

## Glycosylasparaginase-catalyzed Synthesis and Hydrolysis of $\beta$ -Aspartyl Peptides\*

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**$\beta$ -Aspartyl di- and tripeptides are common constituents of mammalian metabolism, but their formation and catabolism are not fully understood. In this study we provide evidence that glycosylasparaginase (aspartylglucosaminidase), an N-terminal nucleophile hydrolase involved in the hydrolysis of the N-glycosidic bond in glycoproteins, catalyzes the hydrolysis of  $\beta$ -aspartyl peptides to form L-aspartic acid and amino acids or peptides. The enzyme also effectively catalyzes the synthesis of  $\beta$ -aspartyl peptides by transferring the  $\beta$ -aspartyl moiety from other  $\beta$ -aspartyl peptides or  $\beta$ -aspartylglycosylamine to a variety of amino acids and peptides. Furthermore, the enzyme can use L-asparagine as the  $\beta$ -aspartyl donor in the formation of  $\beta$ -aspartyl peptides. The data show that synthesis and degradation of  $\beta$ -aspartyl peptides are new, significant functions of glycosylasparaginase and suggest that the enzyme could have an important role in the metabolism of  $\beta$ -aspartyl peptides.**

$\beta$ -Aspartyl and  $\gamma$ -glutamyl peptides are normal constituents of mammalian urine (1, 2) and tissues (3). Although  $\gamma$ -glutamyltransferase (EC 2.3.2.2, GGT) (1)<sup>1</sup> is the key enzyme in the synthesis and hydrolysis of  $\gamma$ -glutamyl compounds such as glutathione in the  $\gamma$ -glutamyl cycle (4), little is known about the metabolism of  $\beta$ -aspartyl peptides.

Glycosylasparaginase (GA; aspartylglucosaminidase;  $N^4$ -( $\beta$ -N-acetyl-D-glucosaminyl)-L-asparaginase; EC 3.5.1.26) is a lysosomal amidase that hydrolyzes the N-glycosidic carbohydrate-to-protein linkage ( $N^4$ -( $\beta$ -N-acetyl-D-glucosaminyl)-L-

asparagine;  $\beta$ -aspartylglucosamine; GlcNAc-Asn) during degradation of glycoproteins. Genetic deficiency of glycosylasparaginase causes a lysosomal storage disease aspartylglycosaminuria (McKusick 208400) that is the most common disorder of glycoprotein degradation in humans and is clinically characterized by severe mental and motor retardation (5, 6). Glycosylasparaginase is a member of the recently described structural superfamily of enzymes termed as N-terminal nucleophile (Ntn) hydrolases (7). The hydrolysis of  $\beta$ -aspartylglycosylamines catalyzed by glycosylasparaginase is initiated by the binding of the  $\beta$ -aspartyl moiety into the active site of the enzyme through its free  $\alpha$ -amino and  $\alpha$ -carboxyl groups (8, 9). The enzyme uses the  $\gamma$ -hydroxyl and  $\alpha$ -amino group of its  $\beta$ -chain N-terminal threonine as an active site nucleophile and general base in the formation of  $\beta$ -aspartyl enzyme, which is subsequently deacylated by water to L-aspartic acid (10, 11). The GA-catalyzed hydrolysis of L-asparagine occurs in a similar manner, resulting in the formation of  $\beta$ -aspartyl enzyme and ammonia (12).

The mechanism of action of glycosylasparaginase and the structural properties of its substrate-binding site (13) led us to consider that the enzyme might have a role in the metabolism of  $\beta$ -aspartyl peptides. In the present study, we demonstrate that synthesis and degradation of  $\beta$ -aspartyl peptides are new significant functions of glycosylasparaginase. This suggests that glycosylasparaginase could have an important role in the metabolism of  $\beta$ -aspartyl peptides present in body fluids and tissues.

### EXPERIMENTAL PROCEDURES

**Materials**—GA was purified to homogeneity from an NIH-3T3 cell line overexpressing recombinant human glycosylasparaginase (14), and the purification protocol included caprylic acid precipitation, affinity chromatography with concanavalin A lectin, gel filtration, hydrophobic interaction chromatography, and anion exchange chromatography (10). The fractions containing GA activity were pooled, and the purity of this enzyme preparation was estimated to be over 90% on SDS-polyacrylamide gel electrophoresis after silver staining, and its glycosylasparaginase activity toward AspAMC (15) was 344 milliunits/mg.  $\beta$ -Aspartame and  $\beta$ -aspartylglycine were purchased from Bachem Feinchemikalien AG (Bubendorf, Switzerland). Other peptides were prepared as described (16, 17), and their chemical and chiral purity was >99% according to HPLC (18), amino acid, and <sup>1</sup>H-NMR (17) analyses. H-Ser-NH<sub>2</sub> and other amides were prepared as described elsewhere (16, 19).  $\beta$ -Glycosylamine (1-amino-N-acetylglucosamine; GlcNAc-NH<sub>2</sub>),  $\beta$ -aspartylglucosamine (GlcNAc-Asn), and aspartic acid  $\beta$ -methyl ester (H-Asp(OMe)-OH) were products of Sigma.

**Enzyme Assays**—Glycosylasparaginase activity of the purified enzyme was measured with a fluorometric method using AspAMC as substrate (15). The kinetic parameters for glycosylasparaginase-catalyzed hydrolysis of  $\beta$ -aspartyl compounds were determined with a spectrophotometric assay (20) as described in detail (21). The least square Lineweaver-Burk analysis was used in the determination of Michaelis constant ( $K_m$ ) and maximum reaction velocity ( $V_{max}$ ).

**Assay of  $\beta$ -Aspartyl Peptides**—Formation of  $\beta$ -aspartyl peptides during their GA-catalyzed synthesis was measured as described (18). Various amounts of  $\beta$ -aspartyl donors and  $\beta$ -aspartyl acceptors were incubated in the presence of glycosylasparaginase (12.2 units/liter) in 50 mM Tris-HCl buffer, pH 7.5, at +37 °C. Aliquots of the incubation mixture were injected onto the HPLC column, and the formation of  $\beta$ -aspartyl peptides was measured. High performance liquid chromatography was performed as described previously (18) using a 250 × 4.6 mm (inner diameter) amino column (Spherisorb-NH<sub>2</sub>, 5- $\mu$ m particles), isocratic elution with 2.5 mM KH<sub>2</sub>PO<sub>4</sub>/acetonitrile (40/60 v/v, pH 4.6), flow rate 1 ml/min and detection wavelength 214 nm.

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<sup>1</sup> The abbreviations used are: GGT,  $\gamma$ -glutamyltransferase; H-Asp(Phe-OMe)-OH,  $\beta$ -aspartame; AspAMC, L-aspartic acid  $\beta$ -(7-amido-4-methylcoumarin); H-Asp(Ser-NH<sub>2</sub>)-OH,  $\beta$ -aspartylserineamide; GA, glycosylasparaginase; GlcNAc-Asn, 2-acetamido-1- $\beta$ -aspartamido-1,2-dideoxy- $\beta$ -D-glucose; HPLC, high performance liquid chromatography; Ntn, N-terminal nucleophile; H-Ser-NH<sub>2</sub>, serineamide.

TABLE I  
Kinetic parameters for glycosylasparaginase-catalyzed hydrolysis of  $\beta$ -aspartyl peptides,  $\beta$ -aspartylglucosamine, and L-asparagine

| Compound                         | $k_{cat}/K_m$   | $V_{max}$                | $K_m$   | Relative rate |
|----------------------------------|-----------------|--------------------------|---------|---------------|
|                                  | $s^{-1} M^{-1}$ | $\mu M \text{ min}^{-1}$ | $\mu M$ | %             |
| H-Asp(GlcNAcNH)-OH               | 6560            | 3.64                     | 250     | 100           |
| H-Asn-OH                         | 1310            | 1.49                     | 538     | 41            |
| H-Asp(Phe-OMe)-OH                | 1240            | 0.84                     | 305     | 23            |
| H-Asp(Ser-NH <sub>2</sub> )-OH   | 220             | 2.58                     | 5315    | 71            |
| H-Asp(Gly-NH <sub>2</sub> )-OH   | 210             | 0.55                     | 1160    | 15            |
| H-Asp(Gly-Gln-OH)-OH             | 90              | 0.98                     | 4880    | 27            |
| H-Asp(Ala-NH <sub>2</sub> )-OH   | 80              | 1.27                     | 7050    | 35            |
| H-Asp(Gly-OH)-OH                 | 50              | 0.11                     | 870     | 3             |
| H-Asp(Gly-Val-OH)-OH             | 40              | 1.78                     | 21590   | 49            |
| H-Asp((Gly) <sub>4</sub> -OH)-OH | 13              | 0.29                     | 1090    | 8             |

## RESULTS

**Glycosylasparaginase-catalyzed Hydrolysis of  $\beta$ -Aspartyl Peptides**—A series of  $\beta$ -aspartyl peptides were tested as potential substrates for glycosylasparaginase. The relative hydrolysis rates of  $\beta$ -aspartyl peptides were lower and Michaelis constants higher than those toward  $\beta$ -aspartylglucosamine, but in most cases the hydrolysis rates were close to that of Asn (Table I). The lowest hydrolysis rates were measured with  $\beta$ -aspartyl glycine peptides. Interestingly, the relative hydrolysis rate of  $\beta$ -aspartylglycineamide was 5-fold higher than that of the corresponding unsubstituted peptide. The  $\beta$ -aspartylphenylalanine methyl ester ( $\beta$ -aspartame) was also hydrolyzed by glycosylasparaginase, suggesting that GA-catalyzed hydrolysis provides a catabolic route for the cleavage of the  $\beta$ -aspartyl residue in this constitutional isomer of the commonly used sweetener  $\alpha$ -aspartame.

**Glycosylasparaginase-catalyzed Synthesis of  $\beta$ -Aspartyl Peptides**—In the presence of serineamide and L-asparagine, glycosylasparaginase catalyzed the formation of  $\beta$ -aspartylserineamide (Fig. 1). In the reaction Asn acted as  $\beta$ -aspartyl donor and serineamide as  $\beta$ -aspartyl acceptor in a kinetically controlled synthesis that closely resembles the glycosylasparaginase-catalyzed synthesis of the N-glycosidic bond by  $\beta$ -aspartylation of glycosylamines (18).  $\beta$ -Aspartylglucosamine and  $\beta$ -aspartame can also function as the  $\beta$ -aspartyl donor as demonstrated by the formation of  $\beta$ -aspartylserineamide in the presence of serineamide as an added nucleophile (Fig. 1). Similar experiments with either L-asparagine or aspartic acid methyl ester (H-Asp(OMe)-OH) as  $\beta$ -aspartyl donors and threonineamide, glycineamide, and leucineamide as  $\beta$ -aspartyl acceptors resulted in the GA-catalyzed synthesis of  $\beta$ -aspartylthreonineamide,  $\beta$ -aspartylglycineamide, and  $\beta$ -aspartylleucineamides, respectively (data not shown). Similarly, the dipeptide H-Gly-Leu-NH<sub>2</sub> was an effective  $\beta$ -aspartyl acceptor in the synthesis of  $\beta$ -aspartylglycylleucine (data not shown), suggesting that larger peptides can also act as nucleophiles in the reaction. On the contrary, no L-asparagine transamidation was detected with glutamic  $\alpha$ -amide as the nucleophile, suggesting that the  $\beta$ -aspartyl acceptor site does not interact favorably with a charged acceptor. No formation of  $\beta$ -aspartyl peptides was detected in the absence of glycosylasparaginase, indicating that the reaction components by themselves are virtually unreactive.

## DISCUSSION

The crystal structure of the glycosylasparaginase-aspartate complex revealed the binding mode of the zwitterionic aspartyl group but implied only that the hydrophobic residues Trp<sup>11</sup> and Phe<sup>278</sup> of the enzyme protein might be involved in the binding of the leaving (oligosaccharidyl) group (13). This kind of hydrophobic and roomy oligosaccharidyl-binding site subsite could

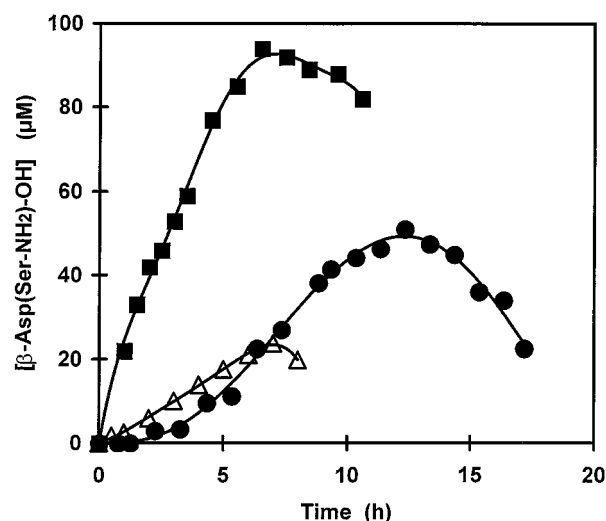


FIG. 1. Time dependence of H-Asp(Ser-NH<sub>2</sub>)-OH formation in the GA-catalyzed  $\beta$ -aspartylation of H-Ser-NH<sub>2</sub>. The reaction mixtures containing GA (12.2 units/liter) were incubated in 50 mM Tris-HCl buffer, pH 7.4, at 37 °C in the presence of H-Ser-NH<sub>2</sub> (100 mM) and Asn (4.4 mM) ( $\Delta$ ), H-Ser-NH<sub>2</sub> (200 mM) and GlcNAc-Asn (13 mM) ( $\blacksquare$ ), or H-Ser-NH<sub>2</sub> (100 mM) and  $\beta$ -aspartame (6 mM) ( $\bullet$ ).

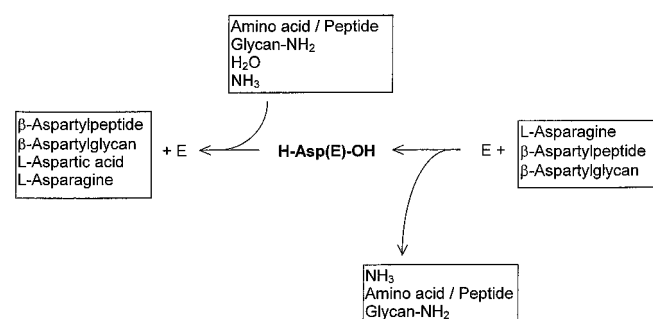


FIG. 2. Hydrolase and transferase activities of human glycosylasparaginase.  $\beta$ -Aspartyl donors and acceptors are shown in the boxes on the right and top, and the synthesis products are shown on the left, respectively.

well accommodate the hydrophobic and bulky leaving group of  $\beta$ -aspartyl peptides. In fact,  $\beta$ -aspartame is hydrolyzed by glycosylasparaginase with kinetic parameters of the same order of magnitude as that of  $\beta$ -aspartylglycosylamine (Table I). Because the reaction components by themselves are virtually unreactive and no formation of  $\beta$ -aspartyl peptides was detected in the absence of glycosylasparaginase, the oligosaccharidyl-binding site in the enzyme must interact with the acceptor nucleophiles in the transamidase and transpeptidase reactions.

Glycosylasparaginase has high activity in brain tissue (22),

and it is tempting to speculate that GA-catalyzed  $\beta$ -aspartylation of amino acids within the tissue would lead to the presence of  $\beta$ -aspartyl peptides such as  $\beta$ -aspartylglycine and  $\beta$ -aspartyltaurine in mammalian brain (3). Whether their presence in the central nervous system reflects another system for the uptake of certain amino acids and/or a system for regulation of excitatory amino acid transmission remains to be investigated.  $\beta$ -Aspartyl peptides are also normal constituents of human urine (2). Tanaka *et al.* (23) reported the formation of  $\beta$ -aspartyl dipeptides in the supernatant of rat kidney, but they were not able to characterize the enzymatic system involved in this reaction. Based on their report and the data presented here, we suggest that the enzyme was glycosylasparaginase. The combined evidence indicates that glycosylasparaginase-catalyzed reactions provide a plausible enzymatic system, which is involved in the metabolism of  $\beta$ -aspartyl peptides present in body fluids and tissues. The demonstration that this enzyme can catalyze the synthesis and degradation of  $\beta$ -aspartyl peptides is important because it implicates that the metabolic defect in aspartylglycosaminuria is not restricted to disorders of glycoprotein degradation (24) but also affects the metabolism of  $\beta$ -aspartyl peptides. How this might relate to the development of the clinical symptoms in aspartylglycosaminuria remains to be investigated. The recently developed glycosylasparaginase-deficient mouse strain (25) will be a valuable experimental tool for the studies of the metabolism and functions of  $\beta$ -aspartyl peptides.

The glycosylasparaginase-catalyzed hydrolase and transferase activities are schematically illustrated in Fig. 2. We propose the name glycosylasparaginase/ $\beta$ -aspartyltransferase for the multifunctional enzyme that catalyzes the enzymatic synthesis and degradation of  $\beta$ -aspartyl peptides, cleaves the *N*-glycosidic bond during degradation of glycoproteins (26), and possesses L-asparaginase activity (12). Because GGT is also an Ntn hydrolase (7) and there are many biochemical similarities between GGT and GA, we predict that the binding mode of the nucleophile amino acids and peptides into the active site of GGT resembles the nucleophile binding to GA. The unique structural features of Ntn hydrolases may account for their

wide substrate specificity and the multifunctional properties found in this superfamily of enzymes.

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