the N74A AS-B mutant to catalyze ammonia-dependent asparagine synthesis relative to the wild-type enzyme argues against this simple interpretation. In addition, LGH does not adversely affect the structure of the GAT domain, being hydrolyzed more effectively than glutamine by wild-type AS-B, as measured by k_{cat}/K_M. A more likely explanation of our results is that the interaction of the Asn-74 side chain with the substrate destabilizes the ES complex. Hence, individual modifications (Asn-74 → Ala or Gln → LGH) are less damaging to binding, since strain arising from enzyme−substrate interactions is lost as well as specific protein−substrate interactions (Mildvan et al., 1992). How-

ever, when both groups are altered simultaneously, the effects of strain can only be lost from one modification. The interaction of Asn-74 with the glutamine substrate has the effect of elevating the energy of the ES complex above that of the free enzyme, lowering the barrier to reaction (Weber et al., 1991). The synergistic effects present in these thermodynamic cycles therefore support our original proposal that Asn-74 is located in the AS-B GAT domain, an idea that is also consistent with the location of the homologous asparagine residue (Asn-102) in the structure of *B. subtilis* glutamine PRPP amidotransferase (Smith et al., 1994).

Implications for the Mechanism of Nitrogen Transfer in AS-B. The ability of AS-B to function as a glutaminase coupled with the observation that ammonia can function as an alternate nitrogen source to L-glutamine in AS-B synthetase activity is consistent with the hypothesis that glutamine-dependent nitrogen transfer is mediated by a molecule of enzyme-bound ammonia (Chaparian & Evans, 1991; Mei & Zalkin, 1989). On this basis, the observations that AS-B catalyzes LGH hydrolysis and that hydroxylamine is an excellent substitute for ammonia in the synthetase activity of the enzyme imply that nitrogen transfer should be mediated by hydroxylamine when LGH is used as the nitrogen source. However, while LGH can be utilized by AS-B to synthesize aspartic acid β-monohydroxamate, a property that currently appears to be unique among *purF* amidotransferases, the turnover number for this synthetase reaction is reduced 10-fold relative to that for glutamine-dependent asparagine synthesis (reaction I). Both LGH and glutamine appear to inhibit the ammonia-dependent synthetase activity of the C1A AS-B mutant, binding to C1A with similar affinities, as measured by their associated K_I values. It is therefore likely that both of these substrates bind at the same site within the AS-B GAT domain. As LGH is more easily hydrolyzed by AS-B than glutamine, the steric bulk of the additional hydroxyl group is tolerated at the glutamine-binding site. One explanation for the reduced turnover number in LGH-dependent synthetase activity might be that LGH is hydrolyzed to yield enzyme-bound hydroxylamine which is released to the solvent due to its inability to bind within an ammonia-binding pocket in the presence of β-aspartyl-AMP. However, this is not consistent with the kinetic similarities of hydroxylamine and ammonia in AS-B synthetase activity. The experimental investigation of this hypothesis is complicated by the high amidohydrolase activity of AS-B which prevents the formation of glutamate and aspartic acid β-monohydroxamate in a 1:1 stoichiometry. Alternatively, hydroxylamine may bind very tightly to the enzyme, curtailing its ability to attack β-aspartyl-AMP. This is again contrary to our observations when free hydroxylamine is used as a substrate.

Given these difficulties in the explanation of the reduced turnover number for LGH-dependent nitrogen transfer, and the chemical issues raised by an ammonia-mediated mechanism (Richards & Schuster, 1992), enzyme-bound hydroxylamine is probably not formed during the synthesis of aspartic acid β-monohydroxamate. This idea is also supported by the heavy atom isotope effects observed for both glutamine-dependent AS-B activities (Stoker et al., 1996). The side chain amide of Asn-74 cannot form identical hydrogen bonds with the primary amide of glutamine and the acylhydroxamate of LGH (Figure 4A,B) unless the latter substrate binds as the less energetically favorable (*E*)-configurational isomer

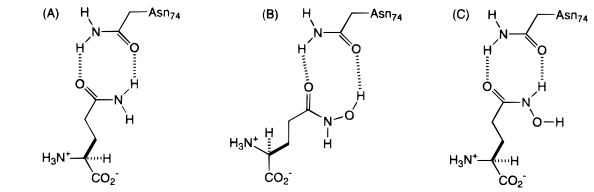

FIGURE 4: Structures of putative hydrogen-bonded complexes involving the Asn-74 side chain and amino acid substrates. Putative hydrogen-bonding interactions between the side chain of Asn-74 and the side chain of (A) substrate glutamine, (B) the (*Z*)-isomer of LGH, and (C) the (*E*)-isomer of LGH.

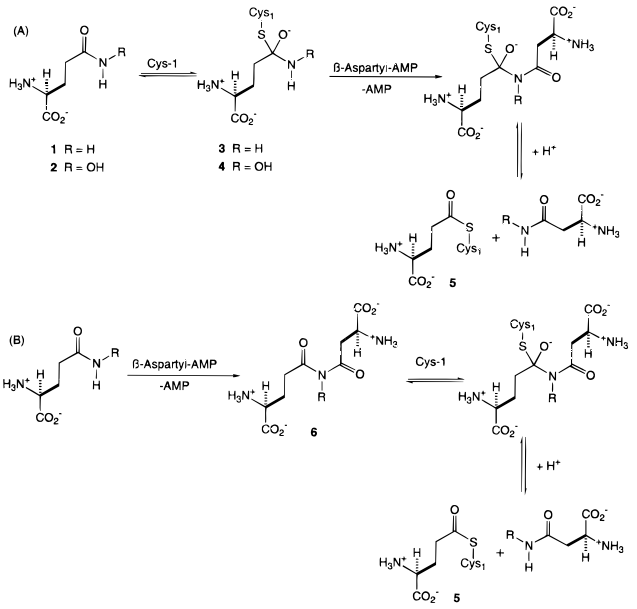

FIGURE 5: Mechanistic alternatives for nitrogen transfer from glutamine or LGH in AS-B. (A) Attack of the thiolate of Cys-1 upon bound glutamine **1** (R = H) or LGH **2** (R = OH) occurs to yield an oxyanion **3** (R = H) or LGH **4** (R = OH) in which the amide nitrogen is activated for nucleophilic attack. (B) Activation of the primary amide of glutamine **1** (R = H) allows direct attack on β-aspartyl-AMP followed by loss of a proton to give an imide intermediate **6**. Subsequent C−N bond cleavage by Cys-1 yields asparagine and the acylenzyme **5**. No stereochemical aspects of the reaction intermediates are implied in either of these two schemes.

(Figure 4C), which is present only in low concentrations in aqueous solution (Challis & Challis, 1979). The acylhydroxamate might not therefore be correctly positioned for direct attack on β-aspartyl-AMP by the nitrogen in the tetrahedral intermediate **4** (Figure 5A), leading to a reduction in k_{cat} for the formation of aspartic acid β-monohydroxamate. In an alternative mechanistic scheme (Figure 5B), which is consistent with recent studies of the mechanism by which asparagine residues in Asn-X-Ser/Thr sequences are glycosylated (Imperiali & Shannon, 1991; Imperiali et al., 1992), Asn-74 might function to activate the amide nitrogen of glutamine, possibly by formation of low-barrier hydrogen bonds (Cleland & Kreevoy, 1994; Gerlt & Gassman, 1993).

In summary, LGH and hydroxylamine can act as alternate substrates to glutamine and ammonia, respectively, in the reactions catalyzed by AS-B. While such an observation has been made for a few members of the *trpG* amidotransferase family (Anderson & Meister, 1966; Li & Buchanan, 1971), our results appear to be the first demonstration that nitrogen transfer can occur from a glutamine analog for a *purF* amidotransferase. In addition, we have been able to

show unambiguously that hydroxylamine is transferred directly from LGH to β-aspartyl-AMP, giving aspartic acid β-monohydroxamate. Finally, nitrogen transfer from LGH proceeds with a turnover number that is 7-fold lower than that observed in glutamine-dependent asparagine synthesis. When combined with our observations of the heavy atom kinetic isotope effects for the glutaminase and glutamine-dependent synthetase activities, this kinetic difference provides further support for the hypothesis that nitrogen transfer is not mediated by enzyme-bound ammonia, at least in the case of AS-B. A more detailed interpretation of these kinetic data, as well as the precise delineation of the catalytic role of Asn-74, awaits the acquisition of a three-dimensional structure for AS-B.

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This paper is dedicated to the memory of Dr. Alton Meister, a pioneer in the study of glutamine metabolism and glutamine-dependent amidotransferases.

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