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Role of the Glycosaminoglycan Microenvironment of Hyaluronidase in Regulation of Its Endoglycosidase Activity

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Abstract—The glycosaminoglycan microenvironment of testicular hyaluronidase was simulated by multipoint covalent attachment of the enzyme to glycans as a result of benzoquinone activation. The efficiency of their binding was assessed using gel chromatography, ultrafiltration, titration of surface amino groups of the enzyme, electrophoresis, as well as judging by the value of residual endoglycosidase activity and its inhibition with heparin. Copolymer glycosaminoglycans, such as dermatan sulfate and heparin, inactivated the endoglycosidase activity as a result the C-5 epimerization of hexuronic acid. It was shown that glucuronic acid and, to a lesser extent, *N*-acetylglucosamine determine the specificity of hyaluronidase. The chondroitin-sulfate microenvironment made the enzyme resistant to heparin inhibition because the equatorial orientation of the OH groups is similar to that in hyaluronic acid. Model experiments with dextran and dextran sulfate showed that sulfation of the glycan chain increased its rigidity, thus hampering the stabilizing effect on hyaluronidase. The effect of chondroitin sulfate on the endoglycosidase activity of hyaluronidase had additive character and did not directly affect the small fragment of the active site of the enzyme located at the bottom of a groove. The glycosaminoglycan microenvironment of hyaluronidase, containing an iduronic acid residue, the α1-3 and α1-4 glycosidic bond, inactivated the hyaluronidase activity of the enzyme, whereas simple polymers (such as gluco- and galactoaminoglycans) potentiated it due to a similar way of linking—β(1e-4e) and β(1e-3e). To understand the nature of these interactions in detail, the effect of oligomeric glycosaminoglycan fragments and their derivatives on hyaluronidase should be studied.

Key words: hyaluronidase, glycosaminoglycans, dermatan sulfate, heparin, chondroitin sulfate, hyaluronic acid, microenvironment, regulation of endoglycosidase activity

Hyaluronidase (HU) is classified with the subclass of enzymes that hydrolyze O-glycosyl-containing compounds [1]. This enzyme is termed hyaluronate-4-glycan hydrolase (the systematic term), or hyaluronoglucosaminidase (the working term) [2]. The main substrate of this enzyme is hyaluronate (HA), a non-sulfated glycosaminoglycan [2, 3]. In the organism, HU interacts with other glycosaminoglycans (GAGs) contained in the extracellular matrix and glycocalyx on the cell surface [1, 4]. The protein–glycan interactions change the microenvironment of the enzyme and may affect its catalytic activity. Regulation of the catalytic activity of HU is interesting both in terms of functioning glycosidases that exhibit endoglycosidase and transglycosylase activities [5, 6] and a controlled change in tissue permeability for therapeutic purposes [7, 8].

The microenvironment of an enzyme is a factor regulating its catalytic activity. The microenvironment of a glycolytic enzyme can be simulated by covalent linking of the enzyme to a glycan. It was shown that, on increasing extent of HU modification with dextran (as a result of multipoint binding of the protein with the polysaccharide), the enzyme becomes resistant to heparin inhibition [9]. The resistance of HU to heparin inhibition depends on the extent of the enzyme modification in a threshold way [10]. The expression of this effect is determined by the hydrophobic interactions and steric hindrances resulting from the enzyme modification [11]. Note that HU statistically (i.e., randomly selecting the cleavage site of the glycan chain) hydrolyzes the β1-4 glycosidic bond between the residues of *N*-acetylglucosamine (GlcNAc) and glucuronic acid (GlcA) in HA [1, 2] and is able to hydrolyze such a bond between the residues of *N*-acetylgalactosamine (GalNAc) and GlcA in chondroitin sulfate

Abbreviations: HU) testicular hyaluronidase; GAG) glycosaminoglycan; DS) dermatan sulfate; Hep) heparin; CHS) chondroitin sulfate; HA) hyaluronic acid; HexA) hexuronic acid; GlcA) glucuronic acid; IdoA) iduronic acid; GlcNAc) *N*-acetylglucosamine; GalNAc) *N*-acetylgalactosamine; Gal) galactose; Glc) glucose.

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(CHS) [4-6]. A possible productive interaction of HU with various GAGs provides the ground for the study of their effect on the endoglycosidase activity of this enzyme.

The purpose of this work was to study the regulatory effect of glycosaminoglycans of various structure on the endoglycosidase activity of testicular hyaluronidase. The microenvironment of the enzyme was simulated using covalent linking of the protein to gluco- and galactoaminoglycans, with control of the extent of the enzyme modification.

MATERIALS AND METHODS

Materials. In this study we used testicular hyaluronidase (EC 3.2.1.35) from bovine testicles (State Enterprise Immunopreparat, Ufa, Russia), which was preliminarily purified by gel filtration on Sephadex G-100 (Pharmacia, Sweden) as described earlier [11]. Potassium hyaluronate from human umbilical cord (average molecular weight 700-800 kD), sodium salt of bovine high-molecular-weight heparin (Hep, 16-18 kD), dermatan sulfate from porcine skin (DS, 20-30 kD), chondroitin-4 sulfate from bovine trachea (CHS, 120-140 kD), trinitrobenzenesulfonic acid, sodium chloride, and sodium dodecyl sulfate were obtained from Sigma (USA). Dextran sulfate (molecular weight 40 kD) was from ICN (USA). All other reagents, the components of buffer solutions (analytical grade), were of domestic production.

Enzymatic activity of HU preparations was determined viscosimetrically as recommended in [1]. For this purpose, we measured the outflow time of the solutions using a B-434 Ostwald viscosimeter (USA) in accordance with the procedure that we developed earlier [9]. Viscosity of solutions was measured at 37°C. The solution contained HA (final concentration 0.06%), HU (4 μg/ml), 0.1 M phosphate buffer (pH 5.5 or 7.5), and 0.15 M NaCl. The activity of the enzyme preparations, evaluated relative to the commercial preparation of bovine testicular HU obtained from Sigma (H 3884), was expressed in National Formulary units (NFU). The specific endoglycosidase activity of a preliminarily purified preparation of native testicular HU was 950-970 NFU/mg protein. The determined value of the endoglycosidase activity of HU derivatives represents the mean of three experimental measurements. The specific activity of preliminarily purified native HU was taken as 100%.

Activation of glycosaminoglycans with benzoquinone was performed as follows [12]. Glycosaminoglycans (10-100 mg) dissolved in 6 ml of 0.02 M phosphate buffer, pH 6.0, was mixed with 100 mg of benzoquinone dissolved in 3 ml of dimethylformamide and incubated at room temperature for 1.5 h in darkness. After incubation, excessive benzoquinone was removed by gel chromatography on Sephadex G-25 (Pharmacia) or dialysis against 0.02 M phosphate buffer, pH 6.0, at room temperature for 8-10 h. The number of hydroquinone attachment sites on the glycan chain of GAG was determined iodometrically [10]. Briefly, the purified solution of the activated GAG was incubated for 0.5 h with titrated iodine solution in the presence of NaOH. After addition of HCl and starch, the mixture was titrated with a solution of sodium thiosulfate (preliminarily titrated with potassium dichromate) until complete decoloration.

Covalent conjugation of HU with activated GAG was performed as described in [13]. Briefly, 18 mg of purified native HU were added to 10 ml of purified benzoquinone-activated GAG, and the pH of the mixture was adjusted to 8.2-8.7 with 1 N NaOH. The mixture was incubated in darkness at room temperature for 18-22 h. The concentrations of HU and activated GAG in the mixture were 20 and 5-10 μM, respectively. The reaction product was purified by gel chromatography on a column packed with Sephadex G-100 equilibrated in 0.025 M phosphate buffer, pH 7.5, supplemented with 0.075 M NaCl. The eluate was lyophilized.

To obtain preparative amounts of HU–GAG conjugates, we used ultrafiltration on an Amicon device (USA) equipped with an XM-100A membrane. The membrane was washed with the solution containing 0.025 M phosphate buffer (pH 7.5) and 0.075 M NaCl until the A_{280} value of the washing solution became less than 3-5% of the initial value. The supernatant was then lyophilized.

The reaction between HU and dextran sulfate was conducted in accordance with the scheme of benzoquinone activation described above and with dextran as described earlier [9, 11].

Protein content in the preparations of native and modified HU was determined by the method of Bradford [14].

Extent of modification of hyaluronidase was determined by titration of the surface amino groups of HU derivatives with trinitrobenzenesulfonic acid in accordance with [15] and has been described in more detail earlier [9, 10].

Resistance to heparin inhibition was estimated by the residual endoglycosidase activity of HU derivatives in the presence of excess Hep. The latter was added (enzyme-to-heparin weight ratio 1 : 100 or 1 : 1000) to the reaction medium in the viscosimeter containing 4 μg/ml of the enzyme, 0.1 M phosphate buffer (pH 5.5 or 7.5), and 0.15 M NaCl. After 0.5-h incubation at room temperature, the endoglycosidase activity of the HU derivatives was determined viscosimetrically as described above. The values shown are the mean of three experimental measurements.

Electrophoretic segregation of the HU derivatives was performed in the presence of SDS and mercaptoethanol in 5-20% polyacrylamide gel [16].

RESULTS AND DISCUSSION

Glycosaminoglycan microenvironment of hyaluronidase. GAGs are heteropolymers consisting of repeating disaccharide units comprised of residues of hexosamine (HexN) and hexuronic acid (HexA) or Gal (Fig. 1). With respect to the repeated units, GAGs are divided into three families: 1) HexA-GalNAc; 2) HexA-GlcNAc; and 3) Gal-GlcNAc. The type 3 disaccharide units are characteristic of keratan sulfate, which was not used in this work because of the structural and compositional differences from other GAG families and the absence of information on its role in homeostasis [17].

The treatment of GAGs with benzoquinone [12] makes these polymers suitable for covalent binding with enzymes [13]. The reduction of quinone to hydroquinone as a result of such treatment is expressed during iodometric titration of benzoquinone-activated GAGs after elimination of excess activator. The number of sites for subsequent binding with the protein amounts to 10-20% (judging by the results of iodometry). The enzyme is covalently linked to the benzoquinone-activated GAG through the surface amino groups. Titration of the surface amino groups allowed us to determine the degree of enzyme modification and to confirm its covalent nature using electrophoresis. It can be assumed that the adducts formed as a result of multipoint interaction of HU with GAG simulate, to a first approximation, the cooperative binding of this enzyme with GAGs in biological systems (glycocalyx, extracellular matrix, etc.). The estimation of comparative endoglycosidase activity of HU derivatives clearly shows the effect of the microenvironment of the covalently linked GAGs on the catalytic function of the enzyme (table). Note that direct treatment of HU with benzoquinone (to 30% modification) under the same conditions and at the same amount that were used in the case of the enzyme interaction with activated GAGs did not significantly alter the endoglycosidase activity and extent of Hep inhibition compared to native enzyme. Thus, the changes in the HU endoglycosidase activity observed in this work can be related mostly to the effect of the GAG microenvironment on the enzyme.

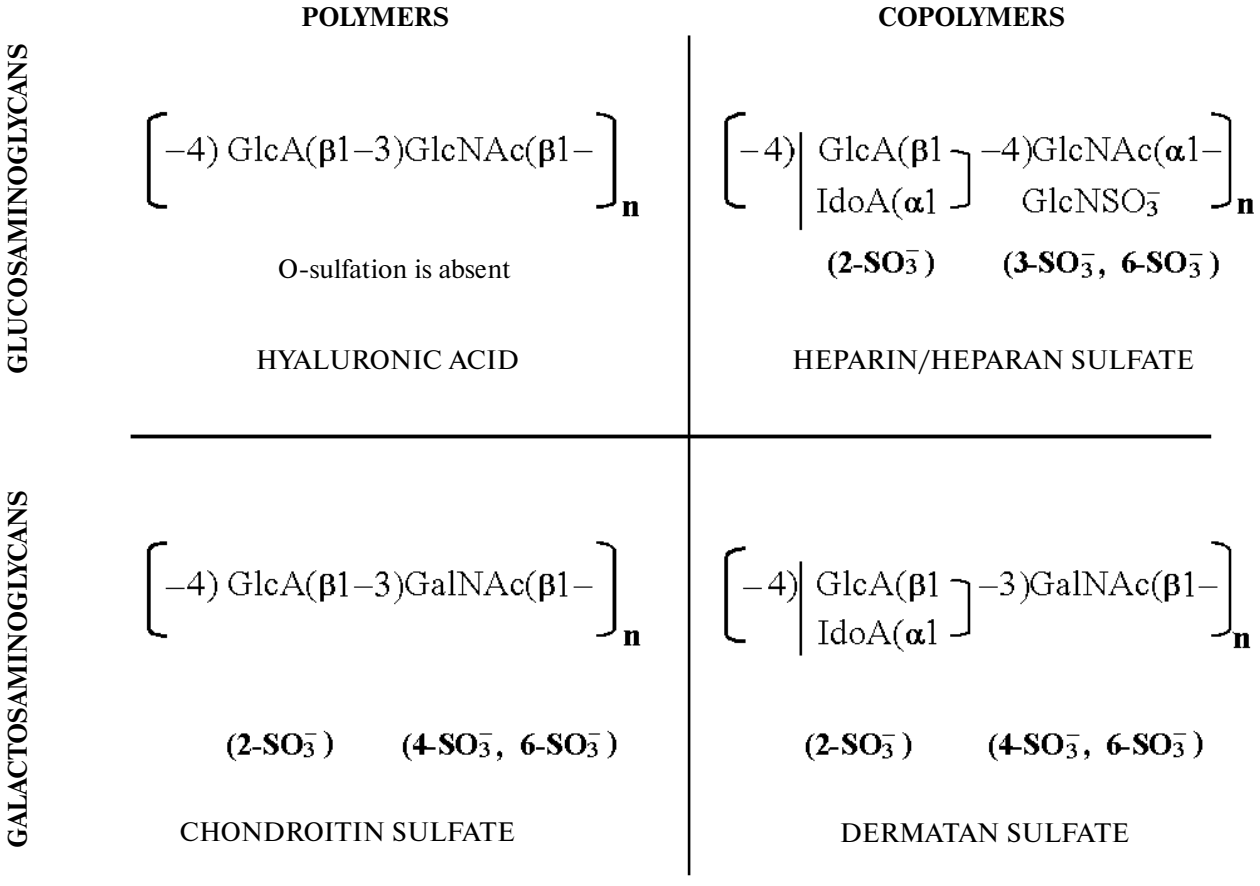

Fig. 1. Formalized presentation of the polymeric units of glycosaminoglycans used in the study. The numbers in parentheses before SO_3^- designate possible positions of sulfation in the residues of HexA or in HexN. Abbreviations are given in the text.

Effect of the glycosaminoglycan microenvironment on the endoglycosidase activity of hyaluronidase

Agent/conjugate	Degree of modification of the amino groups of hyaluronidase, %	Residual activity of hyaluronidase preparations					
		pH 5.5			pH 7.5		
		without heparin, %	with heparin (HU/Hep = 1 : 100), %	with heparin (HU/Hep = 1 : 1000), %	without heparin, %	with heparin (HU/Hep = 1 : 100), %	with heparin (HU/Hep = 1 : 1000), %
Native hyaluronidase	0	100	71-73	38-42	55-57	39-43	35-38
Hyaluronidase with chondroitin sulfate	82-88	76-78	54-62	48-52	63-65	49-53	43-45
Hyaluronidase with heparin	68-70	46-50	38-42	25-29	28-30	13-15	6-8
Hyaluronidase with dermatan sulfate	30-33	25-27	10-12	0	20-22	7-9	0

Effect of copolymeric glycosaminoglycans. We found that GAGs representing copolymers of disaccharide units of differing structure (Hep and DS, Fig. 1) inactivate the enzyme more strongly than a simple polymer (CHS, Fig. 1) comprised of similar units (table). The difference in the formulae of CHS and DS is the presence of an iduronic acid residue (IdoA, Fig. 1) in the glycan chain of the latter. It can be assumed that the presence of this residue largely determines the inactivation of HU. At a low degree of enzyme modification, DS indeed caused a significant drop in the endoglycosidase activity (table). Unlike the GlcA residue, which has a stable $^4C_1$ conformation, the IdoA residue may be present at least in three conformations—$^4C_1$, $^1C_4$, and $^2S_0$ [18]. As a result, DS (unlike CHS) tends to self-associate, with formation of an increased number of intermolecular hydrogen bonds [19]. Possibly, a close interaction of DS with the protein surface during the covalent conjugation enables the formation of a HU-inactivating system of hydrogen bonds. This enhances the specificity of testicular HU to the GlcA residue contained in the polymeric chain, which was reported earlier [20], and points to the importance of the configuration at C-5 HexA (GlcA and IdoA are C-5 epimers) for the catalytic function of HU. This conclusion is not exhaustive, because a comparison of the modifying effects of Hep and DS (table, Fig. 1) shows that the former, at approximately a twofold greater degree of modification, provides almost twice higher level of the residual activity. This is apparently related to the importance of the GlcNAc residue, contained in HA and Hep, for HU (Fig. 1). It has been reported that testicular HU can also degrade CHS containing a GalNAc residue [1, 4-6]. This finding indicates that the specificity of this enzyme to HexNAc residue is lower than to GlcA. Apparently, the location of the substituents at C-4 in GlcNAc weakly affects the specificity of testicular HU. Chondroitin-4 sulfate is sulfated at the C-4 atom in GalNAc, providing a high density of the negative charge in the glycan chain because of the axial location of the sulfonate group relative to the polymeric base [19]. This prevents the self-association of chondroitin-4 sulfate yet does not block its productive interaction with HU. The modification with the GAGs copolymers does not make HU resistant to the Hep inhibition (table) either at pH optimum of the endoglycosidase activity of testicular HU (5.5) or at physiological pH of blood (7.5). Thus, the microenvironment comprised of copolymer GAGs has a denaturing effect on HU, related to the presence in their structure of an IdoA residue attached through the α1-3 and α1-4 glycosidic bonds.

Chondroitin sulfate microenvironment of hyaluronidase. The conjugation of HU with CHS makes it markedly resistant to the Hep inhibition (table). At physiological pH (7.5), the CHS-modified HU is, at least, not less active than the native enzyme. This level is reached at significant degree of HU modification (82-88%). The configurations of simple polymers consisting of similar disaccharide units (Fig. 1), the modifier (CHS), and the substrate (HA) are similar, because their glycosidic bonds fit the same attachment scheme (1e-4e and 1e-3e), where "e" designates the equatorial orientation of hydroxyls [21]. An important difference between HA and CHS are the sulfonate groups (Fig. 1). A direct estimation of their effect using HA is hampered because of its high molecular weight. However, the similarity of hydrolysis of chondroitin (containing no sulfonate groups) and HA by testicular HU has been reported [22]. Dextran and dextran sulfate, which are readily available, are quite suitable for

simple model experiments. The experiments on the HU conjugation with dextran showed that, at high degrees of modification (96-98%), the enzyme gains a pronounced resistance to Hep inhibition [9-11]. The use of benzoquinone-activated dextran sulfate for covalent binding with HU shows that, already at low degrees of modification (approximately 15%), the endoglycosidase activity of HU significantly drops as a result of Hep inhibition (Fig. 2). These data indicate that sulfation of neutral homopolymeric dextran $[Glc(\alpha 1\text{-}6)]_n$ (in general form, $[Glc(\alpha 1\text{-}6)Glc(\alpha 1\text{-}6)SO_3^-\text{-}Glc(\alpha 1\text{-}6)]_n$) makes its structure more rigid due to electrostatic interactions. As a result, the deforming effect of the rigid molecule of dextran sulfate on the catalytic conformation of HU is enhanced, leading to inactivation of the enzyme. In general, sulfation decreases the glycan-chain flexibility, thus hampering endoglycosylation. It was shown earlier that testicular HU hydrolyzes the bonds in HA more rapidly than in CHS [23]. The results of electrophoresis performed under denaturing conditions confirmed the assumption that a certain flexibility of the polyglycan chain is required for the expression of the endoglycosidase activity of HU (Fig. 3). It was found that the HU–CHS conjugate can decompose when stored after lyophilization. The electrophoretic patterns of the conjugate stored for more than three months and a freshly prepared conjugate are shown in Fig. 3 (lanes *7* and *8*). One can see that an increase in the terms of the conjugate storage increases the size of HU–CHS depolymerization. It can be assume that the enzyme optimizes the size and type of its microenvironment for endoglycosidase catalysis. Presumably, this process is promoted by the formation of β-layers both by the anionic GAGs [21] and by the protein preparation after its lyophilization [24]. The specificity of catalytic transformation is apparently determined by the structure of CHS, which is characterized by the hydrophobic interaction increment, similar to that of other GAGs, and a specific network of hydrogen bonds [19], as well as by moistening of the powder during its storage.

Fragment of the active site of hyaluronidase. The endospecificity of glycanases is determined by the topography of their active sites [25]. It was assumed that the active site of HU has the shape of an open groove, in which the substrate molecule (randomly adsorbed along the glycan chain) is attacked by the functional groups of the active site. The major final product of the reaction between HU and HA is either a tetra- or a trisaccharide, depending on whether a GlcA or a GlcNAc residue, respectively, is located at the non-reducing end of the chain [5, 20]. Thus, the minimum fragment of the substrate affected by HU is a fragment consisting of six to seven saccharide residues. It was shown that strong cooperative interactions, indeed, manifest themselves in the solutions of high-molecular-weight HA (comprised of more than 20 disaccharide units) but are absent already in

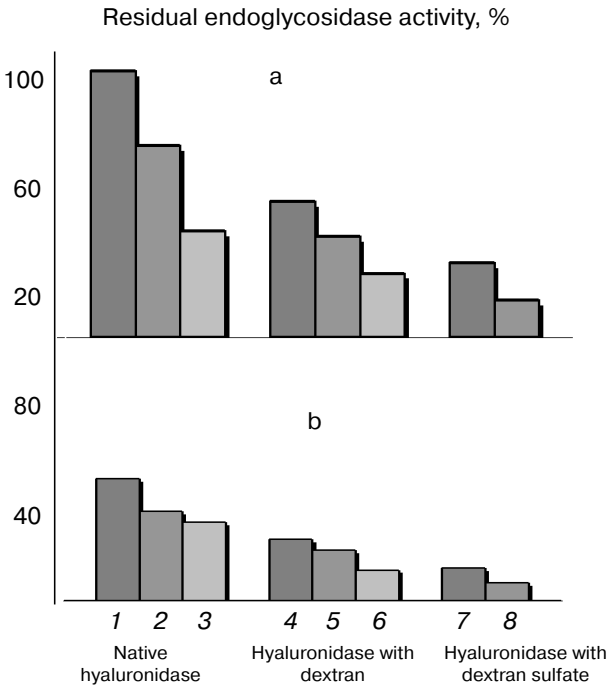

Fig. 2. Diagram comparison of the residual endoglycosidase activity of hyaluronidase preparations in native (*1-3*) and conjugated with dextran (*4-6*) or dextran sulfate (*7*, *8*) form at pH 5.5 (a) and 7.5 (b). The enzymatic activity was determined both in the absence of heparin (*1*, *4*, *7*) in the reaction medium and in the presence of heparin at the HU-to-Hep ratio (w/w) of 1 : 100 (*2*, *5*, *8*) and 1 : 1000 (*3*, *6*). The degree of modification of the amino groups of hyaluronidase with dextran (*4-6*) and dextran sulfate (*7*, *8*) was 96-98 and 14-16%, respectively.

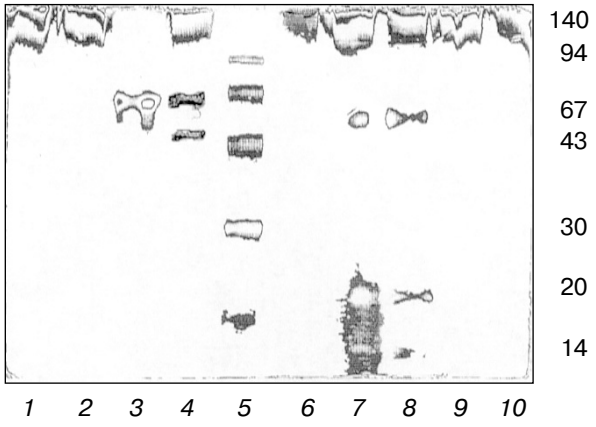

Fig. 3. SDS-PAGE analysis of native hyaluronidase after (*3*) and before (*4*) purification of the commercial preparation; hyaluronidase conjugated with dextran sulfate (*1*), heparin (*2*), chondroitin sulfate (stored for more than three months (*7*) and freshly prepared (*8*)), dermatan sulfate (*9*), and dextran (*10*). Marker proteins are shown in lanes *5* and *6*, their molecular weights (kD) are shown on the right.

HA tetrasaccharides [21]. A productive endoglycosidase effect of HU is determined both by the structure and length of the glycan chain and the size of the active site of the enzyme. The size of the latter is apparently small (according to the results of HU inhibition with flavonoids) [26]. Effective inhibitors were flavones consisting of two condensed cyclic structures of aromatic (A) and maltole (C) types and an aromatic ring (B) attached to the cycle C through an ordinary C–C bond. An increase in the inhibitor size at the expense of glycosidic substituents sharply decreases the inhibition value. The inhibition disappears when the coplanar nature of ring C is disturbed and is maximal when the molecule contains four hydroxyl substituents [26]. This is probably indicative of a fairly flat packing of the bottom of the groove in the HU active site, a limited extension and depth of this groove, and a key role of hydrogen bonds in the proper orientation of reagent in the groove. According to the results of the conjugation of HU with CHS (at the degree of modification of 82-88 and 96-98%), functioning of the active site of the enzyme is not significantly distorted (the residual activity was 77 and 70%, respectively). This finding is indicative of an additive mode of the effect of the CHS microenvironment on the state of the HU active site (i.e., the denaturing effect of the modifier increases with an increase in the degree of modification). On the other hand, the conjugation of HU with DS already at 30% degree of modification markedly denatures the enzyme (table). We can assume that this difference is related to the interaction of a certain site in the enzyme globule (as the degree of modification is low) and DS, as a result of which the active site of the enzyme is deformed. However, the conjugation of HU with Hep (which, similar to DS, does not make HU resistant to inhibition (table) and is characterized by similar positions of the protein bands on the electrophoregram (Fig. 3)), shows that the effect of Hep on HU is more likely cumulative, i.e., the deforming effect is increases on an increase in the degree of modification. A comparison of the oppositely directed effects on HU of dextran sulfate and dextran (Fig. 2) suggests that some sites on the HU surface may possibly restrict its interaction with these polysaccharides. Thus, a small size of the HU active site hampers its direct interaction with GAG; however, in the case of multipoint interaction, allosteric effects that regulate the HU endoglycosidase activity are developed between them. In this case, the proportion of hydrophobic interactions between GAG and HU may be predominant in the overall sum of interactions, but it is apparently similar in various GAGs because of the similarity of their hydrophobic regions [19, 21]. This provides general binding between GAG and HU, when GAG macromolecules act as inhibitors of the interaction between HU and HA [27]. Determination of the association nature (stabilization or inactivation of the enzyme) is related to the realization of fine interactions in the area of the surface contact between HU and GAG. The data obtained do not allow us to state unambiguously whether these thin interactions are realized via a limited site(s) on the protein surface (as may be assumed based on the results obtained using DS and dextran/dextran sulfate) or as a result of an additive interaction (as in the case of CHS and Hep). To make a more precise conclusion, NMR-spectroscopic and molecular computer simulation studies are required.

Concluding remarks. Different GAGs may have oppositely directed effects on the HU endoglycosidase activity. Changes in the local composition of GAGs along the vessel wall [28] or their distribution in the organism with aging [29] or during the development of pathology [30] probably determine their different regulatory effects on the catalytic function of HU. The data available [9-11] and the results of this study led to an assumption that, at high degrees of the HU modification, the CHS chains enable the formation of a relatively extended open and deep groove on the protein surface, with a fragment of the HU active site being located at its bottom. The stabilizing effect of CHS on HU is apparently determined by the possibility of formation of intra-glycan hydrogen bonds between the equatorial OH groups of GlcA and the neighboring groups of the saccharides, which cannot be realized by the axial OH groups of IdoA in the $^1C_4$ conformation of DS [19]. Copolymeric GAGs, which contain IdoA residues attached through α1-3 and α1-4 glycosidic bonds, at different degrees of modification decrease the endoglycosidase activity of HU. Thus, the fine effect of the blocks of GAGs allosterically determines the resulting effect of their interaction with HU [21]. The nature of the interaction may be clarified by a comparative study of the effect on HU of the oligomers of the cellobiose (as in the case of HA), lactose (as in the case of CHS), and maltose (as in the case of Hep) structure. Such a study will be the subject of our further work.

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