Recombinant human glycosylasparaginase catalyzes hydrolysis of L-asparagine

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Received 6 May 1997; revised version received 9 June 1997

Abstract Glycosylasparaginase is a lysosomal amidase involved in the degradation of glycoproteins. Recombinant human glycosylasparaginase is capable of catalyzing the hydrolysis of the amino acid L-asparagine to L-aspartic acid and ammonia. For the hydrolysis of L-asparagine the K_m is 3–4-fold higher and V_{max} 1/5 of that for glycoasparagines suggesting that the full catalytic potential of glycosylasparaginase is not used in the hydrolysis of the free amino acid. L-Asparagine competitively inhibits the hydrolysis of aspartylglucosamine indicating that both the amino acid and glycoasparagine are interacting with the same active site of the enzyme. The hydrolytic mechanism of L-asparagine and glycoasparagines will be discussed.

Key words: Glycosylasparaginase; Lysosomal enzyme; Asparaginase; L-asparagine metabolism

1. Introduction

Bacterial and plant L-asparaginases catalyze the hydrolysis of L-asparagine (Asn) to L-aspartic acid (Asp) and ammonia (Reaction 1) [1]:

$$\text{L-asparagine} + H_2O \rightarrow \text{L-aspartic acid} + NH_3 \quad (1)$$

Glycosylasparaginase (GA; aspartylglucosaminidase; N^4-(β-*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-(β-*N*-acetyl-D-glucosaminyl)-L-asparagine, aspartylglucosamine, GlcNAc-Asn) during the degradation of glycoproteins (Reaction 2) [2,3].

$$N^4\text{-(β-}N\text{-acetyl-D-glucosaminyl)-L-asparagine} + H_2O \rightarrow \text{L-asparticacid} + \text{L-amino-}N\text{-acetylglucosamine} \quad (2)$$

Plant and bacterial L-asparaginases and mammalian glycosylasparaginases have many properties in common including marked sequence homology [4,5] and irreversible inhibition by an L-asparagine analogue, 5-diazo-4-oxo-L-norvaline (DONV) [4,6,7]. The structural properties of the substrate binding site of human glycosylasparaginase [8] led us to reconsider L-asparagine as a potential substrate for human glycosylasparaginase. In the present study, we demonstrate that the hydrolysis of both L-asparagine and glycoasparagines is catalyzed by the recombinant human glycosylasparaginase.

2. Materials and methods

2.1. Materials

GA was purified from an NIH-3T3 cell line overexpressing recombinant human placental GA [4,9], and the purification protocol involved caprylic acid precipitation, affinity chromatography, gel filtration, hydrophobic interaction chromatography and anion exchange chromatography [4]. The fractions containing GA activity were pooled and the purity of this enzyme preparation was estimated to be over 90% on SDS-PAGE electrophoresis after silver staining and its glycosylasparaginase activity was 344 mU/mg towards AspAMC [10]. Total protein was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Aspartylglucosamine, phenylisothiocyanate and carboxymethyl cysteine were products of Sigma Chemical Co., St. Louis, MO, USA. Asparagine and aspartic acid were purchased from E. Merck, Darmstadt, Germany. All the other reagents were of analytical grade and they were used without further purification. High-performance liquid chromatography (HPLC) was carried out with a Merck/Hitachi L-6200 liquid chromatograph (Hitachi Ltd., Tokyo, Japan). The column was Spherisorb S3 ODS2 (150×4.6 mm, i.d.) (Phase Separations Ltd., Deeside, UK).

2.2. Enzyme assays

Glycosylasparaginase assay was based on HPLC analysis of the reaction components GlcNAc-Asn, Asn and Asp after their phenylisothiocyanate (PITC) derivatization [11,12]. Carboxymethyl cysteine (CmCys) was used as an internal standard. The kinetic and inhibition constants were determined at +22°C, and the incubation mixture contained various amounts of GlcNAc-Asn and/or Asn, 0.8 mM CmCys and 60.8 μU GA (0.18 μg) in 50 mM sodium-potassium phosphate buffer, pH 7.5 in a total volume of 50 μl.

The pH optimum of GA hydrolase activity was studied in Britton-Robinson's universal buffer [4,13] over a pH range of 2.75–11.65 in intervals of 0.5 pH units. The incubation mixture consisted either of 1.5 mM Asn, 0.8 mM CmCys and 125 μU of GA (0.36 μg) or 0.6 mM GlcNAc-Asn, 0.8 mM CmCys and 50 μU of GA (0.15 μg) in a total volume of 50 μl, and samples were taken for the HPLC assay usually at 0, 1, 2, 3, 4 and 6 h. The change in the substrate/internal standard ratio during the incubation was measured and compared to the standard curve.

3. Results

Authentic L-asparagine, L-aspartic acid and CmCys were individually derivatized and analyzed by HPLC. Fig. 1A demonstrates that the compounds were well separated from one another. The chromatograms of samples removed at different time points (0, 6 and 22 h) from the reaction mixture initially containing Asn, CmCys and human recombinant GA are presented in Fig. 1B–D. The results demonstrate that L-aspara-

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Abbreviations: Asn, L-asparagine; Asp, L-aspartic acid; AspAMC, L-aspartic acid β-(7-amido-4-methylcoumarin); GA, glycosylasparaginase; AGU, aspartylglycosaminuria; HPLC, high-performance liquid chromatography; SDS-PAGE, sodiumdodecylsulphate polyacrylamide gel electrophoresis; GlcNAc-Asn, N^4-(β-*N*-acetyl-D-glucosaminyl)-L-asparagine, 2-acetamido-1-L-β-aspartamido-1,2-dideoxy-β-D-glucose, aspartylglucosamine; CmCys, carboxymethylcysteine; PITC, phenylisothiocyanate

PII S0014-5793(97)00761-8

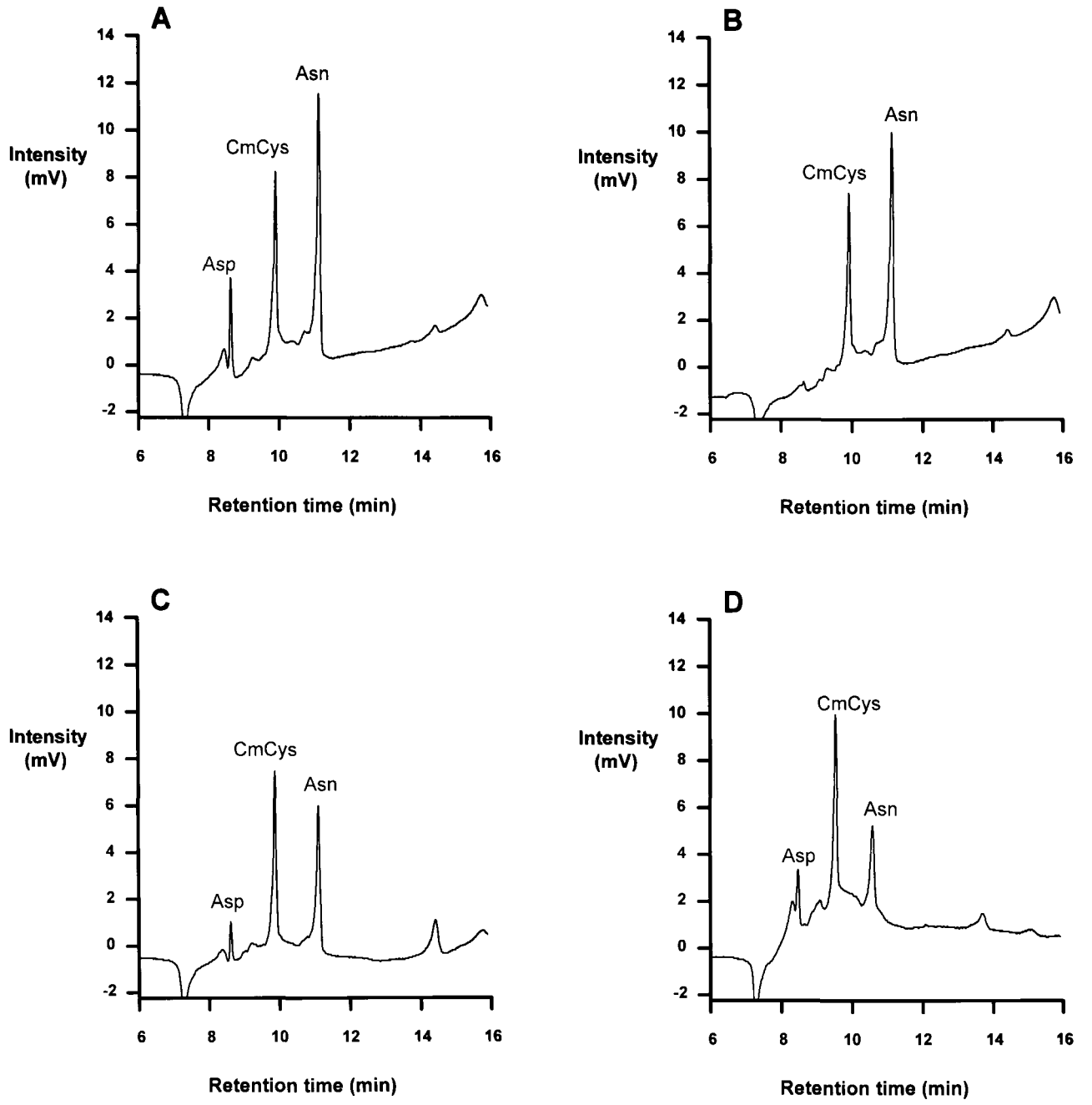

Fig. 1. Characterization of components in the glycosylasparaginase-catalyzed reaction. HPLC chromatogram of a standard mixture containing equimolar amounts of Asp, CmCys and Asn (A), a reaction mixture containing 1.5 mM Asn after incubation at +37°C in the presence of glycosylasparaginase for 0 h (B), 6 h (C) and 22 h (D). Asp, Asn and CmCys eluted at 8.6, 9.9 and 11.2 min, respectively.

gine is hydrolyzed to L-aspartic acid by human glycosylasparaginase, since this conversion was not observed in a blank reaction that was incubated 22 h in the absence of the enzyme (data not shown). In the conditions that were used to determine the kinetic constants for the hydrolysis of Asn, the reaction was linear up to 480 min. The within-day variation of the enzyme assay, based on the substrate/internal standard ratio, was less than 7% ($n = 10$). The GA-catalyzed hydrolysis of both Asn and GlcNAc-Asn showed a wide pH-optimum range over pH 6–9 (Fig. 2). At pH 7.5, the K_m and V_{max} of human recombinant GA for Asn were 656 μM and 1.49 μM min^{-1} and for GlcNAc-Asn 208 μM and 7.23 μM min^{-1}, respectively (Fig. 3). The maximal reaction velocity for the hydrolysis of Asn with GA was 21% of that obtained with the best natural substrate, GlcNAc-Asn. The presence of Asn in the reaction mixture competitively inhibited GlcNAc-Asn degradation by the enzyme with an inhibition constant K_i of 454 μM (Fig. 4).

These facts indicate that the same human enzyme degrades L-asparagine as well as a variety of glycoasparagines. The hydrolytic reactions catalyzed by human glycosylasparaginase are summarized in Fig. 5.

4. Discussion

Tanaka et al. [14] have shown that hog serum and kidney

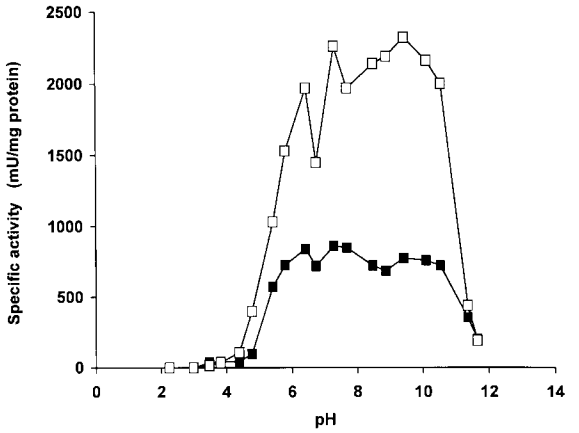

Fig. 2. Effect of pH on the rate of GA-catalyzed hydrolysis of Asn (■) and GlcNAc-Asn (□). The rate of substrate loss is expressed as mU/mg protein.

glycosylasparaginases have L-asparaginase activity whereas glycosylasparaginases from rat liver and kidney do not possess any activity towards the amino acid L-asparagine [15]. Glycosylasparaginases from *Flavobacterium meningosepticum* [16] and insect *Spodoptera frugiperda* [17] also show asparaginase activity. This indicates that there is species variation in the substrate specificity of different glycosylasparaginases. While studying potential substrates of human GA by using a colorimetric assay of L-aspartic acid, the hydrolysis of L-asparagine remained undetected [3]. The three dimensional structure of the active site of GA [8] led us to re-evaluate Asn as a potential substrate of human GA with improved analytical techniques. Both substrate Asn and product Asp in the reaction mixture were characterized and assayed with high-performance liquid chromatography. HPLC possesses several distinct advantages over the earlier used colorimetric assay including separation, identification and quantitation of the reaction components, and it is not subject to errors due to

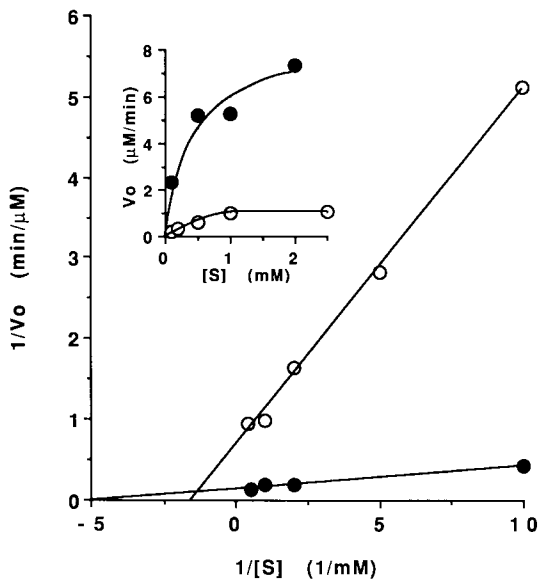

Fig. 3. L-Asparagine and aspartylglucosamine as substrates for human glycosylasparaginase. The effect of Asn (○) and GlcNAc-Asn (●) concentration on the velocity of the glycosylasparaginase-catalyzed reaction (inset) and the corresponding Lineweaver-Burk plots.

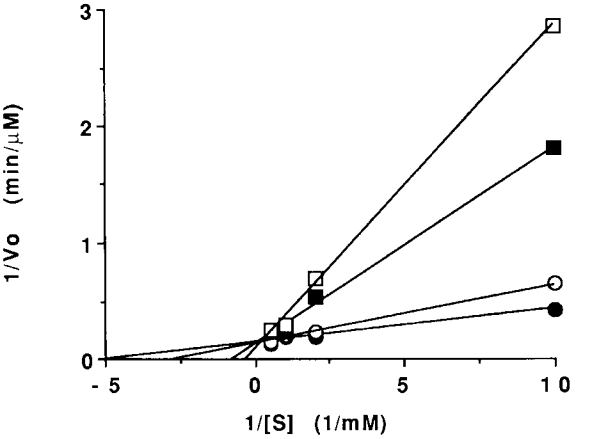

Fig. 4. Determination of the inhibitor constant K_i for Asn with GlcNAc-Asn as a substrate. Various GlcNAc-Asn concentrations were incubated with 5 (□), 2 (■) and 0.5 mM (○) Asn and without Asn (●) A double-reciprocal plots of the reaction velocity versus GlcNAc-Asn concentration are shown.

non-specific color formation which is a major problem with biological specimens.

Human GA catalyzed the hydrolysis of L-asparagine to L-aspartic acid and ammonia. The K_m for the hydrolysis of Asn was 3–4-fold higher than that for glycoasparagines [3] whereas the V_{max} was approximately 1/5 of that obtained for GlcNAc-Asn. This suggests that the full catalytic potential of GA is not used in hydrolysis of Asn since the lack of glycosyl groups prevents effective interaction with the leaving group binding pocket of the enzyme. The pH optimum for GA-catalyzed hydrolysis of Asn was between 6 and 9 that is similar to that of rat [15,18], mouse [18], *Spodoptera frugiperda* [17] and human leukocyte [4] GA in the hydrolysis of GlcNAc-Asn. Such a profile suggests that the enzyme could function outside the lysosomes although it is mainly located within these organelles. Asn competitively inhibited GlcNAc-Asn degradation by GA with an inhibitory constant (K_i = 454 μM) close to the K_m value of GA-catalyzed hydrolysis of GlcNAc-Asn (208 μM) indicating that both compounds are interacting with the same active site of the enzyme. Thus in the GA-catalyzed hydrolysis of glycoasparagines [3,14], Asn is bound into the active site of the enzyme through its free α-amino and α-carboxyl groups. The N-atom attached to C4 of L-asparagine forms a hydrogen bond with Thr-206, which acts as a nucleophile [8,19]. The β-aspartyl intermediate is formed and subsequently L-aspartic acid and ammonia are released.

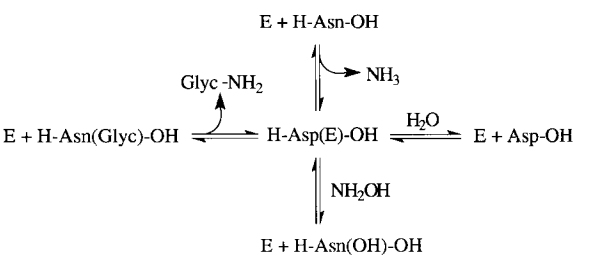

Fig. 5. A scheme for the reactions catalyzed by human glycosylasparaginase. H-Asn-OH, L-asparagine; Asp-OH, L-aspartic acid; NH_3, ammonia; E, glycosylasparaginase; H-Asp(E)-OH, β-aspartyl enzyme intermediate; H-Asn(Glyc)-OH, glycoasparagine; Glyc-NH_2, carbohydrate moiety with 1-amino-*N*-acetylglucosamine; H-Asn(OH)-OH, L-aspartic acid β-hydroxamate [2,3,22].

A deficient activity of GA causes a lysosomal storage disease, aspartylglycosaminuria (AGU; McKusick 208400) (for review: [20]). The present results indicate that the potentially deficient degradation of Asn in AGU must be considered and investigated. The recently described mouse model for AGU [21] will be very helpful in these studies.

Acknowledgements: This work has been financially supported by a grant from the Sigrid Juselius Foundation (I.M.) and the National Research Fund (Bulgaria) Project X-572.

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