Experimental Data in Support of a Direct Displacement Mechanism for Type I/II L-Asparaginases*

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Bacterial L-asparaginases play an important role in the treatment of certain types of blood cancers. We are exploring the guinea pig L-asparaginase (gpASNase1) as a potential replacement of the immunogenic bacterial enzymes. The exact mechanism used by L-asparaginases to catalyze the hydrolysis of asparagine into aspartic acid and ammonia has been recently put into question. Earlier experimental data suggested that the reaction proceeds via a covalent intermediate using a ping-pong mechanism, whereas recent computational work advocates the direct displacement of the amine by an activated water. To shed light on this controversy, we generated gpASNase1 mutants of conserved active site residues (T19A, T116A, T19A/T116A, K188M, and Y308F) suspected to play a role in hydrolysis. Using x-ray crystallography, we determined the crystal structures of the T19A, T116A, and K188M mutants soaked in asparagine. We also characterized their steady-state kinetic properties and analyzed the conversion of asparagine to aspartate using NMR. Our structures reveal bound asparagine in the active site that has unambiguously not formed a covalent intermediate. Kinetic and NMR assays detect significant residual activity for all of the mutants. Furthermore, no burst of ammonia production was observed that would indicate covalent intermediate formation and the presence of a ping-pong mechanism. Hence, despite using a variety of techniques, we were unable to obtain experimental evidence that would support the formation of a covalent intermediate. Consequently, our observations support a direct displacement rather than a ping-pong mechanism for L-asparaginases.

Currently, there still exists a gap in knowledge regarding the enzymatic mechanism by which L-asparaginases hydrolyze the amino acid L-asparagine to L-aspartic acid and ammonia (Fig. 1A). L-Asparaginases have garnered special attention because of their clinical use in the treatment of certain blood cancers (1). In addition, L-asparaginase are also used commercially by the food industry for reducing the production of acrylamide (2), a compound being investigated for its toxicity and carcinogenicity in humans. Hence, understanding the enzymatic mechanism of these important enzymes can be used to inform the design of variants with improved properties. Our specific interest is with the guinea pig L-asparaginase type I enzyme, which holds potential as a replacement for the currently clinically used bacterial enzymes (see more below).

L-Asparaginases can be divided, based on sequence and structural homology, into two unrelated families: type I and II L-asparaginases belong to one family, and type III enzymes belong to another (3). The type I and II enzymes are very similar in terms of overall structure and conservation of active site residues (3). In Escherichia coli, the type I enzyme is cytoplasmic, and type II is periplasmic (3). In addition to having different cellular localization, bacterial type II enzymes have a much lower $K_m$ value for Asn (low micromolar versus millimolar). This ~100-fold lower $K_m$ property is a critical differentiator between the enzymes and has allowed the use of bacterial type II, but not type I, enzymes in cancer chemotherapy. Currently, the E. coli type II enzyme (brand name Elspar®) and that from Erwinia chrysanthemi (brand name Erwinaze®) are Food and Drug Administration-approved for the treatment of certain blood cancers (4). The principle behind the anti-cancer effect of type II L-asparaginases is that certain cancers have become reliant on extracellular sources of Asn. Administration of the bacterial type II enzymes acts to deplete Asn from the blood, and this ultimately induces the death of such cancer cells.

We have recently reported the first structural characterization of a mammalian type I/II L-asparaginase, that from the guinea pig (5). By sequence homology, this guinea pig L-asparaginase would be classified as a type I enzyme; therefore we refer to it as gpASNase1. However, its low micromolar $K_m$ property is consistent with the type II enzymes. The use of bacterial asparaginases can be an impediment to treatment because of a wide range of allergic responses that can result, some of which are quite severe. Furthermore, treatment efficacy can be greatly compromised upon the development of antibodies, which inactivate and deplete the enzyme. Because gpASNase1 is a mammalian L-asparaginase, it would be expected to have lower immunogenicity compared with the Food and Drug Administration-approved bacterial type II enzymes; therefore we suggested that the guinea pig enzyme would make a promising replacement for the bacterial enzymes (5).

Despite being extensively studied, there is still controversy concerning the catalytic mechanism employed by the type I/II enzymes. A reaction such as the hydrolysis of Asn is referred to as a Bi Bi reaction, because two substrates (Asn and water) yield two products (Asp and ammonia). Bi Bi reactions can have
Probing the Mechanism of L-Asparaginases

A schematic of the L-asparaginase reaction. Asparagine (L-ASN) is converted to aspartic acid (L-ASP) with the help of L-asparaginase. A water molecule would release the final Asp product (i.e., ammonia). Such an inhibition pattern is expected when the enzyme uses the ping-pong mechanism (i.e., presence of a covalent intermediate). However, this uncompetitive inhibition behavior will also be observed for a direct displacement mechanism where the substrate must bind to the enzyme prior to inhibitor binding (7). In other words, for the case of L-asparaginases using a direct displacement mechanism, uncompetitive inhibition by hydroxylamine will be observed if the binding of Asn promotes the enzyme state that binds the inhibitor, and as a result, the inhibition cannot be overcome by saturating with Asn. Hence, the observed uncompetitive behavior of hydroxylamine cannot be used to definitively distinguish between the two mechanisms.

In another set of experiments, the researchers incubated 50 mM Asp with 18O-containing water and the enzyme and observed that 0.08% of the Asp became labeled with 18O (6). The incorporation of 18O by the Asp was interpreted as support for the existence of a covalent intermediate, because the breakdown of an enzyme-Asp covalent intermediate by 18O-water would transfer the isotope to the amino acid. However, this result is also compatible with the direct displacement mechanism, where an 18O-water directly attacks Asp. Of note, this work by Ehrman et al. (6) is the one often cited as evidence for the ping-pong mechanism, despite the fact that the data are also compatible with a direct displacement mechanism.

Further support for a ping-pong mechanism came from a study of the L-asparaginase from Saccharomyces cerevisiae (8). These authors also observed uncompetitive inhibition by hydroxylamine and hence came to the conclusion of a ping-pong mechanism. Similarly, Röhm et al. (9) used NMR to study the mechanism of L-asparaginases by probing 18O exchange into Asp catalyzed by the E. coli type II enzyme and came to the same conclusion of a ping-pong mechanism. Notably, the authors concede that “The present oxygen exchange experiments provide results that are consistent with, but do not require, the involvement of a covalent intermediate” (9). Biochemical studies (10, 11) and crystal structures of L-asparaginase (12, 13) identified Thr12 and Thr89 as putative active site residues that would form such a covalent intermediate, consis-
tent with the conservation of these threonine residues in type I/II \(\text{L}\)-asparaginases. Mutagenesis studies of the \(E.\ coli\) type II enzyme revealed that the T12A mutant is still active, albeit with 0.01% (10) to 0.04% (11) activity relative to the wild type enzyme. Mutation of Thr\(^{89}\) to valine resulted in an enzyme with \(\sim 24,000\)-fold reduced rate (14). These experiments reveal critical roles for Thr\(^{12}\) and Thr\(^{89}\) in the \(\text{L}\)-asparaginase reaction but do not necessarily prove one mechanism over the other.

An alternative and complementary method to ascertain the enzyme mechanism is to perform structural studies. Based on the biochemical experiments discussed above, authors of crystallographic studies of \(\text{L}\)-asparaginases interpreted their structural models under the assumption that the enzymes utilize the ping-pong mechanism. Swain \textit{et al.} (13), in reporting the crystal structure of the \(E.\ coli\) type II \(\text{L}\)-asparaginase in complex with the product Asp, surmised that Thr\(^{89}\) is the most likely nucleophile that would form the covalent acyl intermediate. That same year, Miller \textit{et al.} (12) made a similar conclusion regarding the catalytic mechanism, from their analogous Asp complex structure with the \textit{Erwinia} \(\text{L}\)-asparaginase but left doubt regarding whether it was Thr\(^{15}\) (the other conserved threonine) or Thr\(^{89}\) that acts as the nucleophile. For clarity, Table 1 presents the corresponding residue numbers of select conserved active site residues from the two Food and Drug Administration-approved \(\text{L}\)-asparaginases (\(E.\ coli\) and \textit{Erwinia}) and the guinea pig \(\text{L}\)-asparaginase, which is the focus of this study.

The single piece of evidence repeatedly used as the strongest indicator for the ping-pong mechanism for this \(\text{L}\)-asparaginase family comes from a structural study of the T89V mutant of the \(E.\ coli\) type II enzyme (14). Diffraction data collected from a crystal of the T89V mutant grown in the presence of Asp indicated that a covalent bond had indeed formed between the Asp side chain and Thr\(^{12}\). Here, it is important to note that the authors grew the crystals in 100 mM Asp at pH 5.0 over a period of many days. Hence, one cannot entirely discount the possibility that the observed covalent intermediate is an artifact of the crystallization conditions or the fact that the enzyme contained the T89V mutation.

Based on the totality of the literature available at the onset of our studies, we expected that the guinea pig enzyme (gpASNase1) employs the ping-pong mechanism. Our interest in understanding the mechanism of this enzyme stemmed from our goal of designing variants with improved activity. The design criteria for an enzyme that could potentially replace the bacterial \(\text{L}\)-asparaginases in the clinic are low micromolar \(K_m\) value concomitant with high turnover rate (these two criteria will assure complete and rapid depletion of blood Asn levels). Previously we identified the guinea pig \(\text{L}\)-asparaginase that has the required low \(K_m\) property, albeit in the upper range of the desired value, and reported its crystal structure with and without the product Asp (5). A more clinically relevant variant would have even a lower \(K_m\) and an increased catalytic rate.

To guide the design of such a variant, we proceeded to explore the enzymatic mechanism of gpASNase1 and were surprised by recent work of the Ramos group that questioned the long accepted ping-pong mechanism for this \(\text{L}\)-asparaginase family. Using computational methods to study the \(E.\ coli\) type II mechanism, these authors discovered that the energetics are not consistent with the assumed covalent intermediate mechanism but rather with a direct displacement mechanism (15). To shed light on this controversy, we conducted kinetic, mutagenesis, and structural studies on gpASNase1.

Altogether, our work, despite considerable effort, finds no support for the existence of a covalent intermediate. In fact, all of our data are consistent with the direct displacement mechanism, where a water molecule directly attacks the substrate Asn and the active site threonines play a role in transition state stabilization and protonation of the amino leaving group. Additionally, we discovered that the residue with the largest impact on the enzymatic rate is not either of the conserved threonines but rather a conserved active site lysine residue. The likely functions of this lysine residue, in conjunction with an aspartic acid residue, is to activate the water for nucleophilic attack on the substrate and to participate in a proton shuttle that donates a proton to the leaving amino group. In summary, the data presented herein provides experimental results that support a direct displacement mechanism for type I/II \(\text{L}\)-asparaginases.

### Experimental Procedures

**Cloning, Expression, and Purification of Guinea Pig \(\text{L}\)-Asparaginase**—The sequence and cloning of the full-length wild type \textit{Cavia porcellus} (guinea pig) \(\text{L}\)-asparaginase UniProt entry CGC-3 (now Agilent 200523), where the wild type His\(_6\)-TEV-gpASNase1(1–565)-pET14b plasmid acted as the template. For the T19A/T116A double mutant, T19A site-directed mutagenesis was performed using the T116A mutant as the DNA template. Primers for the reaction were designed using the online primer design tool from Agilent for QuikChange II with sequences as follows: T19A-fw (5’-CTACACGGGGTGGCCGCTGGTATGCA-3’), T19A-rv (5’-TGATACCCACGGCGCCACCGGTTAG-3’), T116A-fw (5’-TGTGGTTATCCAGCCGCGGA-TACGATGGCTTCAG-3’), T116A-rv (5’-CTGAAGCCATCGCCGATACGTCGTAACACCAACA-3’), K188M-fw (5’-GCAATTCCGTCACGATGTTGATGACCGA-3’), K188M-rv (5’-CTGGGATACCGCCGATTGCCTG-3’), Y308F-fw (5’-GCGTTACCCTGGGTGTTTGCACGACG-3’), and Y308F-rv (5’-GCTCGTGCCAAACCCTGGGTTA-CGC-3’). Expression and purification of the wild type and mutants followed the same protocol as described by Schalk \textit{et al.} (5) with the following alterations: the buffers for the nickel-Sepharose His-Trap column included 200 mM KCl or 200–500 mM NaCl, and the second wash buffer included 60 mM imidazole to wash off nonspecific proteins. The protein was eluted from the column using buffer containing 300–500 mM imidazole. The buffers used for the nickel-Sepharose His-Trap column had a pH between 7.5 and 8.5. Additionally, initial purifications of the mutants using gel filtration were conducted using 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM DTT and then dialyzed into PBS for subsequent NMR studies. For later purifications, buffer exchange into PBS was carried out during gel filtration. Two versions of PBS were used with varying phosphate or salt concentrations to stabilize the T116A mutant, which would precipitate during dialysis and after several
freeze-thaw cycles. PBS-490 consisted of 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 490 mM NaCl, 10 mM KCl, pH 7.5, and PBS-250 consisted of 20 mM Na₂HPO₄, 5 mM KH₂PO₄, 250 mM NaCl, 50 mM KCl, pH 7.5. After purification, wild type and mutant His₆-TEV-gpASNase1 (1–565) underwent three or four rounds of dialysis against 1 liter of fresh buffer at room temperature, each round typically lasting an hour. A centrifugal concentrator equilibrated three times with fresh buffer was used to concentrate the protein to ~20–30 mg/ml, after which it was flash frozen in liquid nitrogen and stored at ~80 °C.

**Kinetic Assay Methods**—The catalytic activities of the wild type and mutant l-asparaginases were determined using two continuous spectroscopic enzyme-coupled assays. These assays measured the production of the end products of the asparaginase reaction (aspartate and ammonia) through the 1:1 oxidation of reduced NADH to NAD⁺, which is observed spectroscopically as a decrease in absorbance at 340 nm. For the \( k_{\text{obs}} \) rate measurements, we observed the appearance of asparate using 5 units of glutamic-oxaloacetic transaminase (Sigma G2751) and 7 units of malic dehydrogenase (Sigma M2634) as helper enzymes (16, 17). The kinetic assay buffer was comprised of 25 mM Tris, pH 8.0, 300 mM NaCl, 400 \( \mu \)M \( \alpha \)-ketoglutarate, 200 \( \mu \)M NADH. A stock solution of Asn (Sigma-Aldrich A93003) in 25–50 mM Tris, pH 7.5–8.0, was made fresh and used to trigger the reaction. The \( k_{\text{obs}} \) rates were measured at 37 °C at a constant Asn concentration of 1 mM. The final pH of the reaction at 37 °C was ~7.5. Concentrations of the WT and mutant enzymes varied between 0.002 and 50 \( \mu \)M, depending on the enzyme. Concentrations were selected at which the rate and enzyme concentration were linear. All measurements were taken in triplicate. In the blank reaction, buffer was added instead of Asn.

In the burst kinetic assays, 16 units of the helper enzyme glutamic dehydrogenase (Sigma G2751) were used to measure the production of ammonium, the other end product resulting from the hydrolysis of asparagine (18). Experiments were conducted at room temperature, and a final concentration of 150 mM Na₂HPO₄, pH 5.5, was added to the reaction mixture to promote acidification but without causing precipitation of the protein. The low pH was chosen to slow the reaction, and the true pH, as indicated by pH strips, was between pH 6.0 and 6.5.

**Crystallization, Data Collection, and Structure Solution**—Crystals were grown in hanging drops via the vapor diffusion method at 20 °C. The reservoir solution was comprised of 0.1 M HEPES, pH 7.0, and 12–15% PEG 4000. 1 \( \mu \)l of T116A (2.5–5 mg/ml) or T19A (5–12.5 mg/ml) was combined with 1 \( \mu \)l of reservoir solution. 1.25 \( \mu \)l of K188M (50 mg/ml) was combined with 1 \( \mu \)l of reservoir solution. Crystals took from 2 weeks to more than a month to appear. For cryoprotection of the crystals, 27–30% ethylene glycol was added to the reservoir solution. The crystals were soaked in cryoprotectant containing 5 mM Asn (Sigma-Aldrich A93003) for 1–10 min before being flash frozen in liquid nitrogen.

Diffraction data for the T19A, T116A, and K188M complexes with Asn were collected at the Advanced Photon Source at Argonne National Laboratory on the LS-CAT ID-G Beamline (see Table 2 for data collection and refinement statistics). The data were processed using XDS (19). The waters and Asp molecules were removed from the gpASNase1 model (Protein Data Bank code 4R8L) used for the initial refinement by Refmac5 (20). Negative electron density from the \( F_o - F_c \) difference map was observed on the methyl and hydroxyl side chains of Thr¹⁹ or Thr¹¹⁶ that had been mutated to alanine, confirming the presence of an alanine at those positions. Coot (21) was used to build the models, and MacPyMOL (PyMOL Molecular Graphics System, version 1.4; Schroedinger) was used to generate the figures of the structures.

**NMR Methods**—All NMR experiments were performed on a Bruker 900 MHz instrument equipped with a cryogenic triple resonance probe. One-dimensional \(^1\)H NMR measurements were taken at different time points to follow the progress of the asparaginase-catalyzed reaction through measuring the relative amounts of substrate and product. The number of scans in these experiments was set to 64, corresponding to a total acquisition time of 4 min. In all of the reactions 10 mM phosphate buffer, pH 7.4, with 500 mM NaCl in 10% D₂O as used. The sample volume was 200 \( \mu \)l, and 3-mm NMR tubes were used. For the determination of the ability of the WT and mutant (T19A, T116A, K188M, and T19A/T116A) l-asparaginase variants to convert asparagine into aspartate, we used 5 \( \mu \)M enzyme and 50 \( \mu \)M substrate (Asn) and followed the generation of product over time. To test whether the covalent intermediate is formed during the reaction with mutant l-asparaginase variants (T19A, T116A, and K188M), we used equimolar amounts of asparagine and of the enzymes (50 \( \mu \)M). 1 \( \mu \)M WT l-asparaginase was added after the 5 min preincubation with all three mutants to cleave all of the residual, noncovalently bound substrate to form product. For quantification of the amount of aspartate generated, we used the resonances at 2.73, 2.71, 2.61, and 2.59 ppm, all corresponding to two methylene (\( \beta \)) hydrogens of Asp. The data for each individual reaction (all mutants with and without the WT added) were normalized using the NMR spectra acquired at the end of that respective reaction (after 2 h), where essentially all substrate was converted into product (100% Asp).

**Results and Discussion**

**Structural Analysis of gpASNase1 Active Site Mutants Fails to Reveal a Covalent Intermediate**—The single most compelling piece of evidence for the presence of a covalent intermediate in the enzymatic mechanism of type I/II l-asparaginases comes from the aforementioned *E. coli* type II T89V mutant crystal structure obtained from crystals that grew in the presence of the product, Asp, at 100 mM and pH 5.0. In that structure, a covalent bond was observed between Thr¹⁹ and what was the Asp (14) (Fig. 2A). The authors explain the trapping of this covalent intermediate by a deficiency in the deacylation step of the reaction caused by the substitution of Thr⁸⁹ with a valine (14). We reasoned that a more appropriate way to trap such an intermediate is to soak the substrate Asn at physiological pH into a preformed crystal of such a threonine mutant. Therefore, we generated the T116A mutant of gpASNase1 (Thr¹¹⁶ is equivalent to Thr⁸⁹ of the *E. coli* enzyme; Table 1) and crystallized it. The crystals were subsequently transferred to a solution that contained 5 mM Asn, and after a short incubation the crystals were frozen in liquid nitrogen, and diffraction data were col-
FIGURE 2. Observing the substrate Asn bound (but not the covalent intermediate) in our gpASNase1 mutant crystal structures. A, included for comparison is the electron density observed in the E. coli T89V structure (orange; Protein Data Bank code 4ECA). The arrow points to the contiguous density between what was Thr$^{116}$ and Asp, indicating the formation of a covalent intermediate. B, gpASNase1 T116A structure (light blue; Protein Data Bank code 5DND). C, gpASNase1 T19A structure (pink; Protein Data Bank code 5DNC). D, gpASNase1 K188M structure (green; Protein Data Bank code 5DNE). All electron density maps in this figure are $2F_{o} - F_{c}$ maps contoured at 1.5 $\sigma$.

TABLE 1
Numbering of select L-asparaginase active site residues

| E. coli type II residue | Erwinia chrysanthemi type II residue | Guinea pig type I residue |
|------------------------|-------------------------------------|--------------------------|
| Thr$^{126}$             | Thr$^{126}$                         | Thr$^{19}$               |
| Tyr$^{25}$              | Tyr$^{25}$                          | Tyr$^{25}$               |
| Thr$^{116}$             | Thr$^{116}$                         | Thr$^{116}$              |
| Asp$^{90}$              | Asp$^{90}$                          | Asp$^{17}$               |
| Lys$^{162}$             | Lys$^{162}$                         | Lys$^{162}$              |

selected. All data collection and refinement statistics for the structures discussed here are presented in Table 2. Upon inspection of the electron density, it was clear that the substrate Asn did indeed bind at the active site (Fig. 2B). However, it did not form a covalent bond with Thr$^{19}$. In total, we collected 10 data sets on gpASNase1 T116A crystals that were incubated with Asn for a total of at least 10 min. Inspection of the electron densities from all of these crystals failed to reveal a covalent intermediate. For all data sets, we could observe clear electron density for the bound Asn. The one discussed below is a 2.3 Å resolution data set collected on a T116A crystal that was soaked for 5 min in 5 mM Asn.

Because the T116A mutant has residual activity (the kinetic analysis of the various mutants is discussed later), we first had to decide whether the observed electron density represents the substrate Asn versus that of the product Asp. Because these two amino acids are isosteric, the shape of the electron density cannot distinguish between them. We interpret the electron density as representing the substrate Asn based on two considerations: First, because in solution at 37 °C, each turnover for this source of uncertainty by having both threonines present, the presence of the mutation. We reasoned that we could solve this source of uncertainty by having both threonines present and slowing the reaction (to allow for trapping of the covalent intermediate) by some other active site mutation. Lys$^{162}$ is a conserved active site residue in this family of L-asparaginases, and its direct interaction with Thr$^{116}$ (the threonine speculated to be involved in the deacylation step) suggested that its mutation would serve to slow the reaction, and more specifically, the deacylation step (if such occurs). To test this prediction, we generated the K188M variant. We chose to substitute the lysine with a methionine because these amino acids are most similar in shape, but the methionine would not be able to directly interact with Thr$^{116}$. Indeed, kinetic analysis of this mutant shows that it is even slower than either of the threonine mutants discussed above (see below).

Gesto et al. (15) suggested that the covalent intermediate observed in the E. coli T89V structure is an artifact because of the presence of the mutation. We reasoned that we could solve this source of uncertainty by having both threonines present and slowing the reaction (to allow for trapping of the covalent intermediate) by some other active site mutation. Lys$^{162}$ is a conserved active site residue in this family of L-asparaginases, and its direct interaction with Thr$^{116}$ (the threonine speculated to be involved in the deacylation step) suggested that its mutation would serve to slow the reaction, and more specifically, the deacylation step (if such occurs). To test this prediction, we generated the K188M variant. We chose to substitute the lysine with a methionine because these amino acids are most similar in shape, but the methionine would not be able to directly interact with Thr$^{116}$. Indeed, kinetic analysis of this mutant shows that it is even slower than either of the threonine mutants discussed above (see below).

We crystallized the gpASNase1 K188M mutant, transferred a crystal into an Asn-containing solution, and after a short soak froze the crystal and collected diffraction data. Yet again, we failed to observe a covalent intermediate, but rather the substrate Asn (Fig. 2D). In sum, although these structural results do not refute the possibility of a covalent intermediate being formed in the gpASNase1 catalyzed hydrolysis of Asn, despite
Our results do question the existence of such a state.

Very Similar Positioning of Active Site Residues in the Structures of gpASNase1 Mutants in Complex with Asn and That of the Wild Type Enzyme in Complex with Asp—An overlay of the three mutant structures presented here (T19A, T116A, and K188M) with the previously reported wild type structure in complex with Asp (5) reveals a nearly identical positioning of active site residues and of the substrate Asn and the product Asp (Fig. 3). The increase in distance between the carbonyl of Ala142 and the side chain carboxylate of Asp, relative to the distance to the side chain amino group of Asn, was discussed earlier (Fig. 3, arrow 1). Another notable difference is the altered conformation of Lys188 in the T116A mutant (Fig. 3, arrow 2). Because the T116A mutation abolishes the direct interaction between what was Thr116 and Lys188, the ε-amino group of Lys188 loses its anchor. In sum, this analysis shows that the mutations have not significantly perturbed the positioning of the substrate or of the active site architecture. Hence, any difference in the kinetic properties of these mutants is not due to a global structural change but rather to the specific effect of the mutation.

Conserved Positioning of Active Site Water Molecules—The active site of gpASNase1 contains several water molecules. Two of them seem to be conserved between the wild type and mutant gpASNase1 structures and are labeled as W1 and W2 in Fig. 4. We gave particular attention to the active site water molecules, especially those positioned near Asn, because both the direct and double displacement mechanisms (Fig. 1) call for a nucleophilic water that either initiates the hydrolysis reaction or the breakdown of the covalent intermediate, respectively. Although water W1 is closest to the side chain of Asn, its relative position makes it unlikely to be the putative catalytic water. Moreover, with no obvious base nearby, it is hard to imagine significant efforts no covalent intermediate was observed, our results do question the existence of such a state.

Table 2: Data collection and refinement statistics

| Structure | T19A | T116A | K188M |
|-----------|------|-------|-------|
| Protein Data Bank codes | 5DNC | 5DN | 5DNE |
| Data collection statistics | | | |
| X-ray source and detector | LS-CAT ID-G | LS-CAT ID-G | LS-CAT ID-G |
| MARCCD 300 | MARCCD 300 | MARCCD 300 |
| Wavelength (Å) | 0.97857 | 0.97857 | 0.97857 |
| Temperature (K) | 100 | 100 | 100 |
| Resolution (Å) | 2.00 (2.30-2.00) | 2.29 (2.43-2.29) | 2.39 (2.53-2.39) |
| Number of reflections | 775965 (197531) | 347756 (52169) | 184521 (27932) |
| Unique | 97123 (30319) | 66847 (10295) | 57320 (8961) |
| Completeness (%) | 95.9 (88.1) | 98.5 (95.2) | 95.5 (93.8) |
| Rsym (%) | 8.9 (62.1) | 11.9 (75.0) | 11.9 (72.3) |
| Average I/σ(I) | 16.06 (3.54) | 13.38 (2.33) | 9.1 (1.67) |
| Space group | I222 | I222 | I222 |
| Wilson B-factors (Å²) | 32.3 | 32.3 | 32.3 |
| Refinement statistics | | | |
| Refinement program | REFMAC5 | REFMAC5 | REFMAC5 |
| Rcryst (%) | 18.7 | 20.8 | 19.4 |
| Rfree (%) | 21.2 | 24.2 | 23.7 |
| Resolution range (Å) | 30–2.0 | 30–2.3 | 30–2.4 |
| Protein molecules per a.u. | 4 | 4 | 4 |
| Number of atoms | | | |
| Protein | | | |
| ProtA | 2708 | 2700 | 2716 |
| ProtB | 2729 | 2715 | 2722 |
| ProtC | 2719 | 2719 | 2709 |
| ProtD | 2720 | 2726 | 2721 |
| Water molecules | 513 | 399 | 320 |
| Ethylene glycol | 17 | 4 | 9 |
| Asn | 4 | 4 | 4 |
| Root mean square deviation from ideal | 0.010 | 0.010 | 0.010 |
| Bond length (Å) | 1.329 | 1.320 | 1.319 |
| Average B-factors (Å²) | | | |
| Protein | | | |
| ProtA | 36.3 | 37.2 | 38.9 |
| ProtB | 39.0 | 37.9 | 41.1 |
| ProtC | 36.9 | 36.9 | 39.5 |
| ProtD | 40.8 | 40.3 | 42.3 |
| Water molecules | 38.9 | 30.5 | 31.7 |
| Ethylene glycol | 44.1 | 44.0 | 43.4 |
| Asn molecules | | | |
| ProtA, ProtB | 28.9, 27.3 | 28.5, 34.3 | 27.9, 32.6 |
| ProtC, ProtD | 27.3, 26.6 | 33.1, 29.1 | 33.9, 28.3 |
| Ramachandran plot (%) | | | |
| Most favored regions | 98.6 | 98.0 | 98.4 |
| Additionally allowed regions | 1.4 | 2.0 | 1.6 |
| Outlier regions | 0.0 | 0.0 | 0.0 |

*High resolution shell in parentheses.

The table provides a detailed comparison of the data collection and refinement statistics for the three structures of gpASNase1 mutants in complex with Asn, with those for the wild type enzyme in complex with Asp. The data include information on the X-ray source and detector, wavelength, temperature, resolution, number of reflections, completeness, and other relevant parameters. The refinement statistics such as Rcryst, Rfree, resolution range, protein molecules per asymmetric unit, number of atoms in the protein and water molecules, root mean square deviations, and average B-factors are also provided. The table highlights the consistency and quality of the data, indicating that the structures were refined with high accuracy and precision.
how water W1 would be activated, and hence we assign this water molecule a structural role.

In contrast, water W2 is only 2.6 Å from the side chain of Asp\(^{117}\). Although at the observed position of W2 in our structures, it is too far to attack the Asn side chain (\(-4.5\) Å), one can envision that once activated by Asp\(^{117}\), the side chain of Lys\(^{188}\) guides its path to the substrate. A schematic of the interaction that would be made by Asn and gpASNase1, generated by inspecting all of our structures, is presented in Fig. 4B. This shows that water W2, in addition to its short distance to Asp\(^{117}\), makes three additional interactions with nearby residues. Hence, this water molecule seems to be in the appropriate environment for activation.

Residual Enzymatic Activity in gpASNase1 Mutants Suggests against a Covalent Intermediate—The hydrolysis of the Asn side chain as catalyzed by L-asparaginases has been compared with the hydrolysis of peptide bonds by serine proteases (3, 6, 9, 10, 12, 24–26). In such proteases, the side chain of the catalytic serine does indeed form a covalent intermediate with its peptide substrate. Notably, the mutation of the acyl intermediate forming serine dramatically reduces the activity of serine proteases, making the enzyme virtually dead. For example, mutation of this serine to an alanine in serpins results in a protease totally devoid of activity, allowing the determination of the crystal structure of the complex between the inactive mutant serpin and its protein substrate (27, 28). In such proteases, the side chain as catalyzed by L-asparaginases has been compared with the hydrolysis of peptide bonds by serine proteases (3, 6, 9, 10, 12, 24–26).

Residual Enzymatic Activity in gpASNase1 Mutants Suggests against a Covalent Intermediate—The hydrolysis of the Asn side chain as catalyzed by L-asparaginases has been compared with the hydrolysis of peptide bonds by serine proteases (3, 6, 9, 10, 12, 24–26).

An additional structurally conserved active site residue that we explored by mutagenesis is Tyr\(^{308}\) (prime denotes a residue from a neighboring protomer). In gpASNase1, this tyrosine is in proximity (\(-2.9\) Å) to the hydroxyl of Thr\(^{116}\) (Fig. 4B). In the bacterial homologs, the phenolic moiety is brought to a similar position from an N-terminal residue (Table 1) and always interacts with the conserved threonine (Thr\(^{12}\) in E. coli) that was thought to form the covalent intermediate. Hence, a putative role given for this tyrosine is to activate Thr\(^{12}\) so that it could build the covalent intermediate (14). However, upon the mutation of this residue to a phenylalanine, we observe less than 100-fold reduction in rate. This is in line with the observations made with the E. coli enzyme where the mutant Y25F results in an enzyme with \(-1,800\)-fold reduced asparaginase rate (30).

Lastly, type I/II L-asparaginases contain a conserved active site lysine, which has been suggested to act as a base in the rate-limiting step (14). This lysine directly interacts with Thr\(^{116}\) and Asp\(^{117}\). In fact, these three residues have been referred to as the “asparaginase triad” (14). To probe the function of this lysine in gpASNase1 function, we mutated it to a methionine. Surprisingly, the K188M mutant is the slowest variant from all that we tested, being nearly 30,000-fold slower than the wild type enzyme. Notably, the K188M mutant is 60% slower than the T19A mutant, the putative covalent-forming threonine. Because the effect on rate is not due to some structural reason (the K188M structure overlays with a root mean square deviation of 0.42 Å on the wild type structure), we can postulate a critical mechanism-related role for this lysine.

In addition, our results that reveal significant residual activity for the T19A mutant are consistent with experiments performed on the E. coli type II L-asparaginase, where mutation of the presumed covalent intermediate-forming threonine reduced the rate by only 15,000-fold (10). Together, these mutagenesis experiments, by revealing L-asparaginases that have higher than expected residual activity upon mutation of their active site threonine to alanine, question the long held assumption of a ping-pong mechanism.

Lack of Burst Kinetics Indicates the Absence of a Covalent Intermediate—Enzymes that utilize the ping-pong mechanism often show “burst” kinetics. For example, the hydrolysis of p-nitrophenyl acetate by the serine protease chymotrypsin shows a biphasic reaction rate, with an initial pre-steady-state burst that correlates with the concentration of enzyme (31). Because this

FIGURE 3. No large conformational shifts are present at the active site resulting from the T19A, T116A, or K188M mutations. Arrow 1, in the WT structure, the bound product Asp is shifted away from the carbonyl of Ala\(^{142}\) when compared with the bound substrate Asn in the mutant structures. Arrow 2, the ε-amino group of Lys\(^{188}\) turns away from the substrate after its interaction with Thr\(^{116}\) is lost in the T116A mutant.
reaction can be monitored by the appearance of \( p \)-nitrophenol, the observed burst is explained by the rapid formation of the covalent intermediate with the concomitant release of \( p \)-nitrophenol. After the burst, the reaction proceeds at a slower steady-state rate because any further turnover requires the hydrolysis of the covalent intermediate prior to binding of another substrate molecule (i.e. the slower linear appearance of product is determined by the rate-limiting hydrolysis of the covalent acyl intermediate). We reasoned that if gpASNase1 employs the ping-pong mechanism (Fig. 1C), a burst of ammonia should be detectable. Additionally, because we can assay the \( L \)-asparaginase reaction by independently monitoring the rate of ammonia production and the rate of aspartate formation, a ping-pong mechanism would show a lag in the appearance of aspartate (which is formed only after the hydrolysis of the covalent intermediate) relative to the appear-
The fast hydrolysis rate of wild type gpASNase1 (k_{cat} of 58 s^{-1}; k_{obs} at 50 μM of ~10 s^{-1}) precludes the detection of a burst, if it exists, using our enzymatic steady-state assay for ammonia formation. In addition to reducing the assay temperature from 37 °C to room temperature to slow down the reaction rate, we also used the much slower T116A mutant for this experiment. We chose this particular mutant because it is homologous to the E. coli T89V mutant that was shown to form a covalent intermediate with Thr12 (Thr19 in gpASNase1). Hence, the putative covalent intermediate forming threonine Thr19 is present in this clone, and the slow rate caused by the T116A mutation (each turnover takes several minutes) should allow us to detect the burst of ammonia production, if such a burst exists. Moreover, because the magnitude of a burst would be proportional to the amount of enzyme (the burst results from the initial single-turnover), we used a very high concentration of the T116A mutant enzyme (50 μM) with either equimolar or excess amount of the substrate Asn (50 or 250 μM). A single turnover of 50 μM enzyme would result in very noticeable change in the optical density in our assays that monitor the consumption of NADH (see “Experimental Procedures” for details). In addition to trying to observe the burst kinetics by using the assay for ammonia, we concomitantly used the assay that detects aspartate, the other product of the reaction. The rationale for using both assays to follow the reactions that are triggered at the same time is that the aspartate detecting assay should show a lag relative to the ammonia detecting assay, if Asp is only formed upon breakdown of the covalent intermediate. However, we could not detect a burst of ammonia production, and the rate of ammonia formation seemed to be identical to the rate of aspartate formation (Fig. 5).

These results are not consistent with the expected behavior of an enzyme using the ping-pong mechanism. Rather, the data are consistent with an enzyme that employs a direct displacement mechanism. Of note, for an enzyme using the ping-pong mechanism, burst kinetics are only expected if the rate-limiting step is the breakdown of the covalent intermediate. If the rate-limiting step is prior to the formation of the covalent intermediate, these experiments would not be able to distinguish between an enzyme that uses the ping-pong or direct displacement mechanism.

**Monitoring of the gpASNase1 Reaction by NMR Suggests a Direct Displacement Mechanism**—Having failed to obtain evidence for a covalent intermediate from our structural and kinetic studies, we decided to monitor the progression of the reaction by NMR. In all the NMR experiments discussed below, we are only following the resonances from the substrate Asn and product Asp. The advantages of NMR are that it is relatively sensitive and the NMR line widths can provide information about the binding rate and the presence of a covalent intermediate. For example, a covalent intermediate, if formed, would be expected to exhibit the relatively large line widths of the enzyme, and under the experimental conditions used, the substrate resonances would disappear. In contrast, the resonances of a substrate/product that are in rapid equilibrium with the

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**FIGURE 5.** No burst is detected in the hydrolysis of Asn by the T116A gpASNase1 mutant. Experiments were conducted at room temperature at a pH of ~6.0–6.5 and followed by measuring the amount of Asp (red line) or NH₄⁺ (blue line) produced using two continuous spectroscopic enzyme-coupled reactions (see “Experimental Procedures” for details). The substrate Asn was added to 50 μM T116A at a ratio of 1:1 (A) or an excess of Asn at 5:1 (B). No sudden burst of ammonium was observed that would indicate the presence of a covalent intermediate.
enzyme bound and free states (so called fast exchange) would be clearly visible.

First, we used NMR to confirm the residual activity of the mutants. These experiments were performed by adding 5 μM enzyme to 50 μM Asn, with the first spectra being acquired after ~5 min. We monitored the progression of the reaction by collecting spectra also at the 60- and 120-min time points. The disappearance of Asn and appearance of Asp resonances confirmed that all mutants (T19A, T116A, K188M, and T19A/T116A) are able to hydrolyze Asn into Asp (Fig. 6). The relative rate of appearance of the product Asp observed in the NMR experiments (T19A/T116A > T116A > T19A ≈ K188M) is consistent with the relative rates measured using our steady-state kinetic study (Table 3).

Second, we measured the spectra using equimolar concentrations of 50 μM of enzyme and amino acids. Because our goal was to detect the formation of a covalent intermediate, using NMR to investigate an equimolar condition could inform us whether the amino acid is bound to the enzyme or whether it is free in solution. Remember, if a covalent intermediate does form after adding the substrate Asn, the slow tumbling rate of the enzyme-covalent intermediate would make the resonances for the amino acid disappear. This type of experiment is not possible with the wild type enzyme because of its fast enzymatic rate (by the time the first spectra would be acquired, the reaction would have been completed). However, the slow rate of the mutants, especially the K188M mutant, for which each turnover at 37 °C takes about 8 min (we performed the NMR experiments at 25 °C to slow the reaction even further), means that our first spectra taken after a 5-min incubation should observe the state prior to the formation of the product Asp.

Before discussing the result of the K188M mutant with the substrate Asn, it is informative to analyze the spectra of this mutant in the presence of equimolar product, Asp. The spectra of 50 μM K188M mutant plus 50 μM Asp shows clear resonances for the Asp (Fig. 7A). In fact, the control spectra for 50 μM Asp by itself is very similar to the one taken in the presence of the K188M mutant (Fig. 7B). This means that the line-broadening effect on the Asp resonances in the presence of the K188M mutant is not very strong. Put differently, this implies that the product Asp is at fast exchange with the enzyme (i.e. Asp undergoes fast equilibrium between the enzyme-bound and free states). In contrast, the spectra of 50 μM K188M mutant plus 50 μM Asn taken after a 5-min incubation shows very little signal for either Asn or Asp (Fig. 7C). In other words, the resonances for Asn have mostly disappeared, but very weak resonances for the product Asp have appeared in their place (signal for Asp is ~10% of the expected for full conversion of 50 μM Asn to Asp). The previous experiment demonstrated the Asp is at fast exchange with the enzyme (Fig. 7A), so any product formed would be readily and quantitatively observed. Therefore, the lack of Asn resonances implies that either Asn is at intermediate exchange with the enzyme or that it formed the covalent intermediate.

Admittedly, we experienced initial excitement with this result because it is consistent with the formation of the covalent intermediate. To ascertain whether we indeed managed to observe the elusive covalent intermediate, or alternatively, the amino acid Asn is simply bound noncovalently to the enzyme, after the 5-min incubation of equimolar K188M mutant and Asn (both at 50 μM), we added 1 μM wild type enzyme. The rationale for this experimental design is as follows: if indeed a covalent intermediate has formed, then the rate of appearance of the product Asp would be unchanged by the presence of the wild type enzyme. If, on the other hand, Asn has merely formed a noncovalent complex that undergoes intermediate exchange with the free state, the presence of even a small amount of the fast wild type enzyme would result in the rapid conversion of any K188M-released Asn into the product Asp. Fig. 7D shows the results of this experiment; the black curve is the time course for appearance of Asp for the condition without the wild type enzyme. It shows that it takes >40 min for the K188M mutant

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**FIGURE 6.** NMR confirms residual activity of gpASNase1 mutants. One-dimensional NMR spectra of the region used to detect Asn and Asp in the presence of mutant enzymes after 5, 60, and 120 min. Experimental conditions were 5 μM enzyme ± 50 μM Asn or Asp.
to catalyze the hydrolysis of the Asn to Asp. In contrast, and shown in the red curve, addition of wild type enzyme at the 5-min mark (we waited this time to allow for formation of the covalent intermediate), shows that by the time the next spectra at the 10-min mark was acquired, all of the Asn has been hydrolyzed to Asp. This result suggests that the lack of observable signal for Asn at the 5-min mark (i.e. Asn is present in the NMR tube but not observable by NMR) is due to a noncovalent complex between Asn and the K188M mutant enzyme and that this complex undergoes slow equilibrium with the unbound state. Once the amino acid Asn dissociates from the mutant enzyme, it can either rebind or, alternatively, bind to the added wild type enzyme. The latter event causes rapid hydrolysis of the amino acid. Despite a ratio of 1:50 between the wild type and K188M enzyme, 5 min were sufficient to fully hydrolyze Asn into Asp.

This type of experiment (50 μM of enzyme plus 50 μM of Asn, followed at the 5-min mark with the addition of 1 μM wild type gpASNase1) was repeated for the T19A and T116A mutants (Fig. 7, E and F). In agreement with the observation made with the K188M mutant, adding wild type enzyme resulted in full conversion of Asn to Asp. If in any of these experiments a covalent intermediate had formed, the addition of wild type enzyme would have had no effect. The fact that the converse is observed suggests that for none of the mutants tested did a covalent intermediate form.

**Proposed Direct Displacement Mechanism for gpASNase1**—Our inability to prove the existence of a covalent intermediate, together with the residual activity of the threonine mutants support the conclusion made by Gesto et al. (15) for the use of a direct displacement mechanism by type I/II L-asparaginases. Based on our crystal structures of the T19A, T116A, and K188M gpASNase1 mutants in complex with Asn, we prepared a schematic of the active site that represents the putative Michaelis complex (Fig. 4B). The residues that were probed by mutagenesis (Thr<sup>19</sup>, Thr<sup>116</sup>, Lys<sup>188</sup>, and Tyr<sup>308</sup>) are drawn in thick black lines, an additional critical residue in thick gray lines (Asp<sup>117</sup>), and those that have a predominantly substrate-binding role but that do not directly catalyze the reaction are drawn in thin black lines. Importantly, several structurally conserved water molecules are observed in the active site region. The question is, which is the water that attacks the substrate Asn (drawn in red), and how is that water activated?

Clues to answer this question come from our structures. In the structures, we observe two water molecules in the proximity of the substrate, labeled W1 and W2 in Fig. 4B. Although W1 is closest to the γ-amino group, its orientation makes it an unlikely candidate for being the water that attacks the γ-carbonyl group. We suggest that the nucleophilic water is W2. This water makes extensive interactions with the enzyme, the most prominent being with Asp<sup>117</sup>. We envision Asp<sup>117</sup> initiating the hydrolysis reaction by acting as a base and abstracting a proton from water W2, because that activated water molecule attacks the Asn side chain carbonyl (Fig. 8A). The ensuing negative charge of the tetrahedral transition state is then stabilized by the side chain of Thr<sup>19</sup> (Fig. 8B). Collapse of the transition state and the resulting release of the amino group are promoted by donation of a proton from Thr<sup>116</sup>. This threonine acts as the final member of a “triad” proton shuttle that starts with Asp<sup>117</sup> and proceeds via Lys<sup>188</sup> to Thr<sup>116</sup> (Fig. 8C). Hence, the protonated Asp<sup>117</sup> that results from the activation of the catalytic water molecule is the indirect source of the proton that is taken up by the amino leaving group, and its basic character is regenerated when this proton shuttle donates a proton to the amino group as it forms ammonia.

How does this direct displacement mechanism explain the kinetic properties of the mutants? The smallest effect on rate was observed with the Y308F mutant; in the proposed mechanism, this tyrosine plays only a secondary role in positioning Thr<sup>19</sup> so that is can stabilize the transition state. On the other hand, the very slow rate of the T19A mutant is due to its critical aforementioned transition state stabilization function. The less critical effect of the T116A mutant can be explained by a water
molecule that substitutes for the lack of the threonine hydroxyl group. The dramatic effect of the K188M mutant is rationalized by the proposed mechanism to result from the absence of two key functions of this lysine residue: first, to accept the proton from Asp^{117} after it activates the water and, second, to pass that proton along to Thr^{116}. Observing that the T19A/T116A double mutant shows slightly higher activity than each of the individual single mutants was unexpected. One possible explanation for the increase in rate of the double threonine mutant compared with the single threonine mutations is that the double mutant allows for improved access and better positioning of water molecules at the active site. The proposed roles of Thr^{19} (transition state stabilization) and Thr^{116} (protonation of the amino leaving group) are taken up by water molecules when these threonines are mutated to alanine. We speculate that perhaps the double mutant better accommodates water molecules, which act to replace the missing hydroxyl groups of the threonine side chains. The mechanism presented in Fig. 8 is based on the kinetic and crystallographic results of the gpASNase1 mutants. It is nearly identical to the mechanism proposed by Gesto et al. (15), which employed quantum mechanical calculations based from the E. coli type II structure. However, fine differences between the proposed mechanisms do exist. Whereas Gesto et al. assign the active site lysine as the residue that activates the water, we assign this reaction-initiating function to the nearby aspartic acid residue (Asp^{117} in gpASNase1), although the proton abstracted from the water by Asp^{117} is promptly passed on to Lys^{188}. We make this assignment because we observe water W2 to be directly interacting with Asp^{117}. In the mechanism by Gesto et al., a water molecule must move ~2 Å from its position seen in the x-ray structure of the E. coli enzyme. Common to both proposals is the transition state stabilizing role of Thr^{19} (Thr^{12} in the E. coli enzyme) and the proton-donating role of Thr^{116} (Thr^{89}) to the amino leaving group.

Conclusions—The experimental results presented here agree with computational work (15) and altogether suggest that type I/II l-asparaginases do not employ the ping-pong mechanism but rather use a direct displacement mechanism. The covalent intermediate seen in the T89V E. coli structure (14) is probably an artifact of the crystallization conditions that used very high Asp concentrations, low pH, and the long time course of the crystallization process. The uncompetitive behavior of hydroxylamine noted by Ehrman et al. (6), which is often used as an indicator for the ping-pong mechanism, can be explained by the conformational change that occurs to the N-terminal loop upon substrate binding. This conformational change results in the fully formed substrate-binding site and may be a necessary step prior to hydroxylamine binding. Such an ordered binding requirement (Asn prior to the inhibitor hydroxylamine) will also present an uncompetitive inhibition behavior pattern. This experimental support of the direct displacement mechanism can be used to inform the design of variants that have improved properties, such as lower Km and higher kcat, and such variants could be applied to both clinical and industrial uses.
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