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Aspartylglucosaminidase Activity in Rat Central Nervous System — a Histochemical Study

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Aspartylglucosaminidase (AGA, EC 3.5.1.26) is involved in the final stages of glycoprotein hydrolysis in the lysosomes. Its genetically determined deficiency causes the lysosomal storage disease aspartylglucosaminuria (AGU), clinical symptoms of which include progressive psychomotor retardation, diminished communication skills, grotesque facial appearance and skeletal abnormalities, i.e. a number of symptoms pointing out a substantial decline in neuronal function. In this paper, the enzyme distribution in rat central nervous system (CNS) is studied by a newly synthesized artificial substrate — β-Asp-2-naphthylamide used after a simple simultaneous azo-coupling procedure. This method reveals for the first time a high AGA activity in neurons of various regions in rat brain (cerebrum, cerebellum, medulla oblongata) and spinal cord.

Key words: aspartylglucosaminidase, aspartylglucosaminuria, enzyme histochemistry, central nervous system.

Introduction

Aspartylglucosaminidase (glycosylasparaginase, AGA, EC 3.5.1.26) is a lysosomal amidase hydrolyzing the N-glycoside bond between L-asparagine (Asn) and N-acetyl-D-glucosamine (GlcNAc) in the core of N-linked glycoproteins. The enzyme requires free α-amino and α-carboxyl groups of Asn and is less specific towards the carbohydrate moiety attached to it [7]. Thus, AGA is responsible for the very final step of N-glycoproteins digestion in lysosomes. The enzyme has been shown to possess a surprisingly high pH optimum of 7.6 [2]. The discrepancy between lysosomal localization of AGA and its alkaline pH optimum is not explained yet. Genetically determined AGA deficiency leads to a most common lysosomal storage disease that directly involves glycoprotein metabolism, named aspartylglucosaminuria (AGU). AGU is connected with piling up of non-degraded N-acetylglucosaminyl-L-asparagine (GlcNAc-Asn) within lysosomes and its excretion with the urine [11]. Clinical symptoms of AGU are usually developed in the late puberty and include progres-

sive psychomotor retardation, diminished communication skills, grotesque facial appearence and skeletal abnormalities [8]. AGU patients have a relatively long survival — up to 45-50 years of age. Presently, AGU is studied using experimental models of targeted disruption of mice AGA gene (knock-out mice). AGU mice exhibit similar pathophysiology as human patients. Very specific characteristics of those mice brains at autopsy are the enlarged lysosomes in the CNS and loss of Purkinje cells in the cerebellar cortex [5]. Thus, both clinical symptoms and morphological characteristics in the experimental model of AGU point out that AGA might be present in the neurons of CNS. Biochemical experiments of Conchie and Strachan [3], however, have shown that in rat and mouse brain AGA activity is comparatively low. No histochemical study of the enzyme distribution in the CNS of laboratory animals has been performed so far.

The aim of the present study was to develop a specific synthetic substrate for AGA and using it, to study the enzyme activity distribution in the CNS of Wistar rats. The results are expected to help in elucidation of AGA importance for neuronal function and to be useful for the studies of animal models of AGU.

Materials and Methods

Synthesis of AGA substrate — β-Asp-2-naphthylamide. TFA (Asp-NA) and AGA inhibitor - 5-diazo-4-oxo-L-norvaline (DONV). The substrate was synthesized using the ordinary DCC (dicyclohexycarbodiimide) method [1]. In brief, equimolar amounts of 2-naphthylamine (NA, Aldrich), Boc-Asp(OH)-OtBu (Novabiochem) and DCC (Fluka) were mixed in dry tetrahydrofurane for 3 hours at room temperature. Then, the reaction mixture was extracted with ethylacetate and the product — Boc-Asp(2-NA)-OtBu was isolated by solvent evaporation in vacuum. The Boc- and OtBu-protective groups were cleaved simultaneously with trifluoroacetic acid (TFA) for two hours at room temperature and the substrate — Asp-NA was precipitated with diethyl ether as TFA-salt. The AGA specific inhibitor — 5-diazo-4-oxo-L-norvaline (DONV) was synthesized precisely as described by Handschumacher et al. [6].

Animals and tissue treatment. Adult Wistar rats of both sexes were decapitated under deep anesthesia. Cerebrum, cerebellum, medulla oblongata and cervical part of the spinal cord were extracted and fixed in formol-Calcium for one hour at 4°C. Then, the samples were washed in modified Holt's solution (15 % sucrose, 1 % gum arabic) for 36 hours at 4°C and frozen in liquid nitrogen. Serial sections (10 μm) of cerebral cortex, cerebellar cortex, medulla oblongata at the level of hypoglossal nerve and of the cervical region of spinal cord were cut on cryotome Reihert-Jung 2800 (Germany), mounted on gelatinized glass slides, air-dried and covered by 1% celloidin (Fluka) in acetone : diethyl ether : ethanol 4:3:3 for a minute at room temperature.

Visualization of AGA activity and inhibitor controls. Sections were incubated in a substrate solution consisting of 0.3 mM substrate (Asp-NA) and 1.8 ml freshly hexazotized pararosaniline (Merck) in 0.1 M sodium acetate, pH 7.0 for 8 hours at 37°C. The sections were transferred to fresh incubation solution at the fourth hour of incubation. Then, they were post-fixed in 4 % neutral formaline overnight, stained with haematoxyline according to the standard procedure and embedded in glycerol/jelly. Control sections were treated as above, but the incubation medium was supplied with 200 μM AGA inhibitor — DONV.

The examination was made in an Opton IM 35 microscope.

Results and Discussion

AGA possesses the unique ability to cleave off the N-glycoside bond between L-Asn and N-acetylglucosamine in N-linked glycoproteins to give L-Asp and an unstable 2-acetamido-2-deoxy-D-glucopyranosylamine, which hydrolyzes non-enzymatically to N-acetyl-D-glucosamine and ammonium ion (Fig. 1A). The enzyme recognizes L-Asn moiety and acts on its β-amide bond but is not specific towards the carbohydrate chain attached to it. Although the enzyme is classified as an amidohydrolase, its amidase activity is low and even hydrolysis of its natural substrate progresses slowly [4]. Thus far, only one synthetic substrate for AGA — β-Asp-methylcoumarylamide (Asp-AMC) has been synthesized and used for the biochemical analyses of AGA activity in blood samples and cultivated fibroblasts [9]. The enzyme cleaves the amide bond to release a fluorescent compound — aminomethyl coumarine (AMC). Even though the fluorescent analyses are very sensitive, low amidase activity of AGA imposes a problem of a very prolonged incubation time — at least 6 hours are needed for the test. Nevertheless, this test is now extensively used for the diagnosis of AGU [9, 10]. Asp-AMC substrate is not suitable for histochemical studies since the final reaction product of enzyme hydrolysis — the AMC compound is water-soluble.

In the present paper we describe the synthesis of another artificial substrate for AGA - β-Asp-2-naphthylamide (Asp-NA), which can be used for histochemical investigations of the enzyme activity. This substrate possesses a substantial similarity to the natural AGA substrate and is to be used according to the most common histochemical principle of simultaneous azo-coupling and azo-dye formation (Fig. 1B). Its rate of hydrolysis by AGA is also low and the incubation time needed for the

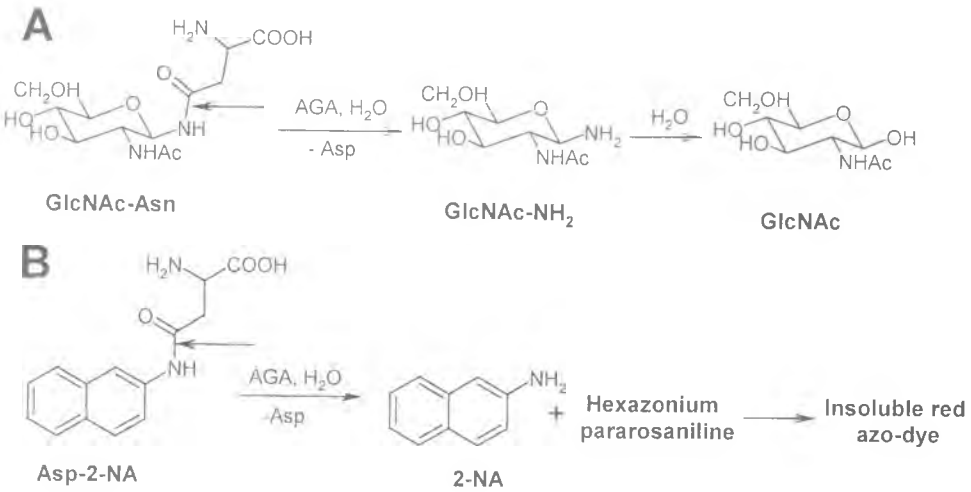

Fig. 1. A: AGA hydrolysis of its natural substrate. The enzyme hydrolyzes N-glycosylic bond in β-N-acetyl-D-glucosaminyl-L-asparagine (GlcNAc-Asn) to give aspartic acid and 2-acetamido-2-deoxy-β-D-glucopyranosylamine (GlcNAc-NH_2); the last compound is later hydrolyzed non-enzymatically to N-acetyl-D-glucosamine (GlcNAc) and ammonium ion; B: Histochemical principle for the visualization of AGA activity. The newly synthesized AGA substrate — Asp-2-NA has a substantial structural similarity to the natural AGA substrate and permits to visualize the enzyme activity according to a common principle of enzyme histochemistry — the simultaneous azo-coupling with diazonium salt to a deeply colored azo-dye

visualization of the enzyme activity is about 8 hours including at least one change of incubation solution. Using our novel AGA substrate we studied the enzyme distribution in the CNS of adult Wistar rats. Preliminary biochemical studies have shown that AGA has a moderate activity in the human brain [4] and comparatively low activity in rat and mouse brains [3]. These studies, however, could not show AGA activity distribution throughout neurons and glial cells of various regions in rat CNS. In our experiments, we found out that AGA has a very high activity in the cell soma of cerebral cortex neurons, Purkinje cells of cerebellar cortex, neurons of the cerebral nuclei of hypoglossal nerve and motor neurons in the cervical part of spinal cord (Fig. 2). The specificity of the enzyme reaction was confirmed by experiments with AGA specific inhibitor DONV. Control sections of all the parts of CNS had no non-specific precipitations. These results gave us grounds to conclude that the enzyme is present and very active in some neurons of the studied parts of rat CNS. This outcome could be expected since genetically determined AGA deficiency leads to symptoms pointing out a substantial impairment of neuronal function. However, the clinical signs of AGU usually start to appear at late juvenile or even adult age. So, the disease follows a very slow progression and it would be interesting to know how late in the pathogenesis of the disease the enzyme begins to be active in the rat CNS. The tracing of AGA activity in the rat CNS during development is going to be performed soon in our laboratory.

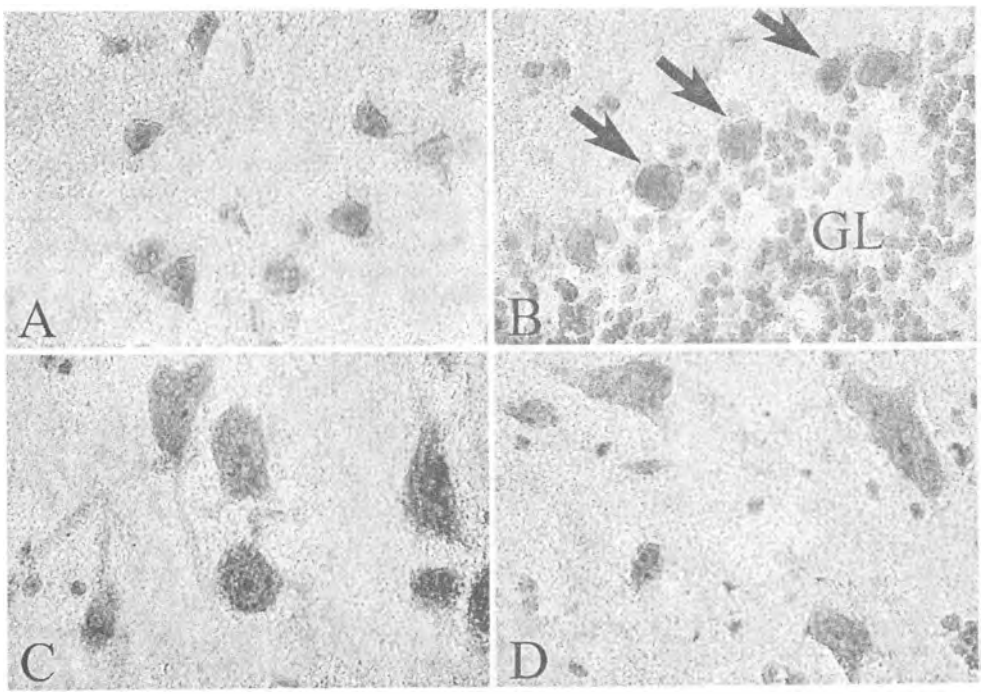

Fig. 2. AGA activity in different parts of rat CNS. A —cerebrum— high enzyme activity in the cytoplasm of cortical neurons; B — cerebellum — Purkinje cells are heavily stained for AGA (arrows), whereas Bergmann's glia and cells in the granulose cell layer (GL) are negative for the enzyme; C — medulla oblongata — neurons of the cerebral nuclei of hypoglossal nerve are highly AGA-positive; D — cervical part of spinal cord — strong reaction for AGA in the cytoplasm of motor neurons. A-D: originally × 400

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