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# Acylation is rate-limiting in glycosylasparaginase-catalyzed hydrolysis of $N^4$-(4′-substituted phenyl)-L-asparagines† ‡

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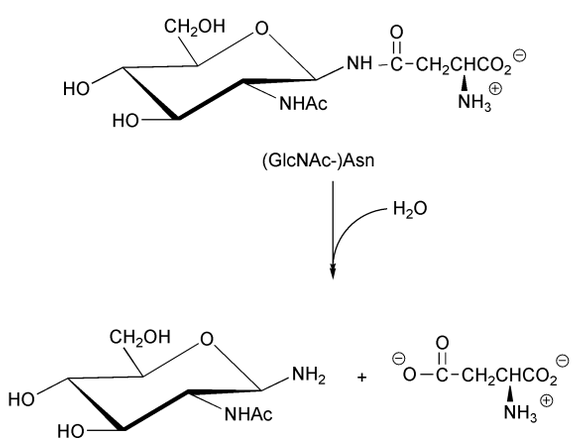
Glycosylasparaginase catalyzes the hydrolysis of the *N*-glycosylic bond between *N*-acetyl-D-glucosamine and L-asparagine in the catabolism of glycoproteins. The mechanism has been proposed to resemble that of serine proteases involving an acylation step where a nucleophilic attack by a catalytic Thr residue on the carbonyl carbon of the *N*-glycosylic bond gives rise to a covalent β-aspartyl–enzyme intermediate, and a deacylation step to give the final products. The question posed in this study was: Is the acylation step the rate-limiting step in the hydrolysis reaction as in serine proteases? To answer this question a series of mostly new substituted anilides was synthesized and characterized, and their hydrolysis reactions catalyzed by glycosylasparaginase from human amniotic fluid were studied. Five $N^4$-(4′-substituted phenyl)-L-asparagine compounds were synthesized and characterized: 4′-hydrogen, 4′-ethyl, 4′-bromo, 4′-nitro, and 4′-methoxy. Each of these anilides was a substrate for the enzyme. Hammett plots of the kinetic parameters showed that acylation is the rate-limiting step in the reaction and that upon binding the electron distribution of the substrate is perturbed toward the transition state. This is the first direct evidence that acylation is the rate-limiting step in the enzyme-catalyzed reaction. A Brønsted plot indicates a small, negative charge (−0.25) on the nitrogen atom of the leaving group anilines containing electron-withdrawing groups, and a small, positive charge (0.43) on the nitrogen atom of the leaving group anilines containing electron-donating groups. The free energy (incremental) change of binding ($\Delta\Delta G_b$) in the enzyme–substrate transition state complexes shows that substitution of a substituted phenyl group for the pyranosyl group in the natural substrate results in an overall loss of binding energy equivalent to a weak hydrogen bond, the magnitude of which is dependent on the substituent group. The data are consistent with a mechanism for glycosylasparaginase involving rapid formation of a tetrahedral structure upon substrate binding, and a rate-limiting breakdown of the tetrahedral structure to a covalent β-aspartyl–enzyme intermediate that is dependent on the electronic properties of the substituent group and on the degree of protonation of the leaving group in the transition state by a general acid.

## Introduction

The predominant *N*-glycosylic[1] bond in nature is between *N*-acetyl-D-glucosamine and asparagine.[2] In the catabolism of N-linked carbohydrate moieties to their constituent molecules, glycosylasparaginase (GA, aspartylglucosaminidase (AGA), $N^4$-(β-*N*-acetyl-D-glucosaminyl)-L-asparaginase; EC 3.5.1.26) catalyzes the hydrolysis of the *N*-glycosylic (amide) bond in β-*N*-acetylglucosaminyl-L-asparagine [(GlcNAc-)Asn] to give aspartic acid and 2-acetamido-2-deoxy-β-D-glucopyranosylamine (Scheme 1), which hydrolyzes non-enzymatically to *N*-acetyl-D-glucosamine (GlcNAc) and ammonium ion.[3] A deficiency, or absence, of GA activity gives rise to aspartylglycosaminuria (AGU), an autosomal recessive inherited lysosomal storage disorder that results in the accumulation of glycoasparagines, particularly (GlcNAc-)Asn, in lysosomes; AGU is the most common disorder of glycoprotein metabolism.[3] A few kinetic studies have been reported for the enzyme.[4] The crystal structures for human GA[5] and *Flavobacterium meningosepticum* GA[6,7] have been reported, and show that GA belongs to the superfamily of N-terminal nucleophile (Ntn) amidohydrolases.[8] Based on some of the kinetic studies and the crystal structure of human GA, a catalytic mechanism was proposed[5] that is analogous to the mechanism for serine proteases (Scheme 2): upon binding of (GlcNAc-)Asn, nucleophilic attack by an N-terminal Thr leads to release of 2-acetamido-2-deoxy-β-D-glucopyranosylamine with formation of a covalent β-aspartyl-threonine ester intermediate, which undergoes hydrolysis to give L-Asp and to regenerate the enzyme. The crystal structures for GA show that the active site is funnel-shaped with binding sites for the α-carboxyl and α-amino groups on Asn at the bottom of the funnel, and the catalytic Thr is located near the top of the funnel. As a result, there is quite a range of structures for the R-group at the $N^4$-nitrogen of Asn which are substrates for GA (Scheme 2): R can be H




**Scheme 1**

† Electronic supplementary information (ESI) available: Supplementary Figures 1–5. See http://www.rsc.org/suppdata/ob/b3/b301513k/

‡ *In memoriam.* Dedicated to the memory of Dr. Terry L. Kruger, Professor of Chemistry, Ball State University (Muncie, Indiana), whose amazing undergraduate organic chemistry lectures changed a career direction, and who was an important mentor in learning to do research (J. M. R.).

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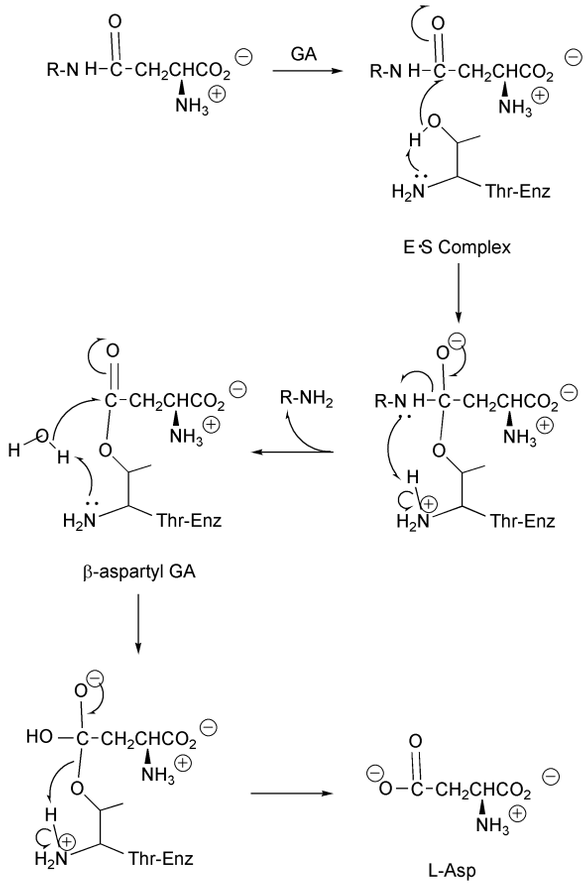


**Scheme 2**

(Asn is a substrate), various monosaccharides to oligosaccharides (high mannose, complex, hybrid structures), methylcoumarin, and various amino acids.[4] Therefore, there is generally little substrate specificity for the amide functional group by GA as long as the α-carboxyl and α-amino groups on Asn are not blocked.[4] In order to support the proposed catalytic mechanism, fundamental studies of the mechanism are required. Evidence thus far would indicate that, in the two-step reaction of acylation and deacylation, the acylation step is probably the rate-limiting step, but there is no direct evidence for this. In this paper we present the first direct evidence that the acylation step is the rate-limiting step in the hydrolysis reaction catalyzed by glycosylasparaginase.

## Results and discussion

### Synthesis of $N^4$-(4′-substituted phenyl)-L-asparagines

Surprisingly, very few $N^4$-(4′-substituted phenyl)-L-asparagines have been reported in the literature, and those that have been are incompletely characterized with reports only of melting points, IR data for one compound, and two optical rotation values for one compound. $N^4$-Phenyl-L-asparagine has been reported,[9–15] racemic mixtures were synthesized for $N^4$-phenyl-, $N^4$-(2′-methylphenyl)-, $N^4$-(3′-methylphenyl)-, $N^4$-(4′-methoxyphenyl)-, $N^4$-(2′-carboxyphenyl)-, and $N^4$-(4′-carboxyphenyl)-D,L-asparagine,[16] and $N^4$-(4′-nitrophenyl)-L-asparagine is commercially available,[17] although no literature citation to its synthesis and properties was found. [$N^2$-Cbz-$N^4$-phenyl-, $N^2$-Fmoc-$N^4$-phenyl-, and $N^2$-Boc-$N^4$-phenyl-L-asparagine and $N^2$-Cbz-$N^4$-(4′-methylphenyl)-L-asparagine have been reported.[18]] We synthesized $N^4$-phenyl-L-asparagine (**2**), $N^4$-(4′-ethylphenyl)-L-asparagine (**3**), $N^4$-(4′-bromophenyl)-L-asparagine (**4**), $N^4$-(4′-nitrophenyl)-L-asparagine (**5**), and $N^4$-(4′-methoxyphenyl)-L-asparagine (**6**); **3** and **4** are new compounds, **5** has only been reported commercially,[17] and **6** has been reported only as a racemic mixture.[16] The general procedure used to synthesize the five compounds was based on a published procedure[9,13] and is shown in Fig. 1. The α-amino group of L-aspartic acid was protected as the $N^2$-phthalyl derivative (Step 1), and the protected acid was converted to $N^2$-phthalyl-L-aspartic anhydride (**1**) (Step 2); reaction of the protected anhydride with the specific aniline (Step 3) and deprotection with aqueous hydrazine (Step 4) gave the product. The use of the phthalyl group as a protecting group has been reported to result in formation only of the $N^4$-asparagine,[9,13] as well as the $N^5$-glutamines,[19] presumably due to steric hindrance that precludes formation of the $N^1$-amides. No attempt was made to optimize yields for the reactions. The syntheses of **2**, **3** and **4** required 12 h, 9 h and 24 h, respectively, for Step 3. The properties of the compounds are consistent with the successful synthesis of each compound. The assignments of the NMR signals are based on the $^1$H and $^{13}$C NMR data for (GlcNAc-)-Asn,[20] the $^1$H NMR data for acetanilides,[21] and the $^{13}$C NMR data for acetanilides.[22] Our data are in general agreement with these data.

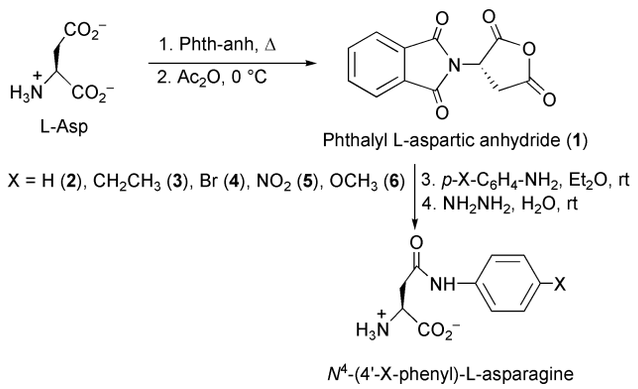


**Fig. 1** The synthesis of $N^4$-(4′-substituted phenyl)-L-asparagines was done in 4 steps. Reaction of L-Asp with phthalic anhydride to protect the α-amino group and reaction with acetic anhydride gave the phthalyl L-aspartic anhydride (**1**). Reaction of the latter with 4-substituted anilines and deprotection of the α-amino group gave the desired compounds, which were characterized.

Numerous attempts to synthesize **5** were not successful with no product detected. The electron-withdrawing nitro group significantly decreases the nucleophilicity of 4-nitroaniline. With an idea to increase the electrophilicity of the β-carbonyl group of the anhydride, and possibly increase the nucleophilicity of 4-nitroaniline, glacial acetic acid (11% v/v) was added to Step 3 in the general procedure. This addition resulted in the formation of **5** after reaction for 8 h. The properties of **5** are consistent with its successful synthesis. We did not pursue a study of the mechanism, but formation of 4′-nitroacetanilide was not detected, which would indicate that a mixed anhydride was not formed in the reaction.

When 4-methoxyaniline was used in Step 3, the product after deprotection with aqueous hydrazine was an approximately equal mixture of **6** and $N^1$-(4′-methoxyphenyl)-L-asparagine (**7**). This was evident from the $^1$H NMR and $^{13}$C NMR spectra (Supplementary Figure 1) where two sets of signals were observed. One set of signals was observed for each isomer in the $^1$H NMR spectrum, while two signals for C-1, C-3, and C-4 (in L-asparagine) were observed for the two isomers in the $^{13}$C NMR spectrum. This is the first observation that the phthalyl group as a protecting group for the α-amino group does not preclude formation of an $N^1$-amide. Presumably this arises from the electron-donating ability of the methoxy group that makes 4-methoxyaniline quite nucleophilic; the reaction required only 4 h, the shortest time for any of the anilines used in this study. However, the formation of the $N^1$-amide may be limited to $N^2$-phthalyl-L-aspartic anhydride because the

**Table 1** Kinetic parameters and relevant data

| Substrate | $k_{cat}/s^{-1}$ | $K_m/\mu M$ | $(k_{cat}/K_m)/\mu M^{-1}\ s^{-1}$ | $\sigma_p^{-}$ [a] | $pK_a$ [b] |
|---|---|---|---|---|---|
| (GlcNAc-)Asn | 5.0 (±0.9) | 215 (±27) | 0.023 | na | na |
| 4-(4′-MeOPh)-L-Asn | 2.0 (±0.2) | 1714 (±112) | 0.0012 | −0.26 | 5.31 |
| 4-(4′-EtPh)-L-Asn | 1.9 (±0.1) | 804 (±35) | 0.0023 | −0.19 | 5.11 |
| 4-(4′-HPh)-L-Asn | 1.2 (±$0.1_5$) | 463 (±46) | 0.0025 | 0.00 | 4.58 |
| 4-(4′-BrPh)-L-Asn | 1.7 (±0.1) | 295 (±15) | 0.0057 | 0.25 | 3.88 |
| 4-(4′-$NO_2$Ph)-L-Asn | 9.0 (±0.9) | 603 (±40) | 0.015 | 1.27 | 1.02 |

[a] Values from reference 25. [b] Values from reference 26.

formation of an $N^1$-amide from reaction of $N^2$-phthalyl-L-glutamic anhydride with 4-methoxyaniline was not reported.[19] Further studies with other anilines containing strongly electron-donating groups are necessary to understand this present result. Separation of the two isomers was achieved quite easily by taking advantage of the limited solubilities of each isomer in aqueous solution at their isoelectric points. The $N^4$-amide (**6**) has a lower isoelectric point (estimated as 5.5) than the $N^1$-amide (**7**) (estimated as 7.5). The difference between the isoelectric points was sufficiently large that the two isomers were separated by dissolving the mixture in aqueous solution at pH 11, and, upon acidification to pH 9, **7** precipitated. Upon further acidification to pH 3, **6** precipitated. The $^1$H NMR and $^{13}$C NMR spectra of **6** are shown in Supplementary Figure 2. The properties of **6** are consistent with its successful synthesis and separation from its isomer.

### Reaction of $N^4$-(4′-substituted phenyl)-L-asparagines with glycosylasparaginase

In the hydrolysis of the $N^4$-(4′-substituted phenyl)-L-asparagines, the released substituted aniline was measured colorimetrically by a diazotization reaction as previously described.[19] Prior to the enzyme kinetic studies, the time and temperature for color development were studied, the absorbance spectrum was recorded to determine the wavelength for measurement, and the molar absorptivity value was calculated from plots of absorbance against concentration. The average of triplicate measurements for 12–16 concentrations of each aniline gave excellent correlation coefficients, $r \geq 0.9994$. The values for each aniline are given in the Experimental section. The values for 4-bromoaniline have not been reported. The other values are in general agreement with those previously reported.[19] The molar absorptivity value for aniline is slightly smaller, while the values for 4-ethylaniline and 4-methoxyaniline are larger; the value for 4-methoxyaniline was checked several times.[23]

In an early study it was reported that $N^4$-phenyl-L-asparagine was not a substrate for GA from hog serum and hog kidney, but was a competitive inhibitor.[12] Recently $N^4$-(4′-nitrophenyl)-L-asparagine (**5**) was shown to be a substrate for GA from recombinant *Flavobacterium meningosepticum*.[24] We found that each of the five anilides was a substrate for GA from human amniotic fluid. The kinetic parameters are given in Table 1; the kinetic parameters for $N^4$-(4′-nitrophenyl)-L-asparagine (**5**) are similar for GA from human amniotic fluid ($K_m$ 603 μM, $k_{cat}$ 9.0 $s^{-1}$) and GA from recombinant *Flavobacterium meningosepticum* ($K_m$ 430 μM, $k_{cat}$ 2.3 $s^{-1}$).[24] No correlations between the kinetic parameters and field/inductive ($\sigma_I$) constants, resonance ($\sigma_R$) constants, Taft steric parameters, and hydrophobic parameters[25] were found.

A Hammett plot of log ($k_{cat}$) *vs.* $\sigma_p^-$ (Fig. 2) is biphasic. The value of $k_{cat}$ varies with substitution on the aniline leaving group. This indicates that acylation is the rate-limiting step in the hydrolysis of these substrates.[27] If deacylation was the rate-limiting step, the value of $k_{cat}$ would be independent of the structure of the aniline leaving group with formation of a β-aspartyl–enzyme intermediate. This is the first direct evidence for a rate-limiting acylation step in the hydrolysis

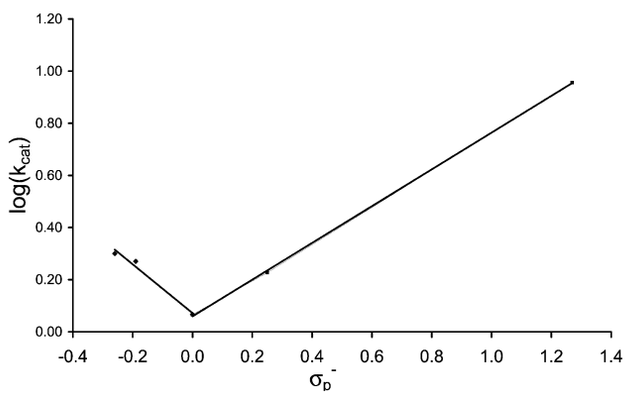


**Fig. 2** A Hammett plot of log ($k_{cat}$) *vs.* $\sigma_p^-$ is biphasic. These data show that acylation is the rate-limiting step in the reaction. (For deacylation as the rate-limiting step, the plot would be linear with a slope = 0.) For substrates containing electron-donating groups the slope $\rho$ is −0.94 ($r = 0.99$) and for substrates containing electron-withdrawing groups the slope $\rho$ is $0.70_5$ ($r = 1.00$).

reaction catalyzed by GA (and an N-terminal amidohydrolase), and strongly implies that acylation is the rate-limiting step in the reaction of GA with (GlcNAc-)Asn. Linear regression of the data for substrates containing electron-donating groups gives a line with a correlation coefficient $r = 0.99$ and a slope $\rho = -0.94$. Linear regression of the data for substrates containing electron-withdrawing groups gives a line with a correlation coefficient $r = 1.00$ and a slope $\rho = 0.70_5$. A Hammett plot of log ($K_m$) *vs.* $\sigma_p^-$ (not shown) is also biphasic, similar to Fig. 2; for substrates containing electron-donating groups the slope $\rho = -1.99$, while for substrates containing electron-withdrawing groups $K_m$ is within experimental error independent of the structure of the aniline leaving group. A Hammett plot of log ($k_{cat}/K_m$) *vs.* $\sigma_p^-$ (Supplementary Figure 3) is linear with a correlation coefficient $r = 0.95$ and a slope $\rho = 0.65$. This second-order rate constant for the reaction of substrate with free enzyme is not affected by nonproductive binding effects. The positive slope is indicative of a favorable effect on the binding step with increasingly electron-withdrawing substituents. The substituent effect on the binding step suggests that the electron distribution of the substrate is perturbed toward the transition state upon binding to the active site.[27]

Because the mechanism for GA has been proposed to be analogous to serine proteases, we compared our results with the most applicable results reported for rate-limiting acylation reactions utilizing anilides for chymotrypsin[28,29] and urokinase.[30] A biphasic Hammett plot for log ($k_{cat}$) *vs.* $\sigma$ as in Fig. 2 was suggested for chymotrypsin by Bundy and Moore.[28] There is an indication of a biphasic plot in the data of Inagami *et al.*[29] similar to our observation in Fig. 2. Inagami *et al.*[29] also reported that a plot of log ($K_m$) *vs.* $\sigma^-$ was scattered, but with the same tendency as the plot of log ($k_{cat}$) *vs.* $\sigma^-$; the plot shows a general trend similar to our observation where $K_m$ is within experimental error independent for electron-withdrawing groups. For urokinase,[30] a plot of log ($k_{cat}$) *vs.* $\sigma^-$ for substrates containing electron-withdrawing groups was linear with a slope $\rho = 0.72$ that is nearly identical to our result

(Fig. 2); no correlation was evident for urokinase in plots of log ($K_m$) *vs.* $\sigma^-$ or log ($k_{cat}/K_m$) *vs.* $\sigma^-$. Therefore, while the kinetic data for GA show some similarities to Hammett correlations in serine proteases, the overall relationship is not particularly strong except for the biphasic relationship of log ($k_{cat}$).

The charge on the nitrogen atom of the aniline leaving group at the transition state may be calculated from the slope of a Brønsted plot.[27] A plot of log ($k_{cat}^X/k_{cat}^H$) *vs.* p$K_a$ for the conjugate acid of the leaving group, *i.e.* the anilinium ion, is shown in Supplementary Figure 4; the plot is biphasic as it must be according to Fig. 2. The charge (slope) on the nitrogen atom in the anilines containing electron-withdrawing groups is $\beta_{lg} = -0.25$ (correlation coefficient $r = 1.00$ for the linear regression) and on the nitrogen atom in the anilines containing electron-donating groups is $\beta_{lg} = 0.43$ (correlation coefficient $r = 0.98$ for the linear regression). These data are similar in magnitude to $\beta_{lg} \approx 0.8$ (calculated)[29] and $\beta_{lg} = 1.27$ for electron-donating groups[31] in the reaction of chymotrypsin, and $\beta_{lg} \approx -0.3$ (calculated)[30] for electron-withdrawing groups in the reaction of urokinase.

The free energy (incremental) change of binding of the substituted anilides relative to the natural substrate in enzyme–substrate transition state complexes, $\Delta\Delta G_b$,[4] was calculated at 37 °C from $\Delta\Delta G_b = -RT\ln[(k_{cat}/K_m)_{aniline}/(k_{cat}/K_m)_{(GlcNAc-)Asn}]$ and are plotted against Hammett constants (Supplementary Figure 5); the plot is linear as it must be according to Supplementary Figure 3, with a correlation coefficient $r = 0.95$ and a slope $\rho = -3.84_5$. Substitution of the pyranosyl group in the natural substrate with a substituted phenyl group results in an overall loss in transition state binding energy between the enzyme and substrate equivalent to a weak hydrogen bond. Interestingly, the magnitude of the loss in binding energy depends on the substituent group on the phenyl ring. For electron-donating groups the loss of binding energy is greater than for electron-withdrawing groups as shown in Supplementary Figure 5. Therefore, in terms of transition state binding energy, these data would indicate that the greater the electron-withdrawing potential on the phenyl ring, the closer the structure of the substituted anilide in the transition state might resemble the natural substrate. The perturbation of electron distribution of the substrates toward the transition state upon binding indicated in Supplementary Figure 3 is supported by the data in Supplementary Figure 5.

These data are consistent with a mechanism for GA similar to that proposed for the transpeptidation reaction of γ-glutamyl transpeptidase based on the electronic properties of the substituent groups and on the degree of protonation of the leaving group in the transition state.[19] For GA (Scheme 2), acylation is the rate-limiting step in the hydrolysis reaction. Nucleophilic attack on the bound substituted anilide by the N-terminal Thr residue is very fast with initial formation of a high energy tetrahedral structure. The binding of this tetrahedral structure perturbs the electron distribution of the substrate toward the transition state. The breakdown of the tetrahedral structure is general acid-catalyzed where proton transfer to the nitrogen of the leaving group aniline occurs simultaneously with C–N bond cleavage. The α-amino group on the catalytic Thr residue (*i.e.*, the N-terminal group of the subunit containing the catalytic Thr residue) has been proposed to act as a general acid.[5] The degree of proton transfer and the degree of C–N bond cleavage depend on the electronic properties of the substituent group on the aniline. Electron-withdrawing groups favor C–N bond cleavage which results in significant bond stretching in the transition state, and make the nitrogen atom less basic which results in less proton transfer in the transition state. Electron-donating groups favor C–N bond cleavage which results in less bond stretching in the transition state, and make the nitrogen atom more basic which results in greater proton transfer in the transition state. The formation of the covalent β-aspartyl–enzyme intermediate is therefore the rate-limiting step in this hydrolysis reaction. These results are in agreement with data for chymotrypsin that support a rate-limiting breakdown of the tetrahedral structure for anilide substrates to the acyl enzyme.[32,33] Additional studies will clarify further aspects of the mechanism.

## Experimental

### Materials and methods

Chemicals purchased from the following suppliers were: phthalyl anhydride, aniline, 4-ethylaniline, 4-bromoaniline, 4-nitroaniline, 4-methoxyaniline, and *N*-(1-naphthyl)ethylenediamine dihydrochloride from Acros; L-aspartic acid from Spectrum Chemical; deuterium oxide (99.9 atom% $^2$H), dimethyl sulfoxide-$d_6$ (99.9 atom% $^2$H), and acetone-$d_6$ (99.9 atom% $^2$H) from Cambridge Isotopes; sodium lauryl sulfate, Concanavalin A-Sephrose 4B, Sephadex G-100, and bovine serum albumin (fraction V) from Sigma; $N^4$-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-L-asparagine from Bachem (California); 4-dimethylaminobenzaldehyde from Mallinckrodt. All other chemicals were at least analytical grade. Human amniotic fluid was obtained from the Chemistry Laboratory of the Carolinas Medical Center (Charlotte, North Carolina), and was stored at −20 °C.

A GE 300 spectrometer was used to record NMR spectra. $^1$H NMR spectra were recorded at 300.2 MHz in a 5 mm probe at ambient temperature with a 5000 Hz sweep width, 30° pulse angle, and an 8k (real) data block; no line-broadening factor was applied to the accumulated FID. Natural abundance $^{13}$C NMR spectra were recorded at 75.5 MHz in a 5 mm probe at ambient temperature with a 20000 Hz sweep width, 30° pulse angle, and an 8k (real) data block; protons were broad-band decoupled and a line-broadening factor of 2.0 Hz was applied to the accumulated FID. The error in the measured chemical shifts was ±0.002 ppm for $^1$H NMR and ±0.033 ppm for $^{13}$C NMR; *J* values are given in Hz and the error in the measured coupling constants was ±0.61. The $(CD_3)_2SO$–NaOD solvent was prepared by adding a 1 M NaOD–$D_2O$ solution to dimethyl sulfoxide-$d_6$ to give a ratio of $(CD_3)_2SO$ to NaOD–$D_2O$ of 95:5 (v/v). Elemental analyses were done at Atlanta Microlabs, Inc. (Norcross, Georgia).

**$N^2$-Phthalyl-L-aspartic anhydride 1.** The synthesis of **1** was carried out as described with slight modification.[13] Phthalic anhydride (37.0 g, 250 mmol) was added over 5 min to a stirred suspension of L-aspartic acid (33.3 g, 250 mmol) in pyridine (400 ml) at room temperature. The mixture was heated under reflux for 4 h, after which the solution was cooled to room temperature. Any solid material was removed by filtration. The filtrate was evaporated under reduced pressure to give a light yellow oil. Acetic anhydride (200 ml) was added to the oil. After stirring the mixture for 1 h at room temperature, the solution was cooled at 4 °C for 2 h. The resulting precipitate was collected by filtration, washed with anhydrous diethyl ether (3 × 50 ml), and recrystallized from 1,4-dioxane (300 ml) to give **1** (25.5 g, 42%) as white crystals. Mp 224–226 °C (from 1,4-dioxane) (lit.,[13] 224–226 °C); $\delta_H$(300.2 MHz; $CD_3COCD_3$; acetone at 2.05 ppm) 3.45 (1H, dd, $J_{2,3a}$ 6.10 and $J_{3a,3b}$ −18.92, 3a-H), 3.65 (1H, dd, $J_{2,3b}$ 9.77 and $J_{3a,3b}$ −18.92, 3b-H), 5.80 (1H, dd, $J_{2,3a}$ 6.10 and $J_{2,3b}$ 9.77, 2-H), 7.95 (4H, s, Ph) (lit.,[13] $\delta_H((CD_3)_2SO)$ 3.53 (2H, d, 3-H), 5.77 (1H, t, 2-H), 8.00 (4H, s, Ph)); $\delta_C$(75.5 MHz; $(CD_3)_2SO$–NaOD; dimethyl sulfoxide at 39.50 ppm) 33.36 (C-3), 47.28 (C-2), 123.75 (2 × C-3′), 131.07 (2 × C-2′), 135.11 (2 × C-4′), 166.57 (2 × C-1′), 169.33 (C-1), 169.95 (C-4).

**$N^4$-(4′-Substituted phenyl)-L-asparagines.** The synthesis of **2**, **3**, **4**, **5**, and **6** was a modified procedure of those reported.[13,19] To a suspension of **1** (3.0 g, 12.2 mmol) in anhydrous diethyl

ether (80 ml) was added the (substituted)aniline (12.2 mmol). The solution was stirred at room temperature (for the time given below), after which the solvent was evaporated under reduced pressure. The solid was dissolved in 0.1 M NaOH (10 ml), the solution was acidified with 5 M HCl to pH 2.0, and the resulting precipitate was collected and dissolved in methanol (100 ml). Any insoluble material was removed by filtration. Hydrazine (10 ml) was added and the mixture was stirred at room temperature for 24 h. After the solvent and excess hydrazine were evaporated under reduced pressure, 0.5 M HCl (150 ml) was added and the pH was adjusted to 1.0 with 5 M HCl. The resulting precipitate was removed by filtration. To the filtrate was added solid $NaHCO_3$ to bring the pH to 5.0, and the solution was set at 4 °C overnight. The precipitate was collected by filtration and washed with acetone.

***N*⁴-Phenyl-L-asparagine 2.** Aniline (1.1 g, 12.2 mmol) was added and the reaction was stirred for 12 h to give **2** (0.81 g, 33%) as a white powder. Mp 247–248 °C (lit.,[9] 251–252 °C; lit.,[10] 250–252 °C; lit.,[11] 241–242 °C; lit.,[13] 248–249 °C; lit.,[16] 255 °C (D,L mixture)) (Found: C, 57.75; H, 5.87; N, 13.48. Calc. for $C_{10}H_{12}N_2O_3$: C, 57.69; H, 5.77; N, 13.46%); $\delta_H$(300.2 MHz; $(CD_3)_2SO$–NaOD; dimethyl sulfoxide at 2.50 ppm) 2.17 (1H, dd, $J_{2,3a}$ 7.33 and $J_{3a,3b}$ −15.80, 3a-H), 2.36 (1H, dd, $J_{2,3b}$ 5.50 and $J_{3a,3b}$ −15.80, 3b-H), 3.56 (1H, dd, $J_{2,3a}$ 7.33 and $J_{2,3b}$ 5.50, 2-H), 7.00 (1H, t, $J_{3',4'}$ 7.93, 4′-H), 7.26 (2H, dd, $J_{2',3'}$ 7.93 and $J_{3',4'}$ 7.93, 2 × 3′-H), 7.57 (2H, d, $J_{2',3'}$ 7.93, 2 × 2′-H); $\delta_C$(75.5 MHz; $(CD_3)_2SO$–NaOD; dimethyl sulfoxide at 39.50 ppm) 43.80 (C-3), 53.60 (C-2), 119.45 (2 × C-2′), 123.40 (C-4′), 129.03 (2 × C-3′), 139.41 (C-1′), 174.69 (C-4), 175.92 (C-1).

***N*⁴-(4′-Ethylphenyl)-L-asparagine 3.** 4-Ethylaniline (1.5 g, 12.2 mmol) was added and the reaction was stirred for 9 h to give **3** (0.62 g, 22%) as a white powder. Mp 225–226 °C (Found: C, 60.84; H, 6.79; N, 11.77. Calc. for $C_{12}H_{16}N_2O_3$: C, 61.02; H, 6.78; N, 11.86%); $\delta_H$(300.2 MHz; $(CD_3)_2SO$–NaOD; dimethyl sulfoxide at 2.50 ppm) 1.10 (3H, t, $J_{5',6'}$ 7.93, 6′-H), 2.04 (1H, dd, $J_{2,3a}$ 9.76 and $J_{3a,3b}$ −14.60, 3a-H), 2.34 (1H, dd, $J_{2,3b}$ 4.27 and $J_{3a,3b}$ −14.60, 3b-H), 2.48 (2H, q, $J_{5',6'}$ 7.93, 5′-H), 3.45 (1H, dd, $J_{2,3a}$ 9.76 and $J_{2,3b}$ 4.27, 2-H), 6.95 (2H, d, $J_{2',3'}$ 8.00, 2 × 2′-H), 7.30 (2H, d, $J_{2',3'}$ 8.00, 2 × 3′-H); $\delta_C$(75.5 MHz; $(CD_3)_2SO$–NaOD; dimethyl sulfoxide at 39.50 ppm) 16.32 (C-6′), 28.03 (C-5′), 45.07 (C-3), 54.78 (C-2), 122.11 (2 × C-2′), 127.52 (2 × C-3′), 135.93 (C-4′), 144.69 (C-1′), 175.13 (C-4), 177.62 (C-1).

***N*⁴-(4′-Bromophenyl)-L-asparagine 4.** 4-Bromoaniline (2.1 g, 12.2 mmol) was added and the reaction was stirred for 24 h to give **4** (0.95 g, 27%) as a white powder. Mp 250–251 °C (Found: C, 41.87; H, 3.80; Br, 27.82; N, 9.75. Calc. for $C_{10}H_{11}BrN_2O_3$: C, 41.83; H, 3.83; Br, 27.85; N, 9.76%); $\delta_H$(300.2 MHz; $(CD_3)_2SO$–NaOD; dimethyl sulfoxide at 2.50 ppm) 1.95 (1H, dd, $J_{2,3a}$ 10.37 and $J_{3a,3b}$ −14.04, 3a-H), 2.37 (1H, dd, $J_{2,3b}$ 3.05 and $J_{3a,3b}$ −14.04, 3b-H), 3.40 (1H, dd, $J_{2,3a}$ 10.37 and $J_{2,3b}$ 3.05, 2-H), 7.07 (2H, d, $J_{2',3'}$ 8.55, 2 × 3′-H), 7.37 (2H, d, $J_{2',3'}$ 8.55, 2 × 2′-H); $\delta_C$(75.5 MHz; $(CD_3)_2SO$–NaOD; dimethyl sulfoxide at 39.50 ppm) 45.81 (C-3), 55.64 (C-2), 108.62 (C-4′), 125.86 (2 × C-2′), 129.93 (2 × C-3′), 152.77 (C-1′), 176.89 (C-4), 178.90 (C-1).

***N*⁴-(4′-Nitrophenyl)-L-asparagine 5.** 4-Nitroaniline (1.7 g, 12.2 mmol) and glacial acetic acid (10 ml) were added, and the reaction was stirred for 8 h to give **5** (0.31 g, 10%) as a slightly yellow powder. Mp 244–245 °C (Found: C, 44.73; H, 4.75; N, 15.35. Calc. for $C_{10}H_{11}N_3O_5 \cdot 0.9H_2O$: C, 44.52; H, 4.76; N, 15.58%); $\delta_H$(300.2 MHz; $(CD_3)_2SO$–NaOD; dimethyl sulfoxide at 2.50 ppm) 2.69 (1H, dd, $J_{2,3a}$ 6.40 and $J_{3a,3b}$ −16.50, 3a-H), 3.05 (1H, dd, $J_{2,3b}$ 6.40 and $J_{3a,3b}$ −16.50, 3b-H), 3.68 (1H, dd, $J_{2,3a}$ 6.40 and $J_{2,3b}$ 6.40, 2-H), 7.83 (2H, d, $J_{2',3'}$ 8.55, 2 × 3′-H), 8.17 (2H, d, $J_{2',3'}$ 8.55, 2 × 2′-H); $\delta_C$(75.5 MHz; $(CD_3)_2SO$–NaOD; dimethyl sulfoxide at 39.50 ppm) 38.24 (C-3), 50.30 (C-2), 118.71 (2 × C-2′), 124.85 (2 × C-3′), 141.96 (C-4′), 145.42 (C-1′), 169.49 (C-4), 169.97 (C-1).

***N*⁴-(4′-Methoxyphenyl)-L-asparagine 6.** 4-Methoxyaniline (1.5 g, 12.2 mmol) was added and the reaction was stirred for 4 h to give an approximate equimolar mixture of **6** and *N*¹-(4′-methoxyphenyl)-L-asparagine (**7**) (0.96 g) as a white powder. The powder was dissolved in water (20 ml) and the solution was adjusted to pH 11 with 6 M NaOH. The solution was adjusted to pH 9.0 with 5 M HCl, at which point **7** precipitated and it was collected by filtration. The filtrate was adjusted to pH 3.0 with 5 M HCl, at which point **6** precipitated. The precipitate was collected by filtration and dried in an oven at 60 °C (2 h) to give **6** (0.36 g, 12%) as a white powder. Mp 225–226 °C (lit.,[16] 265 °C (D,L mixture)) (Found: C, 55.19; H, 6.09; N, 11.64. Calc. for $C_{11}H_{14}N_2O_4$: C, 55.46; H, 5.88; N, 11.76%); $\delta_H$(300.2 MHz; $(CD_3)_2SO$–NaOD; dimethyl sulfoxide at 2.50 ppm) 2.03 (1H, dd, $J_{2,3a}$ 8.55 and $J_{3a,3b}$ −14.60, 3a-H), 2.31 (1H, dd, $J_{2,3b}$ 4.27 and $J_{3a,3b}$ −14.60, 3b-H), 3.42 (1H, dd, $J_{2,3a}$ 8.55 and $J_{2,3b}$ 4.27, 2-H), 3.68 (3H, s, 5′-H), 6.71 (2H, d, $J_{2',3'}$ 9.15, 2 × 2′-H), 7.39 (2H, d, $J_{2',3'}$ 9.15, 2 × 3′-H); $\delta_C$(75.5 MHz; $(CD_3)_2SO$–NaOD; dimethyl sulfoxide at 39.50 ppm) 44.80 (C-3), 54.24 (C-2), 55.18 (C-5′), 113.43 (2 × C-3′), 121.94 (2 × C-2′), 138.21 (C-1′), 153.67 (C-4′), 174.40 (C-4), 176.41 (C-1).

## Kinetic studies

Human glycosylasparaginase (dimeric form) was isolated from human amniotic fluid as described earlier[4,34] utilizing the exceptional resistance to sodium dodecyl sulfate (SDS):[35] solubilization and centrifugation of amniotic fluid, ammonium sulfate precipitation, 3% SDS–35% ammonium sulfate, concanavalin A affinity chromatography, gel-exclusion chromatography. To inhibit any protease activity present in the amniotic fluid, disodium ethylenediaminetetraacetic acid (EDTA) and phenylmethylsulfonyl fluoride (PMSF), as well as sodium azide, were included in the solubilizing buffer; these chemicals have no affect on GA activity. Only GA activity was measured in the protein sample at the end; we are also not aware of another enzyme capable of catalyzing the hydrolysis of *N*⁴-(4′-substituted phenyl)-L-asparagines. GA activity for (GlcNAc-)Asn was assayed overnight in 0.09 M citrate–0.06 M phosphate buffer at pH 5.6, 37 °C, where optimal activity was observed, and the *N*-acetyl-D-glucosamine released during the reaction was measured using the Morgan–Elson reaction.[36] Protein concentration was measured with the Coomassie blue (Bradford) protein assay, using bovine serum albumin as the standard.[37] The kinetic parameters used for the natural substrate, (GlcNAc-)Asn, were $K_m$ 215 (±27) μM and $k_{cat}$ 5.0 (±0.9) $s^{-1}$.

The concentrations of the *N*⁴-(4′-substituted phenyl)-L-asparagines in 0.09 M citrate–0.06 M phosphate buffer at pH 5.6, 37 °C ranged from approx. 0.1 to 7 times $K_m$, and a single quantity of enzyme was used in each assay. Following overnight incubation, the reaction was quenched with 40% trichloroacetic acid. The release of the substituted aniline was measured colorimetrically by a diazotization reaction as previously described.[19] The time and temperature for color development, wavelength for measurement of the product, and the molar absorptivity value calculated by a linear regression analysis (with a correlation coefficient, $r \geq 0.9994$) from triplicate measurements for each aniline were: 4-nitroaniline 10 min, 25 °C, 548 nm, 55.7 $mM^{-1}$ $cm^{-1}$; 4-bromoaniline 10 min, 25 °C, 562 nm, 44.4$_5$ $mM^{-1}$ $cm^{-1}$; aniline 15 min, 25 °C, 558 nm, 38.9 $mM^{-1}$ $cm^{-1}$; 4-ethylaniline 60 min, 25 °C, 572 nm, 42.6 $mM^{-1}$ $cm^{-1}$; 4-methoxyaniline 60 min, 37 °C, 588 nm, 31.9 $mM^{-1}$ $cm^{-1}$. The average of triplicate measurements was used to calculate the kinetic parameters by regression analysis.

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## Notes and references

1 The IUPAC-IUBMB Joint Commission on Biochemical Nomenclature: Nomenclature of Carbohydrates (Recommendations 1996) (www.chem.qmul.ac.uk/iupac/2carb/) states that the term "*N*-glycoside" is improper and does not recommend its use, but "glycosyl" is recommended. Therefore, "glycosylic" is used in this paper, rather than "glycosidic".
2 C. M. Smith, *Tetrahedron*, 1998, **54**, 11317–11362.
3 N. N. Aronson, Jr., *Biochim. Biophys. Acta*, 1999, **1455**, 139–154.
4 J. M. Risley, D. H. Huang, J. J. Kaylor, J. J. Malik, Y.-Q. Xia and W. M. York, *Arch. Biochem. Biophys.*, 2001, **391**, 165–170.
5 C. Oinonen, R. Tikkanen, J. Rouvinen and L. Peltonen, *Nature Struct. Biol.*, 1995, **2**, 1102–1108.
6 J. Xuan, A. L. Tarentino, B. G. Grimwood, T. H. Plummer, Jr., T. Cui, C. Guan and P. Van Roey, *Protein Sci.*, 1998, **7**, 774–781.
7 H.-C. Guo, Q. Xu, D. Buckley and C. Guan, *J. Biol. Chem.*, 1998, **273**, 20205–20212.
8 C. Oinonen and J. Rouvinen, *Protein Sci.*, 2000, **9**, 2329–2337.
9 F. E. King and D. A. A. Kidd, *J. Chem. Soc.*, 1951, 2976–2978.
10 F. Weyand, P. Klinke and I. Eigen, *Chem. Ber.*, 1957, **90**, 1896–1905.
11 J. Kovacs, H. Nagy Kovacs, I. Konyves, J. Csaszar, T. Vajda and H. Mix, *J. Org. Chem.*, 1961, **26**, 1084–1091.
12 M. Tanaka and I. Yamashina, *Carbohydr. Res.*, 1973, **27**, 175–183.
13 M. J. Murphy and J. F. Stubbins, *J. Pharm. Sci.*, 1980, **69**, 553–555.
14 W. E. Adams, E. W. Snook and T. J. Curphey, *J. Labelled Compd. Radiopharm.*, 1980, **18**, 991–997.
15 D. Nisato, J. Wagnon, G. Callet, D. Mettefeu, J.-L. Assens, C. Plouzane, B. Tonnerre, V. Pliska and J.-L. Fauchere, *J. Med. Chem.*, 1987, **30**, 2287–2291.
16 Y. Liwschitz, A. Zilkha and E. Nemes, *Bull. Res. Counc. Isr., Sect. A*, 1961, **10**, 107–110.
17 BACHEM, #L-1690.
18 X. Huang, X. Luo, Y. Roupioz and J. W. Keillor, *J. Org. Chem.*, 1997, **62**, 8821–8825.
19 A. Menard, R. Castonguay, C. Lherbet, C. Rivard, Y. Roupioz and J. W. Keillor, *Biochemistry*, 2001, **40**, 12678–12685.
20 J. F. G. Vliegenthart, L. Dorland and H. van Halbeek, *Adv. Carbohydr. Chem. Biochem.*, 1983, **41**, 209–374.
21 J. Bennett, M. Delmas and J. C. Maire, *Org. Magn. Reson.*, 1969, **1**, 319–327.
22 C. J. O'Connor, D. J. McLennan, D. J. Calvert, T. D. Lomax, A. J. Porter and D. A. Rogers, *Aust. J. Chem.*, 1984, **37**, 497–510.
23 Unlike the other four anilines, color development for 4-methoxyaniline increased up to at least 72 h at 37 °C. We found that plots of absorbance *vs.* concentration at any time, *e.g.*, after 60 min, 120 min, 180 min, *etc.*, were linear with excellent correlation coefficients. Therefore, it was important to measure the absorbance immediately at a fixed incubation time. We chose a 60 min incubation time that may account for the major difference between the molar absorptivity value reported earlier for a 30 min incubation [19] and our value. It is not clear that the same phenomenon may account for the differences in molar absorptivity values for 4-ethylaniline, which we did not pursue experimentally. Whether this phenomenon may be true for anilines containing electron-donating groups, in general, is also not clear from these data.
24 Y. Liu, C. Guan and N. N. Aronson, Jr., *J. Biol. Chem.*, 1998, **273**, 9688–9694.
25 C. Hansch and A. Leo, *Substituent Constants for Correlation Analysis in Chemistry and Biology*, John Wiley & Sons, New York, 1979.
26 X. Huang, N. Day, X. Luo, Y. Roupioz, M. Seid and J. W. Keillor, *J. Pept. Res.*, 1999, **53**, 126–133.
27 W. P. Jencks, *Cold Spring Harbor Sym. Quant. Biol.*, 1971, **36**, 1–11.
28 H. F. Bundy and C. L. Moore, *Biochemistry*, 1966, **5**, 808–811.
29 T. Inagami, A. Patchornik and S. S. York, *J. Biochem.*, 1969, **65**, 809–819.
30 D. Petkov, E. Christova, I. Pojarlieff and N. Stambolieva, *Eur. J. Biochem.*, 1975, **51**, 25–32.
31 L. Parker and J. H. Wang, *J. Biol. Chem.*, 1968, **243**, 3729–3734.
32 M. Caplow, *J. Am. Chem. Soc.*, 1969, **91**, 3639–3645.
33 E. C. Lucas and M. Caplow, *J. Am. Chem. Soc.*, 1972, **94**, 960–963.
34 J. M. Risley, D. H. Huang, J. J. Kaylor, J. J. Malik and Y.-Q. Xia, *J. Enzyme Inhib.*, 2001, **16**, 269–274.
35 T. Heiskanen, O. K. Tollersrud, M. Zhao and L. Peltonen, *Protein Expression Purif.*, 1994, **5**, 205–210.
36 J. L. Reissig, J. L. Strominger and L. F. Leloir, *J. Biol. Chem.*, 1955, **217**, 959–966.
37 C. M. Stoscheck, *Methods Enzymol.*, 1990, **182**, 50–68.