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Glycosylasparaginase Inhibition Studies: Competitive Inhibitors, Transition State Mimics, Noncompetitive Inhibitors

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Glycosylasparaginase catalyzes the hydrolysis of the *N*-glycosylic bond between asparagine and *N*-acetylglucosamine in the catabolism of *N*-linked glycoproteins. Previously only three competitive inhibitors, one noncompetitive inhibitor, and one irreversible inhibitor of glycosylasparaginase activity had been reported. Using human glycosylasparaginase from human amniotic fluid, L-aspartic acid and four of its analogues, where the α-amino group was substituted with a chloro, bromo, methyl or hydrogen, were competitive inhibitors having K_i values between 0.6–7.7 mM. These results provide supporting evidence for a proposed intramolecular autoproteolytic activation reaction. A proposed phosphono transition state mimic and a sulfo transition state mimic were competitive inhibitors with K_i values 0.9 mM and 1.4 mM, respectively. These results support a mechanism for the enzyme-catalyzed reaction involving formation of a tetrahedral high-energy intermediate. Three analogues of the natural substrate were noncompetitive inhibitors with K_i values between 0.56–0.75 mM, indicating the presence of a second binding site that may recognize (substituted)acetamido groups.

Keywords: Glycosylasparaginase, Inhibition, Transition state analogues, Aspartic acid analogues

INTRODUCTION

The predominant *N*-glycosylic bond in nature is between *N*-acetyl-D-glucosamine and asparagine.[1] 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 [**1**, (GlcNAc-)Asn] to give aspartic acid and 2-acetamido-2-deoxy-β-D-glucopyranosylamine, which hydrolyzes non-enzymatically to *N*-acetyl-D-glucosamine (GlcNAc) and ammonium ion.[2] 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

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metabolism.[2] A catalytic mechanism involving an *N*-terminus Thr that is somewhat analogous to the mechanism in serine proteinases has been proposed[3] and involves formation of a tetrahedral structure (Figure 1). Only a few compounds have been reported to inhibit GA activity. Competitive inhibitors include 3-hydroxybutanone (K_i 4.1 mM),[4] β-L-aspartyl hydroxamate (K_i 0.005 mM),[5] and L-Asn (K_i 0.454 mM).[6] The only noncompetitive inhibitor of GA activity is *N*-acetylcysteine (K_i 3.2 mM).[4] The asparagine analogue 5-diazo-4-oxo-L-norvaline (DONV) is an irreversible inhibitor of GA activity[7–11] and forms an α-ketone ether linkage with the hydroxyl group of an *N*-terminus Thr residue (K_i 0.080 mM).[9] Interestingly, neither a product of the hydrolysis reaction, L-aspartic acid, nor a compound that mimics the proposed transition state have been studied as inhibitors of GA activity. We studied the inhibition of human GA activity, (a) by L-Asp and some analogues of L-Asp, (b) by analogy with serine proteinases, a phosphono analogue and a sulfo analogue as transition state mimics, and (c) by some analogues of (GlcNAc-)Asn. The structures of the compounds used in this study are shown in Figure 2, and the results of this study are reported below.

MATERIALS AND METHODS

N^4-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-L-asparagine (**1**) was purchased from Bachem (CA). L-Aspartic acid (**2**), 3-phosphono-D,L-2-aminopropionic acid (**7**), L-cysteic acid (**8**) and L-cysteine (**9**) were purchased from Sigma Chem. Co. D,L-2-Methylsuccinic acid (**5**) and succinic acid (**6**) were purchased from Aldrich Chem. Co. L-2-Chlorosuccinic acid (**3**) and L-2-bromosuccinic acid (**4**) were synthesized as described.[12] N^1-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)glycinamide (**10**), *N*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)chloroacetamide (**11**) and *N*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)bromoacetamide (**12**) were synthesized as described.[13–15] Human glycosylasparaginase (dimeric form) was isolated from human amniotic fluid[16–18] using the exceptional resistance to SDS[19] (K_m for (GlcNAc-)Asn 0.116 mM). Glycosylasparaginase activity was measured in citrate-phosphate buffer at pH 5.8, at 37 °C. *N*-Acetyl-D-glucosamine released during the reaction was measured using the Morgan-Elson assay.[20] Inhibition studies were conducted using three or four concentrations of (GlcNAc-)Asn and three to five concentrations of the inhibitor under investigation. Since it has been shown that GA does not recognize and bind the D-enantiomer,[5] in the equimolar racemic D,L-mixtures of **5** and **7**, the structure-activity studies considered only the L-enantiomers. The average of triplicate measurements was used to calculate the kinetic parameters by least-squares regression analysis of $1/\nu_0$ *vs.* $1/[S]_0$ plots and Dixon ($1/\nu_0$ *vs.* [I]) plots; errors were calculated by progression of errors analysis.

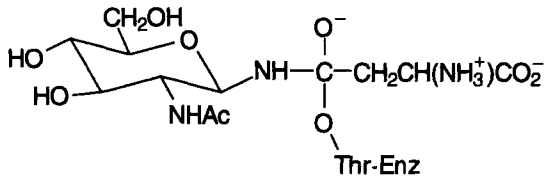
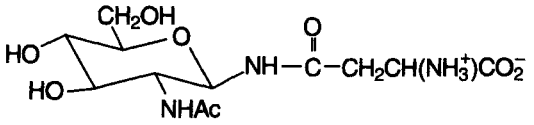


FIGURE 1 Structure of the tetrahedral intermediate in the hydrolysis reaction of the *N*-glycosylic bond catalyzed by glycosylasparaginase.

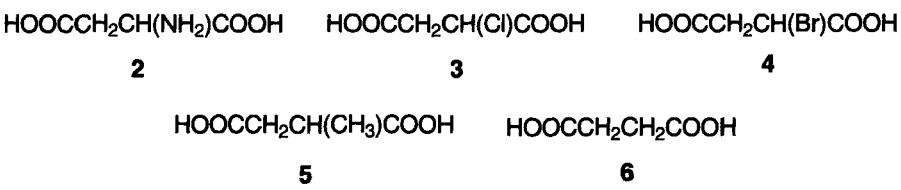
RESULTS

The results of the inhibition studies for compounds **2–12** are given in Table I. L-Asp (**2**) and its four analogues (**3–6**) are all competitive inhibitors of GA activity. Figure 3 shows the Dixon plot for **3**. The two transition state mimics, **7** and **8**, were also competitive inhibitors of GA activity; L-Cys (**9**) was used as a basis for comparing the thiol group in **9** to the sulfo group in **8** and was not an inhibitor of GA activity. The



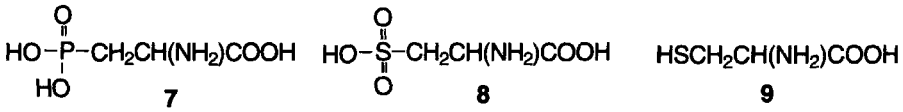
1

L-Asp and analogues



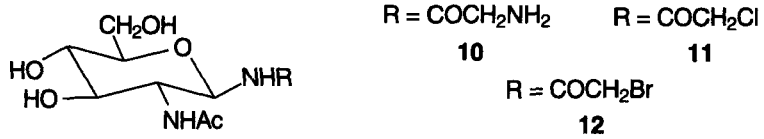
2 **3** **4** **5** **6**

transition-state mimics



7 **8** **9**

GlcNAc-Asn analogues



10 **11** **12**

FIGURE 2 Structures of compounds used in this study.

three analogues of (GlcNAc-)Asn, **10–12**, were noncompetitive inhibitors of GA activity. Figure 4 shows the double reciprocal and Dixon plots for **11**.

TABLE I K_i values for inhibition of glycosylasparaginase activity by compounds

Compound	Inhibition	K_i (mM)
2	Competitive	0.6 ± 0.1
3	Competitive	7.7 ± 1.0
4	Competitive	2.7 ± 0.2
5	Competitive	0.7 ± 0.1
6	Competitive	5.0 ± 1.0
7	Competitive	0.9 ± 0.2
8	Competitive	1.4 ± 0.3
9	None	–
10	Noncompetitive	0.75 ± 0.11
11	Noncompetitive	0.64 ± 0.06
12	Noncompetitive	0.56 ± 0.05

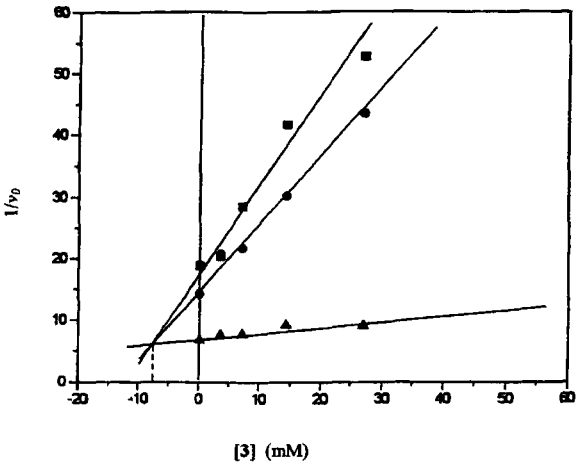


FIGURE 3 The Dixon plot for competitive inhibition of glycosylasparaginase activity by L-2-chlorosuccinic acid (**3**). The concentrations of (GlcNAc-)Asn used were 0.113 mM, 0.151 mM, and 0.376 mM. The K_i value is 7.7 mM.

DISCUSSION

To date, only five compounds have been shown to inhibit glycosylasparaginase activity – three competitive inhibitors, one noncompetitive inhibitor, and one irreversible inhibitor. Of the three competitive inhibitors, β-L-aspartyl hydroxamate and L-Asn are also substrates.[2,5] One product, L-aspartic acid, of the hydrolysis reaction catalyzed by GA had not previously been studied as an inhibitor of GA activity; however, GA had been reported[5] to catalyze an L-aspartic acid C-4 β-carboxyl(oxygen)-water exchange reaction, which was used to support a mechanism analogous to the mechanism in serine proteinases by formation of a tetrahedral structure (Figure 1). We found that L-Asp (**2**) is a competitive inhibitor of GA activity with a K_i 0.6 mM, and, therefore, L-Asp is both a substrate and a competitive inhibitor of GA as are β-L-aspartyl hydroxamate and L-Asn. The value of K_i for L-Asp (0.6 mM) is comparable to that for L-Asn (0.454 mM). We also studied as inhibitors four analogues of L-Asp where the α-amino group was replaced with an α-chloro (**3**), α-bromo (**4**), α-methyl (**5**), and hydrogen (**6**). Each of these molecules is a competitive inhibitor of GA activity, as illustrated in Figure 3 with the Dixon plot for the α-chloro analogue (**3**). These molecules are poorer inhibitors than L-Asp with K_i values slightly greater, except for L-2-methyl-succinic acid (**5**) which has a K_i value (0.7 mM) comparable to L-Asp. These results show that groups of smaller or comparable size to the α-amino group may be substituted for the α-amino group, and still compete effectively with substrate for the active site. While at first this appears to be surprising, it is consistent with a proposed intramolecular autoproteolytic reaction. GA is synthesized as a 346 amino acid zymogen that undergoes post-translational processing to form a heterodimeric structure consisting of a 24 kDa heavy (H)-subunit (α-subunit) and a 17 kDa light (L)-subunit (β-subunit) at maturation.[2] The formation of the two subunits is proposed to occur as an intramolecular autoproteolytic reaction[21] that involves His-204, Asp-205, and Thr-206, which are absolutely conserved in all sequences of GA known.[22–24] The side chain of Asp-205 is proposed to move into the binding pocket of the enzyme, which places the side chains of His-204 and Thr-206 in the correct positions to autocatalyze the hydrolysis of the peptide bond between Asp-205 and Thr-206 to give the two subunits. Site-directed mutagenesis studies provide strong supporting evidence for this reaction.[25,26] The side chain β-carboxyl group of Asp-205 would occupy the position where the α-carboxyl group of the substrate binds, but there is, of course, no β-amino group that would occupy the binding site where the α-amino group of the substrate binds. In this proposed autoproteolytic activation reaction an amino group is not required to be present, but a carboxyl group is necessary for the reaction. Our results are additional supporting evidence for this proposed intramolecular autoproteolytic reaction. Another proposed intramolecular proteolytic mechanism[27] where Asp-205 acts as a general base is not precluded by our results. Substrate analogues synthesized using **3**, **4**, **5**, and **6** would be expected to be substrates for GA, and, in fact, each of the N^4-GlcNAc analogues of **3**, **4**, **5**, and **6** are substrates for GA (unpublished results).

Evidence thusfar supports a mechanism for glycosylasparaginase analogous to serine proteinases. This involves formation of a tetrahedral structure (Figure 1) during the hydrolysis reaction. A wide variety of transition state isosteres for hydrolysis of the peptide-amide bond has been described, and in particular phosphono and sulfo analogues.[28,29] Those that have been studied as transition state mimics of the tetrahedral structure in proteinase reactions have been shown to be inhibitors of the hydrolysis reaction at values of K_i ranging from nM to mM. We studied the inhibition of the phosphono analogue **7** and sulfo analogue **8** as transition state mimics of the GA reaction. Each of these

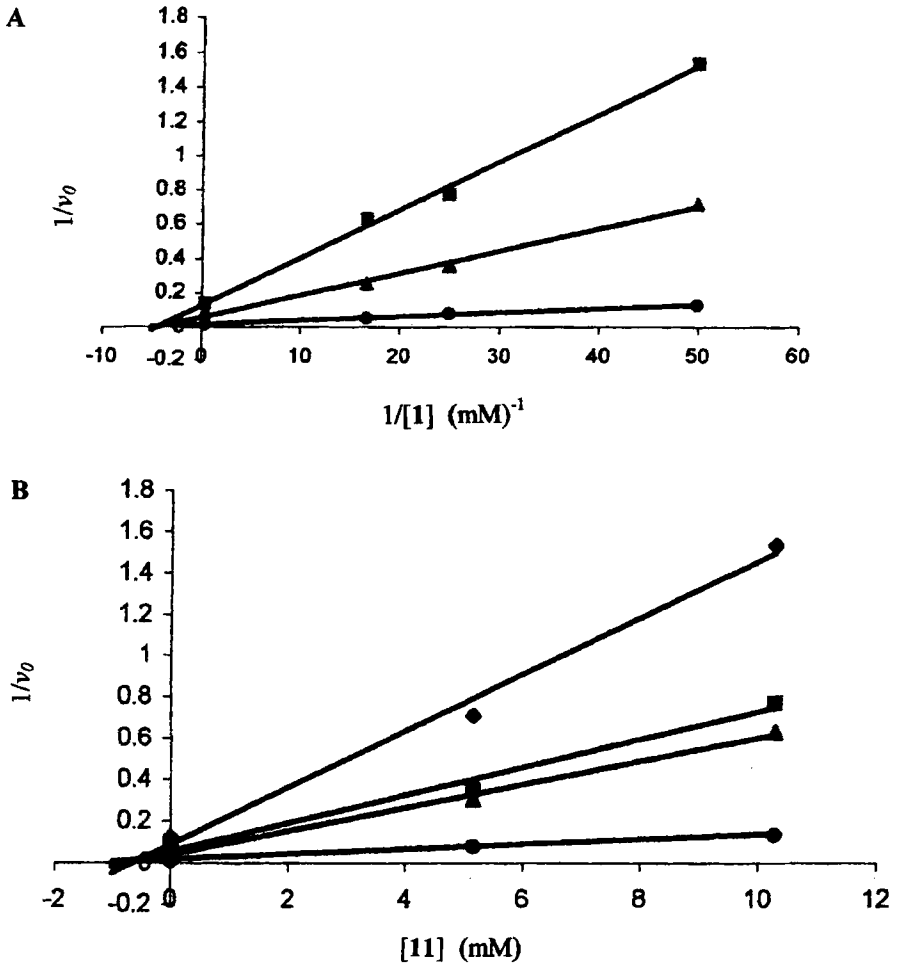


FIGURE 4 The double reciprocal plot (A) and Dixon plot (B) for noncompetitive inhibition of glycosylasparaginase activity by *N*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)chloroacetamide (**11**). The concentrations of (GlcNAc-)Asn used were 0.02 mM, 0.04 mM, 0.06 mM, and 2.8 mM. The K_i value is 0.64 mM.

molecules was a competitive inhibitor of GA activity with K_i's (0.9 mM and 1.4 mM, respectively) of the same order as for L-Asp and L-Asn. L-Cys (**9**) was used as a reference standard for the inhibition of the sulfo analogue **8**; L-Cys is not an inhibitor of GA activity, and, therefore, the inhibition by L-cysteic acid (**8**) arises as a result of the oxidation of the thiol of **9**. While good inhibitors of GA activity, these transition state mimics are somewhat poorer inhibitors than might be expected. This is particularly surprising since **7** and **8**, if not mimicking the transition state of the natural substrate exactly, should mimic the transition state of the L-aspartic acid C-4 β-carboxyl(oxygen)-water exchange reaction catalyzed by GA. However, many inhibitors of serine and cysteine proteinases have been found to be rather poor inhibitors of proteasomes,[30] which is a member of the *N*-terminal nucleophile amidohydrolases superfamily, as is GA.[2] Therefore, this particular catalytic mechanism of GA may have an effect on the inhibition reaction at the active site compared with amidohydrolases not in this superfamily.

Previously only *N*-acetylcysteine had been reported to act as a noncompetitive inhibitor of

GA activity. We found that three analogues of (GlcNAc-)Asn, **10–12**, were noncompetitive inhibitors of GA activity, as illustrated in Figure 4 with the double reciprocal and Dixon plots for analogue **11**. The K_i values (0.75 mM for **10**, 0.64 mM for **11**, 0.56 mM for **12**) are of the same order of magnitude as for *N*-acetylcysteine (3.2 mM). Thus, there appears to be a separate binding site on GA to modify the catalytic process. The structural similarity between these four molecules is the (substituted)acetamido group, for which there may be a strong affinity at a second binding site. The 2-acetamido group on GlcNAc would appear not to be in the correct orientation for GlcNAc to act as a noncompetitive inhibitor since GlcNAc has been reported not to be an inhibitor of GA activity.[31] Additional studies of this inhibition are warranted to clarify further the nature of this second binding site.

Acknowledgments

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