Formation and Isolation of a Covalent Intermediate during the Glutaminase Reaction of a Class II Amidotransferase†

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ABSTRACT: Incubation of *Escherichia coli* asparagine synthetase B (AS-B) with [^{14}C]-L-glutamine gives a covalent adduct that can be isolated. Radiolabeled protein is not observed (i) when the wild-type enzyme is incubated with 6-diazo-5-oxo-L-norleucine (DON) prior to reaction with [^{14}C]glutamine or (ii) when the C1A AS-B mutant is incubated with [^{14}C]-L-glutamine. Both of these alterations eliminate the ability of the enzyme to utilize glutamine but do not affect ammonia-dependent asparagine synthesis. Formation of the covalent adduct therefore depends on the presence of the N-terminal active site cysteine, which has been shown to be essential for glutamine-dependent activity in this and other class II amidotransferases. The amount of covalent adduct exhibits saturation behavior with increasing concentrations of L-glutamine. The maximum observed quantity of this intermediate is consistent with its involvement on the main pathway of glutamine hydrolysis. The chemical properties of the isolable covalent adduct are consistent with those anticipated for the γ-glutamyl thioester that has been proposed as an intermediate in the AS-B-catalyzed conversion of glutamine to glutamate. The covalent adduct is acid-stable but is labile under alkaline conditions. On the basis of the measured rates of formation and breakdown of this intermediate, it is kinetically competent to participate in the normal catalytic mechanism. These studies represent the first description of a thioester intermediate for any class II amidotransferase and represent an important step in gaining further insight into the kinetic and chemical mechanisms of AS-B.

Numerous clinical studies have correlated the importance of asparagine biosynthesis with the ability of T-cell leukemias to develop resistance to combination therapies employing L-asparaginase (*1*). In eukaryotes, glutamine-dependent asparagine synthetase (AS)[1] mediates asparagine production, and specific, potent inhibitors that prevent asparagine formation in tumor cells therefore have potential as therapeutic adjuncts to treatments employing L-asparaginase (*2*, *3*). Efforts to develop such inhibitors using rational methods have been hampered by an incomplete understanding of the chemical and kinetic mechanism of human AS (*4*). In addition, extensive screening studies have failed to elicit any useful lead structures for the development of potent AS inhibitors (*5*−*8*). Our efforts to probe the molecular details of glutamine-dependent asparagine synthesis have primarily employed *Escherichia coli* AS-B (*9*), encoded by the *asnB* gene (*10*), as a model for human AS. The *E. coli* enzyme can be obtained in large amounts using standard protein expression protocols (*9*), and catalyzes the ATP-dependent synthesis of asparagine utilizing either ammonia (reaction 1) or glutamine (reaction 2) as a nitrogen source. In the absence of aspartate, AS-B catalyzes the hydrolysis of glutamine to glutamate and ammonia (reaction 3), a reaction that can be stimulated by the addition of ATP (*11*, *12*).

reaction 1: L-Asp + NH_3 + ATP → L-Asn + AMP + PP_i

reaction 2: L-Asp + L-Gln + ATP → L-Asn + L-Glu + AMP + PP_i

reaction 3: L-Gln → L-Glu + NH_3

Importantly, the thiolate side chain of the conserved, N-terminal cysteine (Cys-1) must be present for both glutamine-dependent activities of AS-B (*9*), and other glutamine-dependent asparagine synthetases (*13*, *14*), placing these enzymes in the class II (formerly *PurF*) superfamily of amidotransferases (*15*, *16*). Other class II amidotransferases include GPA (*17*), GFAT (*18*), and glutamate synthase (*19*). The functional roles in glutamine binding and activation of Cys-1 and other N-terminal, GAT domain residues conserved throughout class II amidotransferases

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[1] Abbreviations: AS, asparagine synthetase; AS-B, asparagine synthetase B; GAT, glutamine amide transfer; GPA, glutamine 5′-phosphoribosylpyrophosphate amidotransferase; GFAT, glutamine fructose-6-phosphate amidotransferase; WT, wild-type; DON, 6-diazo-5-oxo-L-norleucine; LGH, L-glutamic acid γ-monohydroxamate; TCA, 2,2,2-trichloroacetic acid; SA, specific activity; BSA, bovine serum albumin; GnHCl, guanidinium-HCl; CAD, carbamoyl phosphate synthetase−aspartate transcarbamoylase−dihydroorotase; CPS, carbamoyl phosphate synthetase; SDS, sodium dodecyl sulfate; GMPS, guanosine monophosphate synthase; PABA, *p*-aminobenzoic acid; KIE, kinetic isotope effect; Ntn, N-terminal nucleophile.

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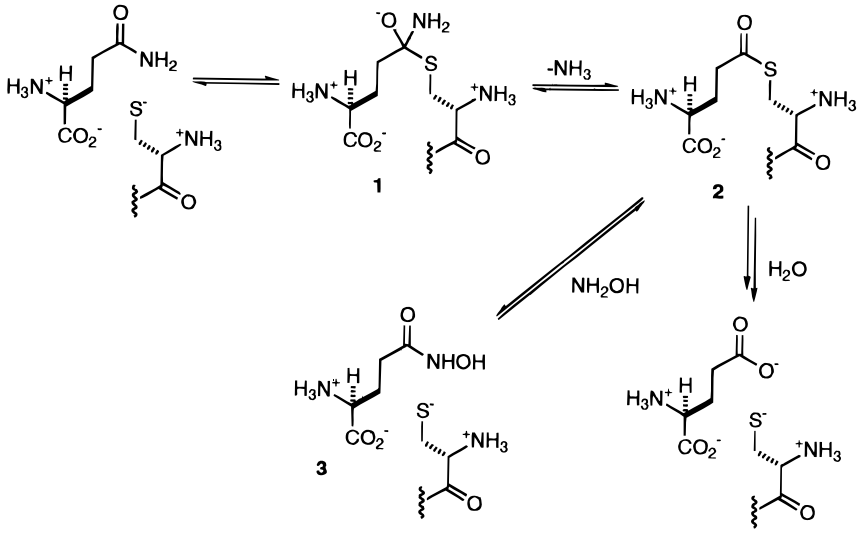
Scheme 1: Minimal Mechanism for the Formation of a Putative γ-Glutamyl Thioester Intermediate in AS-B Glutaminase Activity and Its Partitioning in the Presence of Hydroxylamine



have been defined in an extensive series of studies (*4*, *9*, *11*, *12*).

Experiments to define the molecular details by which the amide nitrogen is removed from glutamine and transferred to β-aspartyl-AMP, an intermediate formed in a second active site in the C-terminal domain of the enzyme (*4*), are complicated by the fact that AS-B exhibits two competing glutamine-dependent activities. We have therefore undertaken to define the glutaminase and synthetase activities of AS-B in separate systematic studies. In particular, the mechanistic relationship between amide hydrolysis in class II amidotransferase and thiol proteases (*20*, *21*) is intriguing given subtle differences in the catalytic functional groups employed by these two families of enzymes (*22*). Thioesters are proposed to be intermediates in thiol-catalyzed amide hydrolysis (Scheme 1), and evidence for their isolation in glutamine breakdown by class I amidotransferases has been reported (*23*−*26*). In contrast, and despite substantial effort, no thioester intermediate has yet been characterized for any class II amidotransferase. We now report experimental conditions for the isolation of a covalent adduct that is formed when AS-B is incubated with glutamine. The chemical properties of this adduct, together with the observation that the thiolate side chain of Cys-1 is required for its formation and other kinetic evidence, are consistent with the hypothesis that it represents the γ-glutamyl thioester intermediate formed during AS-B-catalyzed glutamine hydrolysis. The isolation of this intermediate represents an important step in gaining further insight into the details of the kinetic and chemical mechanisms of nitrogen transfer in AS-B and other class II amidotransferases.

MATERIALS AND METHODS

Enzymes and Reagents. Recombinant WT AS-B and the C1A AS-B mutant, in which Cys-1 is substituted by alanine, were constructed, expressed, and purified using published procedures (*9*). Protein concentrations were determined using a BioRad assay kit with known amounts of AS-B, calibrated using amino acid analysis of several samples, by the Protein Chemistry Core Facility of the Interdisciplinary Center for Biotechnology Research at the University of Florida. [U-^{14}C]-L-Glutamine (277 mCi/mmol) was purchased from Amersham (Arlington Heights, IL). 6-Diazo-5-oxo-L-norleucine (DON) and Sephadex G-50 Fine were obtained from Sigma Chemical Co.

Partitioning the γ-Glutamyl Thioester Intermediate to L-Glutamic Acid γ-Monohydroxamate by Addition of Hydroxylamine. Reaction mixtures containing WT AS-B (9.3 μg), 50 mM glutamine, 100 mM Bis-Tris, Tris-HCl (pH 8), and variable concentrations of hydroxylamine (300-μL total volume) were incubated at 37 °C for 45 min. The reaction was terminated by the addition of 100 μL of 16% TCA. An aliquot (50 μL) was removed and used in an end-point assay employing glutamate dehydrogenase in the presence of NAD^+ to measure the concentration of glutamate formed (*27*). Coupling reagent (300 mM glycine, 250 mM hydrazine, pH 9, 1 mM ADP, 1.6 mM NAD^+, 2.2 units of glutamate dehydrogenase) was added to the aliquot and the resulting mixture incubated for 10 min at room temperature. The absorbance at 340 nm was measured and the glutamate concentration determined by comparison to a standard curve. LGH formation was measured in a final volume of 500 μL by addition of a solution containing 80% TCA, 6 N HCl, and 10% $FeCl_3$ in 0.02 N HCl to the remaining reaction solution. After centrifugation of the samples to remove particulates, the absorbance of the resulting hydroxamate−$FeCl_3$ complex at 540 nm was determined. A standard curve using a stock solution of authentic LGH was used to quantitate the product formed in these reactions.

Isolation of the Putative γ-Glutamyl Thioester Formed by Incubation of AS-B with [^{14}C]Glutamine. WT AS-B (0.74 nmol), or the C1A AS-B mutant (0.74 nmol), was incubated with 1 mM [U-^{14}C]-L-glutamine (SA 22 000 dpm/nmol) in 100 mM Tris-HCl (pH 8.0) at room temperature (100-μL total reaction volume). After 30 s, the reaction was quenched in 8% TCA (1 mL) and 100 μL of BSA (10 mg/ml) was added. After 2 min in the quench solution the samples were filtered under vacuum on a 2.5-cm nitrocellulose filter (0.45-μm porosity), and the filter was washed with 50 mL of 1 N HCl. The filter was transferred to 5 mL of scintillation fluid (ScintiVers II*) and the ^{14}C activity measured on a Beckman LS6000IC scintillation counter. This procedure was repeated

in the absence of enzyme to measure the nonspecific binding of L-glutamine to the filter.

Stability of the Putative γ-Glutamyl Thioester Formed by Incubation of AS-B with [^{14}C]Glutamine. WT AS-B (4.8 nmol) was incubated at room temperature for 30 s with 1.5 mM [U-^{14}C]-L-glutamine (SA 25 633 dpm/nmol) in 100 mM Bis-Tris and Tris-HCl (pH 8) (100-μL total volume). The enzyme-catalyzed reaction was terminated by the addition of 22 μL of 0.5 M sodium acetate (pH 4) in 5% SDS. The reaction solution was divided into two portions, and protein was separated from free L-glutamine on a Sephadex G-50 Fine spin column equilibrated in (1) 0.1 M sodium phosphate (pH 12) in 1% SDS or (2) 0.1 M sodium phosphate (pH 2) in 1% SDS. The amount of radiolabeled enzyme that passed through the column was determined by liquid scintillation counting, and the total protein concentration was measured using a standard BioRad assay kit. The percentage of radiolabeled enzyme was calculated as an average of values from six separate experiments.

Base Lability of the Covalent Adduct Isolated by Gel Filtration. In all reactions, WT AS-B (4.8 nmol) was incubated for 30 s at room temperature with 1.5 mM [^{14}C]-glutamine (SA 25 633 dpm/nmol) in a reaction containing 100 mM Bis-Tris and Tris-HCl (pH 8). Reactions were terminated by the addition of 22 μL of 0.5 M sodium acetate (pH 4) in 5% SDS. To determine the stability of the putative γ-glutamyl thioester intermediate at pH 2 and 12, protein was separated from free glutamine (*21*) using a Sephadex G-50 Fine spin column equilibrated in 0.1 M sodium phosphate (either pH 2 or pH 12) in 1% SDS. The amount of radiolabeled enzyme in 70 μL of the solution which passed through the column was then determined by scintillation counting, and the protein concentration was measured using an assay kit supplied by BioRad. Average values, together with the standard error, given here were derived from six independent assays.

Glutaminase Assays. Initial rates of L-glutamine hydrolysis were determined by a modified procedure which uses glutamate dehydrogenase in the presence of NAD^+ to measure glutamate concentration (*27*). Reaction mixtures (100 μL) contained 100 mM Bis-Tris and Tris-HCl (pH 8.0) each, 8 mM $MgCl_2$, and various concentrations of glutamine and were initiated by the addition of wild-type AS-B and incubated at various temperatures (4−40 °C). A control reaction lacking enzyme was used to measure glutamate formed from spontaneous breakdown. The reactions were terminated by the addition of 20 μL of 1 N acetic acid. A coupling reagent (300 mM glycine, 250 mM hydrazine, pH 9, 1 mM ADP, 1.6 mM NAD^+, 2.2 units of glutamate dehydrogenase) was added and incubated for 10 min at room temperature. The absorbance at 340 nm was measured, and the concentration of glutamate was determined by comparison to a standard curve. Initial velocities were measured at nine different substrate concentrations, and each velocity was an average of three measurements. Stability of the wild-type AS-B at each temperature was confirmed by incubation in buffer of the appropriate temperature for various amounts of time followed by an assay of activity at pH 8. Initial velocity conditions were confirmed at each pH by linear plots of velocity versus time and velocity versus enzyme concentration. The pH of the buffer was adjusted to pH 8 at each temperature studied to account for the pK_a change with temperature.

Data Manipulation. As shown in the equation below, the value for $-E_a/RT$ can be derived from the slope of a plot of the log k_{cat} versus $1/T$ (K) where A is a constant which is related to the collision frequency and steric factors.

$$k = Ae(-E_a/RT) \quad (2)$$

Values for the energy, enthalpy, and entropy of activation then were calculated using the following equations:

$$\Delta H^* = E_a - RT \quad (3)$$

$$\Delta G^* = RT \ln(k_B T/h) - RT \ln(k) \quad (4)$$

$$\Delta G^* = \Delta H^* - T\Delta S^* \quad (5)$$

where ΔH^* is the enthalpy of activation, R is the gas constant, k_B is Boltzman's constant, h is Planck's constant, k is the forward rate constant, ΔG^* is the free energy of activation, and ΔS^* is the entropy of activation.

Rate of Deacylation of the Thioester Intermediate. A glutamyl−enzyme adduct was formed at 5 °C by incubating 0.74 nmol of WT AS-B with 1.5 mM L-glutamine (SA 20 000 dpm/nmol) in a 100-μL reaction containing 100 mM Bis-Tris and Tris (pH 8.0). After the reaction was incubated for 1 min, 100 μL of 200 mM nonradioactive glutamine was added to each sample to dilute the unreacted radioactive glutamine. The samples were quenched at various time intervals after the dilution as described above, and the amount of radioactivity associated with the protein was determined by scintillation counting.

RESULTS

Hydroxylamine has been widely used to provide indirect evidence for the formation of acylenzyme intermediates in amide hydrolysis reactions catalyzed by serine proteases (*29, 30*) and the class I amidotransferases CAD (*23*) and CPS (*25*). Addition of 5 mM hydroxylamine to a solution containing WT AS-B (9.3 μg) and 30 mM glutamine, followed by incubation at pH 8 and 37 °C for 45 min, produced both LGH and glutamate in a ratio of 0.16. The amount of LGH formed in the partitioning experiments with WT AS-B is consistent with the formation of a γ-glutamyl thioester in the glutaminase activity of this class II amidotransferase.

To establish that the adduct formed from L-glutamine and WT AS-B represented a true intermediate in the AS-B-catalyzed glutaminase reaction, quantitative kinetic measurements were undertaken. We examined the behavior of the covalent adduct in filter binding assays that had been used by others in successful attempts to isolate similar reaction intermediates (*24*). In these experiments, enzyme was mixed with [U-^{14}C]-L-glutamine for 30 s and then denatured with TCA to give a precipitate that was filtered and washed with 1 N HCl. Radioactivity remaining on the filter was measured using liquid scintillation counting. Approximately 37.5% of WT AS-B was radiolabeled under these experimental conditions (Table 1). Two control experiments demonstrated that radioactivity associated with the filter did not arise from nonspecific binding. First, no radiolabeled adduct was observed when the C1A AS-B mutant was incubated with [U-^{14}C]-L-glutamine in place of the wild-type enzyme (Table 1). This site-specific mutant, while retaining the ability to

Table 1: Isolation of Putative γ-Glutamyl/AS-B Adduct by Filter Binding Assay[a]

incubation sample	dpm_{av}	% enzyme labeled
WT AS-B + [^{14}C]Gln	6504 ± 183	37.5
WT AS-B + DON + [^{14}C]Gln	185 ± 55	
C1A + [^{14}C]Gln	290 ± 115	
[^{14}C]Gln only	384 ± 187	

[a] In the experiments employing WT AS-B (0.74 nmol) that was covalently modified by 1 mM DON for 30 min (second entry), DON was diluted by addition of saturating [^{14}C]glutamine and the amount of radiolabeled protein was determined as described for the filter binding method (see Materials and Methods). Each dpm_{av} value represents the average of three assays together with the standard error.

employ ammonia in the synthetase reaction, lacks both of the glutamine-dependent activities of the WT enzyme. In addition, the C1A AS-B mutant binds L-glutamine with a higher affinity than WT AS-B (*5*). In the second set of experiments, WT AS-B was initially treated with DON (*31*), an irreversible inhibitor of the glutamine-dependent activity of class II amidotransferases. In the case of DON-modified AS-B, while retaining full ammonia-dependent synthetase activity (data not shown), no radiolabeled covalent adduct was formed when this protein was incubated with [U-^{14}C]-L-glutamine under standard conditions (Table 1). Hence, the thiolate side chain of Cys-1 is necessary for formation of radiolabeled material. DON inactivation can be blocked by glutamine suggesting that this reagent forms a covalent bond specifically with the side chain of Cys-1, a hypothesis that has been confirmed crystallographically for *E. coli* GPA (*32*, *33*). Chemical modification of mammalian asparagine synthetases with DON gives enzymes that maintain their ability to catalyze ammonia-dependent asparagine synthesis (*34*, *35*).

Examination of the effect of glutamine concentration on the steady-state amounts of the covalent adduct revealed a saturation curve in which the maximum molar ratio of adduct to enzyme was 0.37 (Figure 1). Further, the glutamine concentration at half-saturation was 0.14 mM. Under steady-state conditions, the K_M for glutamine in the WT AS-B glutaminase activity is 0.17 mM (data not shown), supporting the hypothesis that the isolated adduct was an intermediate in AS-B-catalyzed glutamate formation. Further indirect evidence that the covalent adduct isolated in these assays was indeed the γ-glutamyl thioester intermediate (Scheme 1) was obtained in studies of the stability of the radiolabeled enzyme as a function of pH. A gel filtration protocol was used to determine the stability of the radiolabeled protein. Briefly, WT AS-B was incubated with [U-^{14}C]-L-glutamine under our standard conditions, and then the resulting adduct was subjected to gel filtration chromatography columns equilibrated at either low or high pH. Approximately 36 ± 4% and 6 ± 2% of radiolabeled WT AS-B was obtained after gel filtration at pH 2 and 12, respectively. This demonstrates that while the intermediate was acid-stable, breakdown occurred under basic conditions. The reliability of this procedure is further indicated by the observation that the amount of radiolabeled enzyme obtained by gel filtration at pH 2 is identical to that observed at saturating glutamine concentration in the filter binding measurements. Each measurement was repeated six times.

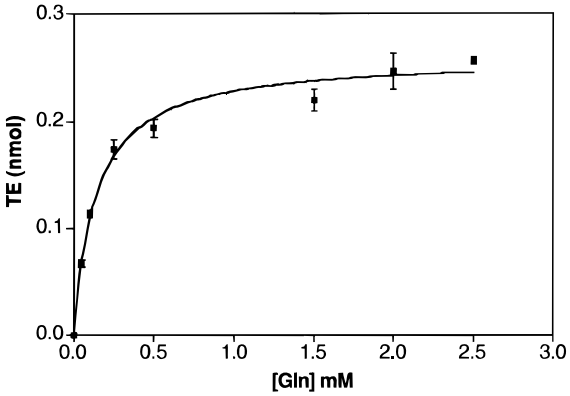


FIGURE 1: Effect of glutamine on the steady-state concentration of the putative γ-glutamyl thioester intermediate. The steady-state concentration of the putative thioester intermediate was measured at 5 °C by incubating WT AS-B (0.74 nmol) with various concentrations of [^{14}C]glutamine in 100 mM Tris-HCl (pH 8) (100-μL total volume). After 30 s, the reaction was quenched and radiolabeled enzyme was trapped using a filter binding protocol (see Materials and Methods). The glutamine concentration at half-saturation was determined from nonlinear regression analysis using the Prism software package, supplied by GraphPad, Inc. (San Diego, CA).

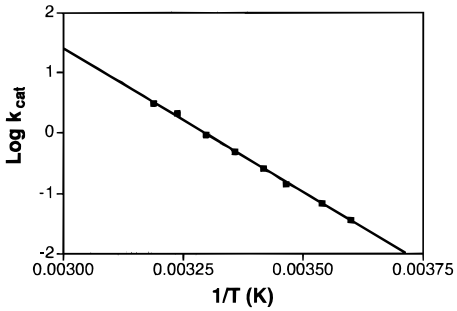


FIGURE 2: Temperature dependence of glutamine hydrolysis catalyzed by WT AS-B. The initial rates were determined at pH 8.0 in 100 mM Bis-Tris and Tris-HCl as described in Materials and Methods.

To demonstrate that the adduct is kinetically competent, it is necessary to show that both its formation and breakdown occur at rates at least as fast as the k_{cat} for the overall reaction. These measurements were performed at 5 °C in order to slow the rates and permit their measurements by manual mixing techniques. Because other studies had been performed at 37 °C, it was important to ensure that the nature of the rate-limiting step(s) was unchanged between 37 and 5 °C. The steady-state kinetic parameters for the glutamine hydrolysis were therefore determined at temperatures within this range. The thermodynamic properties of glutamine hydrolysis catalyzed by AS-B were as follows: ΔG^*, 167 kJ/mol (37 °C); ΔH^*, 89 kJ/mol (37 °C); ΔS^*, −252 J/mol K^{-1} (5−40 °C); $T\Delta S$, −78 kJ/mol (37 °C). The Arrhenius plot (Figure 2) derived from these data was linear, indicating that the nature of the rate-limiting step was unchanged over this temperature or that multiple mechanistic steps coincidentally possessed mutually compensating changes with temperature. We favor the former explanation.

Attempts to measure the rate constant for formation of the putative thioester were unsuccessful. Even at 5 °C, formation of the intermediate was complete within 10 s. This indicates that the reaction had proceeded for at least 3 half-

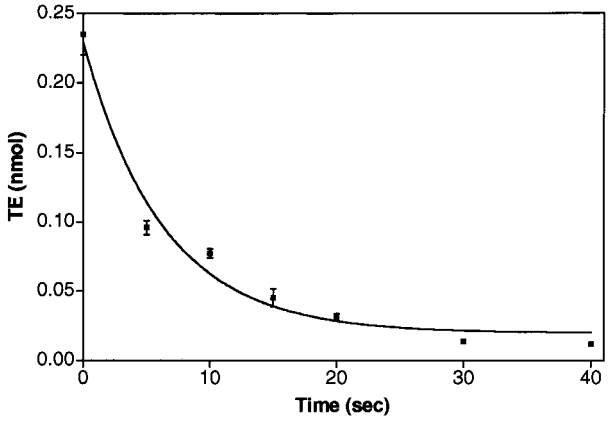


FIGURE 3: Rate of deacylation of the thioester intermediate formed during WT AS-B-catalyzed glutamine hydrolysis.

lives within this period; the rate constant associated with formation of the intermediate was therefore <0.2 s^{-1}. The rate constant associated with loss of bound radioactivity from the enzyme was determined by incubating the preformed complex with a large excess of unlabeled glutamine. The disappearance of the protein-associated radioactivity followed a monoexponential pattern, and a rate constant of 0.016 ± 0.02 s^{-1} was obtained (Figure 3). This value is 4 times higher than the k_{cat} value of 0.043 ± 0.007 s^{-1} measured for the glutamine hydrolysis reaction.

DISCUSSION

Glutamine-dependent amidotransferases have been classified into two families, class I (formerly *TrpG*) and class II (formerly *PurF*), based on multiple sequence alignment of GAT domains containing the catalytic residues that are involved in glutamine activation (*15*). Crystal structures obtained for the class I amidotransferases GMPS (*36*) and CPS (*37*) both show the presence of a catalytic triad of conserved cysteine, histidine, and aspartate residues. These residues are therefore thought to mediate glutamine hydrolysis in a manner similar to analogous residues in thiol proteases such as papain (*21*). In this mechanism, initial attack of the thiolate on the amide yields a tetrahedral intermediate (**1**) that collapses to give a γ-glutamyl thioester (**2**) and ammonia (Scheme 1). Acid−base catalysis by the active site histidine plays a prominent role in this pathway. Subsequent hydrolysis of the reactive thioester regenerates the enzyme and produces glutamate. In the case of class I amidotransferases, direct evidence for this mechanism has been provided by isolation of the thioester for CAD (*23*), the PabA subunit of the *E. coli* PABA synthase (*24*), and CPS (*25*, *26*). Isolation of these covalent intermediates has contributed significantly to our understanding of the mechanism of glutamine hydrolysis by these enzymes. In particular it has allowed the use of a minimal model to examine the rate-determining step of the reaction, as well as the effects of other substrates on this step.

While the mechanistic relationship between class I amidotransferases and thiol proteases is now firmly established, the situation is less clear for class II amidotransferases. Although Cys-1 is essential for glutamine-dependent activity in this family of enzymes (*4*, *18*, *38*), class II amidotransferases exhibit significant differences in their ability (i) to hydrolyze glutamine in the absence of other substrates and (ii) to employ ammonia as an alternate nitrogen source (*4*). Although early experiments on site-specific GPA mutants suggested the presence of a catalytically active histidine residue (*38*), subsequent mutagenesis experiments on AS-B (*9*), and crystal structures of various class II amidotransferases (*32*, *33*, *39*, *40*), have failed to identify histidines that participate in acid−base catalysis. It has therefore been proposed that the N-terminal amino group of Cys-1 performs the role of an active site histidine, placing class II amidotransferases in the Ntn hydrolase superfamily (*41*), which includes the 20S proteasome (*42*) and aspartylglucosaminidase (*43*). Unambiguous kinetic evidence defining the participation of the N-terminal amine in catalysis, however, has yet to be reported. Finally, heavy-atom KIE determinations revealed subtle differences in the detailed kinetics of the underlying mechanisms of amide hydrolysis employed by AS-B and papain (*44*). On the other hand, the glutamine binding and activation site of class II amidotransferases does possess some characteristic features observed in thiol proteases, including a conserved residue (Asn-74) that appears to be involved in defining an oxyanion hole (*11*, *22*, *32*, *40*).

In light of these structural and kinetic differences, it is crucial to validate the existence of the putative γ-glutamyl thioester intermediate in AS-B-catalyzed glutamine hydrolysis. The demonstration of the formation of LGH upon addition of hydroxylamine to WT AS-B in the presence of glutamine supports the notion that a thioester is formed during AS-B-catalyzed glutamate formation. In principle, an anhydride or ester would also be consistent with these observations. However other observations argue against these types of intermediates. For example, reaction of hydroxylamine with an anhydride intermediate might generate an acylhydroxamate derivative of the enzyme, resulting in time-dependent inhibition by the added nucleophile. No such inhibition of AS-B in the presence of hydroxylamine was observed under our reaction conditions. Further, no catalytically important GAT domain aspartate or glutamate has been identified. The involvement of an ester intermediate is also unlikely since added hydroxylamine causes partitioning of AS-B glutaminase activity even at pH 6.

On the basis of these results, we propose that the covalent intermediate represents a γ-glutamyl thioester with the side chain of Cys-1 (Scheme 1). Several lines of evidence support this notion. Filter binding experiments employing [U-^{14}C]-L-glutamine gave a radiolabeled derivative of AS-B. Radiolabeled enzyme was not obtained when the C1A AS-B mutant (in which Cys-1 is replaced by an alanine residue) was incubated with [^{14}C]-L-glutamine in place of wild-type enzyme. While it lacks both glutamine-dependent synthetase and glutaminase activity, the C1A AS-B mutant retains ammonia-dependent synthetase activity and exhibits a higher affinity for glutamine than WT AS-B (*9*). Hence, the lack of a radiolabeled adduct with the C1A AS-B mutant strongly suggests that covalent modification occurs via the thiolate side chain of Cys-1. The failure of the C1A AS-B mutant to form a covalent derivative is also evidence that nonspecific glutamine binding does not account for the radioactivity observed in our experiments employing WT AS-B. The specific requirement for the thiolate side chain of Cys-1 in formation of the radiolabeled enzyme was also supported by experiments employing WT AS-B that had been previously inactivated by incubation with DON. In addition, the

amount of the isolated covalent adduct saturated with increasing glutamine, half-saturation being observed at a glutamine concentration that is very similar to the K_M of this substrate in steady-state studies of the AS-B glutaminase reaction. The intermediate was acid-stable but labile in alkaline solution, which is consistent with the chemical behavior of thioesters. Taken together, these observations are most easily explained by the intermediacy of a γ-glutamyl thioester. While the presence of such an intermediate has long been suspected, this is the first direct demonstration of its existence in a class II amidotransferase.

The stoichiometry, saturation behavior, and rates of formation and breakdown of the covalent intermediate are consistent with its participation in the normal glutamine hydrolysis mechanism. Moreover, the observation that the rates of formation and breakdown of the covalent intermediate were >4.7- and 4-fold greater that the steady-state k_{cat} value for glutamine hydrolysis indicates that one or more steps after glutamate formation within the enzyme active site are largely rate-limiting. The observation of nearly identical k_{cat} values for the enzyme-catalyzed hydrolyses of glutamine and more reactive analogues such as LGH and glutamate γ-methyl ester further supports this proposal (see ref *12* and unpublished data). While the rates at which these substrates form the covalent γ-glutamyl thioester intermediate may differ, the overall turnover rate is limited by subsequent events that are common to all three.

The covalent γ-glutamyl thioester occupies a key position in the mechanism of glutamine cleavage by AS-B. The ability to directly detect the formation and breakdown of this species provides opportunities to characterize the wild-type enzyme more completely and to assess the effects of mutations on elementary steps. These studies will also reveal the parallels between the glutamine hydrolysis portion of the AS-B mechanism and those of thiol proteases and other GAT enzymes.

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