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Resistance of Dextran-Modified Hyaluronidase to Inhibition by Heparin

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Abstract—Properties of native and aldehyde dextran-modified hyaluronidase (with surface amino group modification about 98%) were investigated. Optimal endoglycosidase activity of the native enzyme was observed at 0.15 M NaCl and pH 5.5 and electrostatic interactions influenced the enzyme activity. The inhibitory effect of heparin on hyaluronidase activity slightly differed at pH 5.5 (1.5-fold inhibition) and 7.5 (1.2-fold inhibition). Ionic strength of the reaction medium only slightly influenced the effect of heparin. Modification of hyaluronidase with dextran increased hydrophobic interactions and steric hindrance. Conjugation with dextran increased the resistance of hyaluronidase activity to denaturing agents (urea, guanidinium hydrobromide) and extended the optimal conditions for maximal endoglycosidase activity (pH 4.5-6.5, the range of NaCl concentration from 0.1 to 0.3 M). The conjugation also reduced electrostatic effects on the active site of hyaluronidase and efficacy of heparin inhibition. At pH 7.5 the enzyme was almost insensitive to heparin. The resistance of dextran-modified hyaluronidase to heparin points to approaches for subsequent studies of the heparin-binding site of this enzyme and biomedical trial of the stabilized enzyme for the treatment of acute cardiovascular lesions.

Key words: hyaluronidase, dextran, chemical modification, heparin, inhibition, enzyme stabilization

The carbohydrate component often determines the behavior of glycolipids, glycoproteins, and proteoglycans in biological systems. Glycosylation of proteins may significantly influence their properties [1, 2]. Studies of changes in composition and length of polysaccharides during biological conversions is one of the most important goals of intensively developing glycobiology. Hyaluronidase is one of the important enzymes that influence the carbohydrate component of biological macromolecules. However, little is known about the regulation of hyaluronidase activity. Such study is interesting from both basic and practical viewpoints. Hyaluronidase is a potential agent for reducing the size of myocardial infarction [3]; this effect can be attributed to hydrolytic cleavage of oligosaccharides accompanied by improvement of tissue permeability. Reduction of myocardial infarction size is an important factor for the survival of a patient. The present period is characterized by certain changes in the strategy of medical treatment of myocardial infarction. Before the "era of thrombolytic therapy" (which started in sixties and seventies), medical treatment was focused mainly on the limitation of myocardial working load and suppression of arrhythmia. Later the treatment was focused on the fastest rechanneling of the thrombosed blood vessel. Modern strategy also includes the development of accompanying (adjacent) therapy [4]. This includes the use of acidic polysaccharides (e.g., heparin) as anticoagulants and maintenance of sufficient tissue permeability [5] required for normalization of microcirculation and functional recovery of the myocardium [6] after acute cardiovascular dysfunction. Optimization and stabilization of hyaluronidase administered to patients is an important task because some polysaccharides such as heparin markedly inhibit the native enzyme infused into the bloodstream [7]. Previously we demonstrated that covalent conjugation of hyaluronidase with dextran modulated the inhibitory effect of heparin [8]. Increasing the modification of surface amino groups reduced the sensitivity of hyaluronidase to inhibition by heparin. In the present study, we investigated the nature of interactions responsible for the resistance of modified hyaluronidase to inhibition by heparin. For this purpose, we compared some properties of native and dextran-modified hyaluronidase. The enzyme with surface amino group modification of about 98% is almost insensitive to heparin.

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MATERIALS AND METHODS

Chemicals. Hyaluronidase (EC 3.2.1.35) isolated from bovine testicles was produced by Immunopreparat (Russia). Dextran T-40 (40 kD) and Sephadex G-100 were produced by Pharmacia (Sweden). Human umbilical hyaluronic acid (potassium salt) of 700-800 kD, trinitrobenzenesulfonic acid, sodium periodate, bovine high molecular weight heparin (sodium salt), sodium borohydride, sodium chloride, and SDS were purchased from Sigma (USA). All other chemicals of analytical grade (components of buffer solutions) were produced by Reakhim (Russia).

Enzyme assay. Hyaluronidase activity was assayed viscosimetrically [9] at 37°C using an Ostwald B-434 viscosimeter (USA) as described previously [8]. Concentrations of potassium hyaluronate and hyaluronidase were 0.02% and 4 μg protein/ml, respectively [8]. The enzyme activity was expressed in Sigma units; bovine testicular hyaluronidase purchased from Sigma (H 3631) was used as the reference preparation. According to the Sigma definition, one unit of hyaluronidase activity corresponds to the amount of enzyme catalyzing formation of 1 μg glucuronic acid determined colorimetrically by reaction with 3,5-dinitrosalicylic acid.

Hyaluronidase modification. Electrostatically neutral dextran was converted to the aldehyde dextran form by partial periodate oxidation; the number of aldehyde groups (20-22 per 100 monosaccharide units) was determined iodometrically. Covalent attachment of native hyaluronidase to this aldehyde dextran was carried out at 4°C for 18 h using 0.15 M NaCl and pH 8.3 [8]. The resulting adduct was reduced by sodium borohydride, isolated by gel filtration on Sephadex G-100, and lyophilized.

Protein content was determined by the Bradford method [10].

Modification degree of hyaluronidase was evaluated spectrophotometrically by titrating surface amino groups of the enzyme with trinitrobenzenesulfonic acid [11]. All details were described earlier [8]. Since interaction with aldehyde dextran is accompanied by reduction of amino groups, a decrease of number of titrable amino groups implies an increase in modification degree of hyaluronidase. The initial parameter corresponds to the native enzyme; lack of titrable amino groups means total (100%) modification of hyaluronidase.

Effects of pH, salt concentration, and heparin on hyaluronidase activity were studied using identical enzyme concentration in the reaction medium. Acetate and phosphate buffers (0.02 M) were used in the pH ranges of 4.0-6.0 and 6.0-8.0, respectively. The ionic strength of the reaction medium was increased by adding appropriate concentrations of sodium chloride or calcium chloride to buffer solutions containing substrate (potassium hyaluronate) and the enzyme. Inhibition of hyaluronidase by heparin was evaluated at enzyme/heparin ratio 1 : 100 (w/w) after preincubation for 0.5 h before assay of hyaluronidase activity [8].

Spectrofluorimetric study of hyaluronidase preparations was carried out using a Hitachi F-4010 spectrofluorimeter (Japan) at excitation and emission wavelengths of 280 and 348 nm, respectively. The enzyme concentration in the cuvette was always the same. Effects of heparin (at enzyme/heparin ratio 1 : 100, w/w) and sodium chloride were studied at room temperature and pH 5.5 (in 0.02 M acetate buffer) or 7.5 (0.02 M phosphate buffer) by the intensity of the intrinsic protein fluorescence.

Effects of urea and guanidine hydrobromide on hyaluronidase activity were studied in 0.02 M acetate buffer, pH 5.5, at 37°C with and without heparin (ratio of concentrations hyaluronidase/heparin 1 : 100, w/w).

Electrophoresis of hyaluronidase preparations was carried out as described by Laemmli [12] using 5-20% polyacrylamide gel.

RESULTS

Enzyme preparations. Figure 1a (curve *1*) shows a gel-filtration profile of Lidase preparation (a commercial native testicular hyaluronidase). The major proportion of hyaluronidase activity was localized within peak II (not shown). Its isolation and subsequent lyophilization yielded a purified preparation of native hyaluronidase (table). During gel filtration it is eluted as single asymmetrical protein peak (Fig. 1a, curve *2*). The position of this peak on the elution profile is consistent with peak of hyaluronidase activity of this preparation (Fig. 1a, curve *3*). Thus, a single gel filtration of commercial

Some parameters of lyophilized hyaluronidase preparations

Preparation	Protein content, %	Specific activity, U per mg protein	Degree of modification of the enzyme, %
Commercial preparation Lidase	20	17,500	0
Purified native hyaluronidase	2	50,000	0
Dextran-modified hyaluronidase	1.4	29,000	98

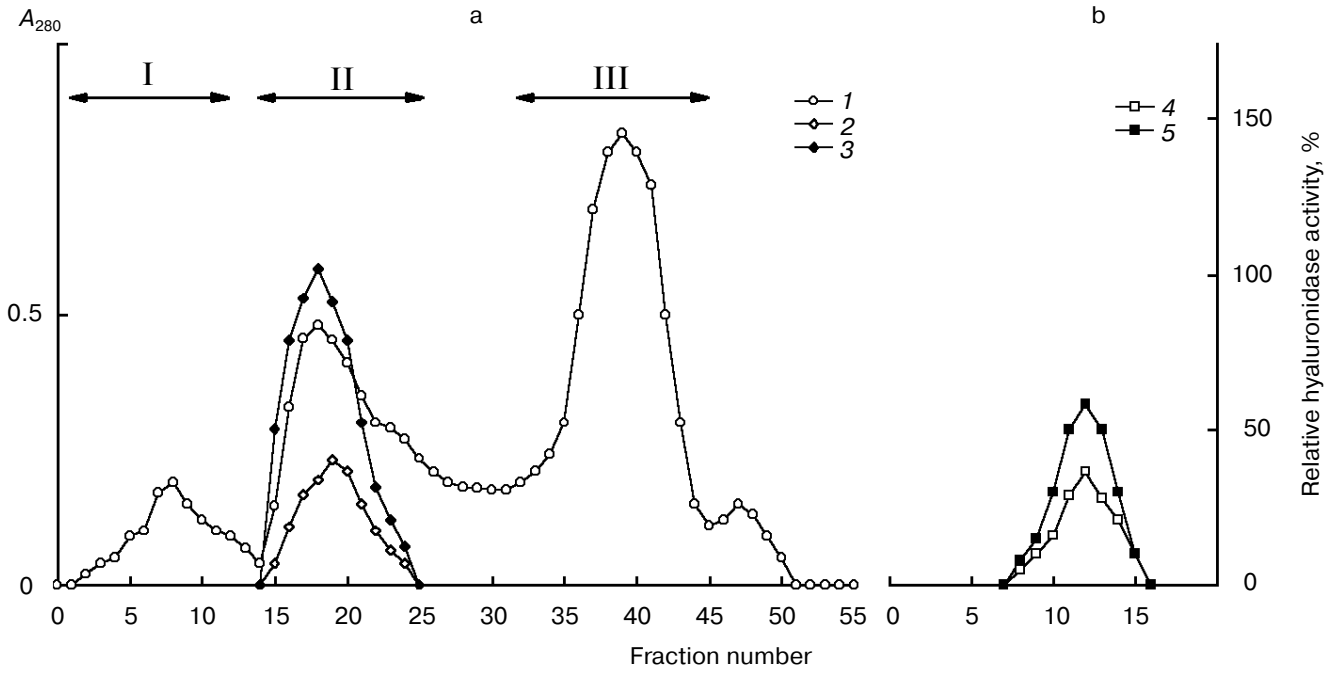

Fig. 1. Gel filtration of hyaluronidase preparations on Sephadex G-100 equilibrated with 0.02 M phosphate buffer, pH 7.5, containing 0.15 M NaCl. a) Absorbance (A_{280}) of native hyaluronidase of a commercial Lidase preparation (*1*) (numbers I-III represent its collected peaks), purified enzyme (re-chromatography of peak II) (*2*), and its hyaluronidase activity (*3*); b) absorbance (A_{280}) of the dextran-modified enzyme (*4*) and its relative hyaluronidase activity (*5*).

hyaluronidase is sufficient to obtain a nearly homogenous preparation (Fig. 2). This preparation was used in subsequent experiments. Modification of this preparation with dextran changed the position of the protein (Fig. 1b, curve *4*) and hyaluronidase activity (Fig. 1b, curve *5*) peaks on the elution profile. Data of electrophoresis under denaturing conditions support the covalent nature of the modification (Fig. 2). This yielded hyaluronidase

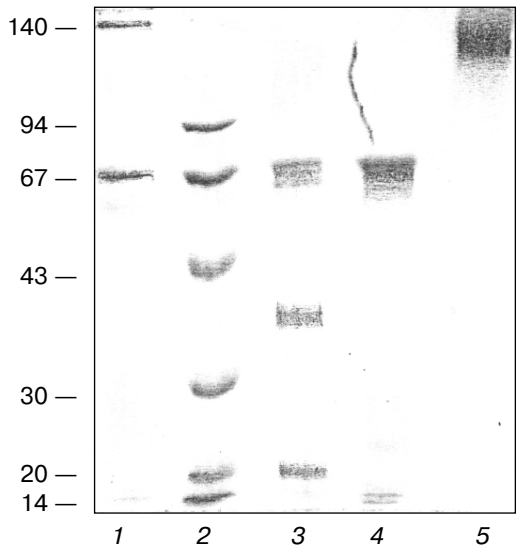

Fig. 2. SDS-Electrophoresis of hyaluronidase preparations: *1*, *2*) position of marker proteins (with indicated molecular masses in kD); *3*) commercial native hyaluronidase (Lidase); *4*) purified native, and *5*) dextran-modified hyaluronidase.

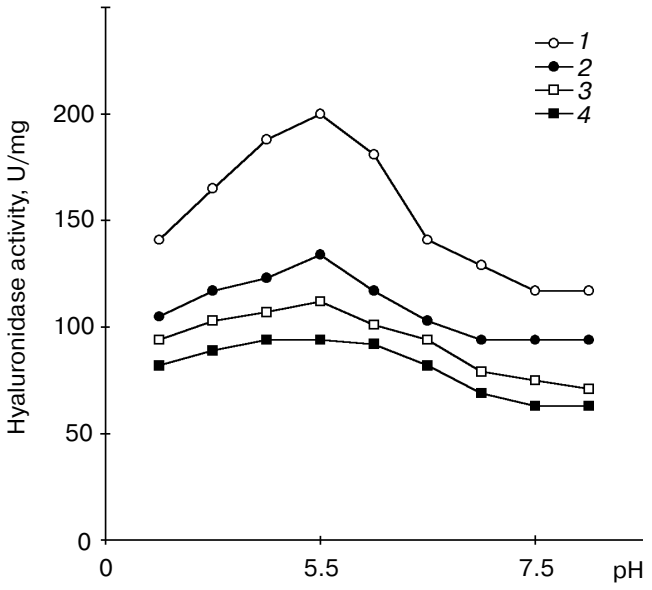

Fig. 3. Dependence of hyaluronidase activity on pH in the absence (*1*, *3*) and in the presence of heparin (*2*, *4*) (enzyme/heparin ratio 1 : 100, w/w); *1*, *2*) native hyaluronidase; *3*, *4*) dextran modified hyaluronidase.

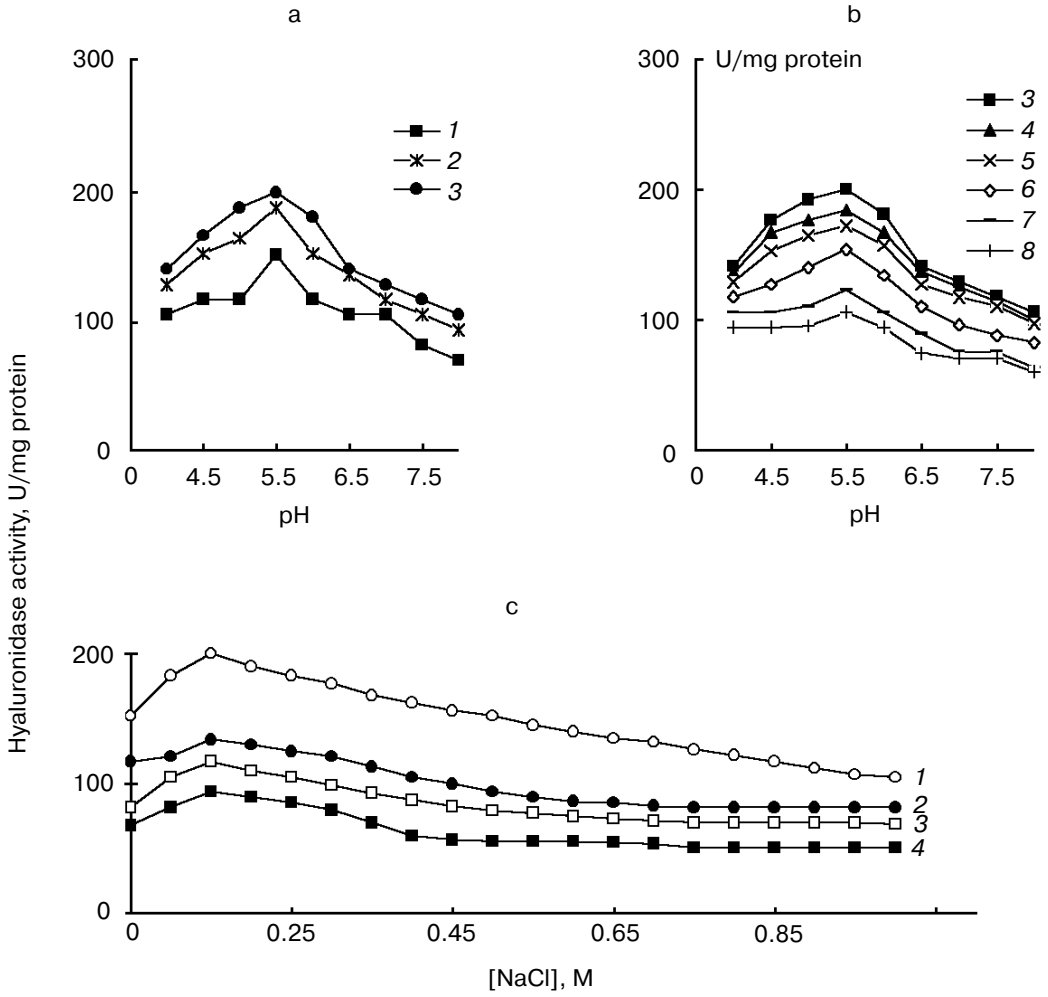

Fig. 4. Effect of NaCl on the activity of native hyaluronidase assayed in the pH range 4.0-8.0 (a, b) and at pH 5.5 and 7.5 (c). a, b) Hyaluronidase activity (U/mg) at various NaCl concentrations (M): *1*) 0.01; *2*) 0.1; *3*) 0.15; *4*) 0.2; *5*) 0.3; *6*) 0.5; *7*) 0.75; *8*) 1.0; c) effect of NaCl on hyaluronidase activity (U/mg) at pH 5.5 (*1*, *2*) and pH 7.5 (*3*, *4*) assayed in the absence (*1*, *3*) and the presence (*2*, *4*) of heparin.

having 98% of its titrable amino groups modified; this dextran-modified hyaluronidase retained 58% of the specific activity of the native purified enzyme (expressed as 100%, Fig. 1). This modified enzyme was used in subsequent experiments.

Effect of pH on catalytic activity. Native hyaluronidase exhibits maximum activity at pH 5.5 (Fig. 3). Conjugation of the enzyme with dextran smoothed the pH dependence of its catalytic activity and the pH optimum became broader (4.5-6.5). Heparin inhibited the catalytic activity of native hyaluronidase (Fig. 3) but did not change its pH optimum (5.5). Under these conditions, the pH dependence of catalytic activity of the modified enzyme remained essentially unchanged.

Effect of ionic strength. Increasing NaCl concentration to 0.15 M in the reaction medium was accompanied by the increase in catalytic activity of the native enzyme over the whole pH range (Fig. 4a). Further increase in NaCl concentrations decreased the hyaluronidase activity (Fig. 4b). Thus, 0.15 M NaCl is optimal for manifestation of catalytic activity of native hyaluronidase both at pH 5.5 and 7.5 (Fig. 4c). (Although at pH 7.5 NaCl-dependent changes were less pronounced, this pH value is close to that of blood [13]). The effect of ionic strength on the behavior of dextran-modified hyaluronidase (Fig. 5, a and b) was similar to that of the native biocatalyst (Fig. 4, a and b) but was less pronounced. The latter resulted in almost total insensitivity of dextran-modified hyaluronidase to variation of NaCl concentration at pH 7.5, whereas at pH 5.5 a peak of hyaluronidase activity of the modified enzyme was clearly detected (Fig. 5c). In the presence of calcium chloride, the optimum of hyaluronidase activity of native and dextran-modified enzyme was detected in the range of 0.01-0.1 M concentrations. Further increase of $CaCl_2$

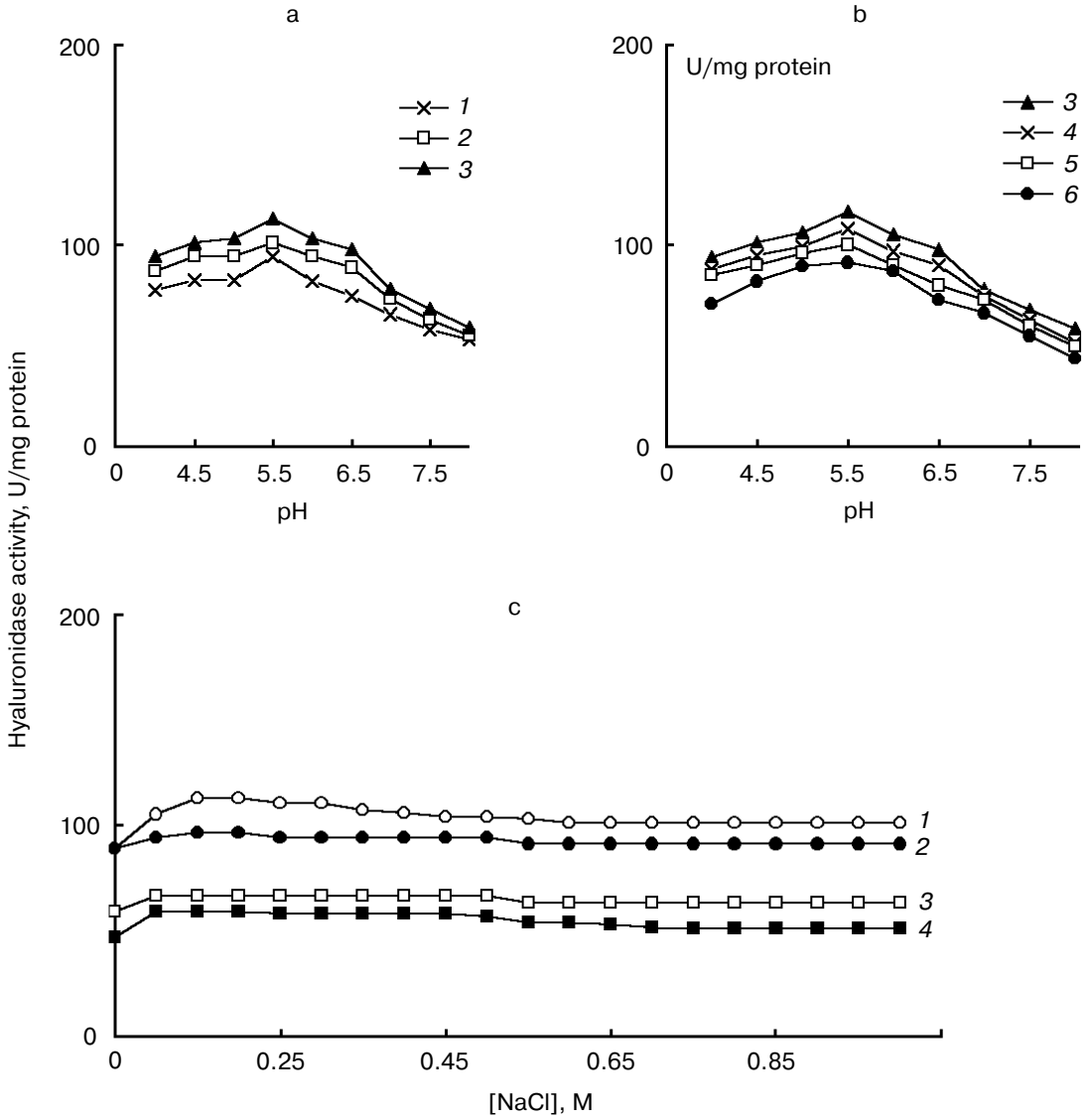

Fig. 5. Effect of NaCl on the activity of dextran-modified hyaluronidase assayed in the pH range 4.0-8.0 (a, b) and at pH 5.5 and 7.5 (c). a, b) Hyaluronidase activity (U/mg) at various NaCl concentrations (M): *1*) 0.01; *2*) 0.1; *3*) 0.15; *4*) 0.5; *5*) 0.75; *6*) 1.0; c) effect of NaCl on hyaluronidase activity (U/mg) at pH 5.5 (*1*, *2*) and pH 7.5 (*3*, *4*) assayed in absence (*1*, *3*) and the presence (*2*, *4*) of heparin.

concentration was accompanied by a decrease in hyaluronidase activity.

Inhibition by heparin. Heparin markedly reduced the catalytic activity of native hyaluronidase both at pH 5.5 (pH optimum) and 7.5 (Fig. 4c). The effect of heparin on catalytic activity of modified hyaluronidase was less evident over the whole pH range. The activity of the modified hyaluronidase assayed in the absence or in the presence of heparin was also less sensitive to variations of NaCl concentrations (Fig. 5c). At these pH values, the dependence of catalytic activity of native and modified hyaluronidase on $CaCl_2$ concentration (assayed in the presence and in the absence of heparin) is also symbatic.

Fluorescent study. Increase of NaCl concentration in the cuvette reduced the intensity of intrinsic fluorescence of native hyaluronidase (Fig. 6). Although in the presence of heparin the initial value of intrinsic protein fluorescence (at identical protein concentration) was lower, NaCl addition had a minor effect. The same behavior was also characteristic for dextran-modified hyaluronidase (Fig. 6). It should be noted that the native and modified hyaluronidases are characterized by similar dependences of intrinsic fluorescence on heparin and NaCl at pH 5.5 and 7.5 (Fig. 6).

Inactivation of hyaluronidase by urea and guanidine hydrobromide. Increasing the denaturing agent concentration caused progressive decrease in catalytic activity of

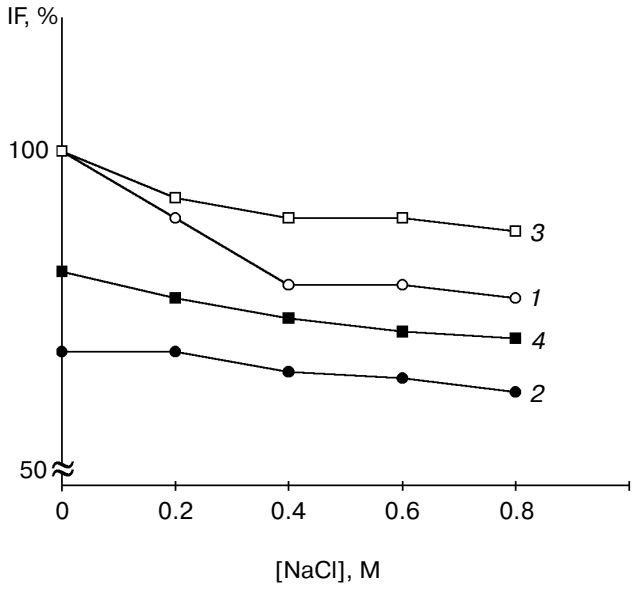

Fig. 6. Dependence of relative intensity (%) of hyaluronidase intrinsic fluorescence on NaCl concentration in the absence (*1*, *3*) and in the presence (*2*, *4*) of heparin: *1*, *2*) native hyaluronidase; *3*, *4*) dextran-modified hyaluronidase.

both native and dextran-modified hyaluronidase (Fig. 7). Heparin potentiated this inactivation. The urea concentrations required for 50% inactivation of hyaluronidase activity (IC_{50}) were 4.0 and 5.0 M for the native and dextran-modified enzymes, respectively. In the presence of heparin, IC_{50} values for inactivation of the native and dextran-modified enzymes were somewhat lower (2.6 and 4.4 M, respectively) (Fig. 7). Guanidine hydrobromide exhibited similar effects (with almost identical shape of inactivation curves as in Fig. 7). However, 50% reduction of hyaluronidase activity of the native and dextran-modified enzymes was observed at significantly lower concentrations (0.3 and 0.4 M). The IC_{50} values for inhibition of the native and modified hyaluronidases in the presence of heparin were 0.25 and 0.3 M, respectively.

DISCUSSION

Native testicular hyaluronidase. The hyaluronidase preparation produced in Russia under the commercial name Lidase is used in medical practice for local administration [13]. Single-stage gel filtration of this preparation yields highly purified enzyme with apparent molecu-

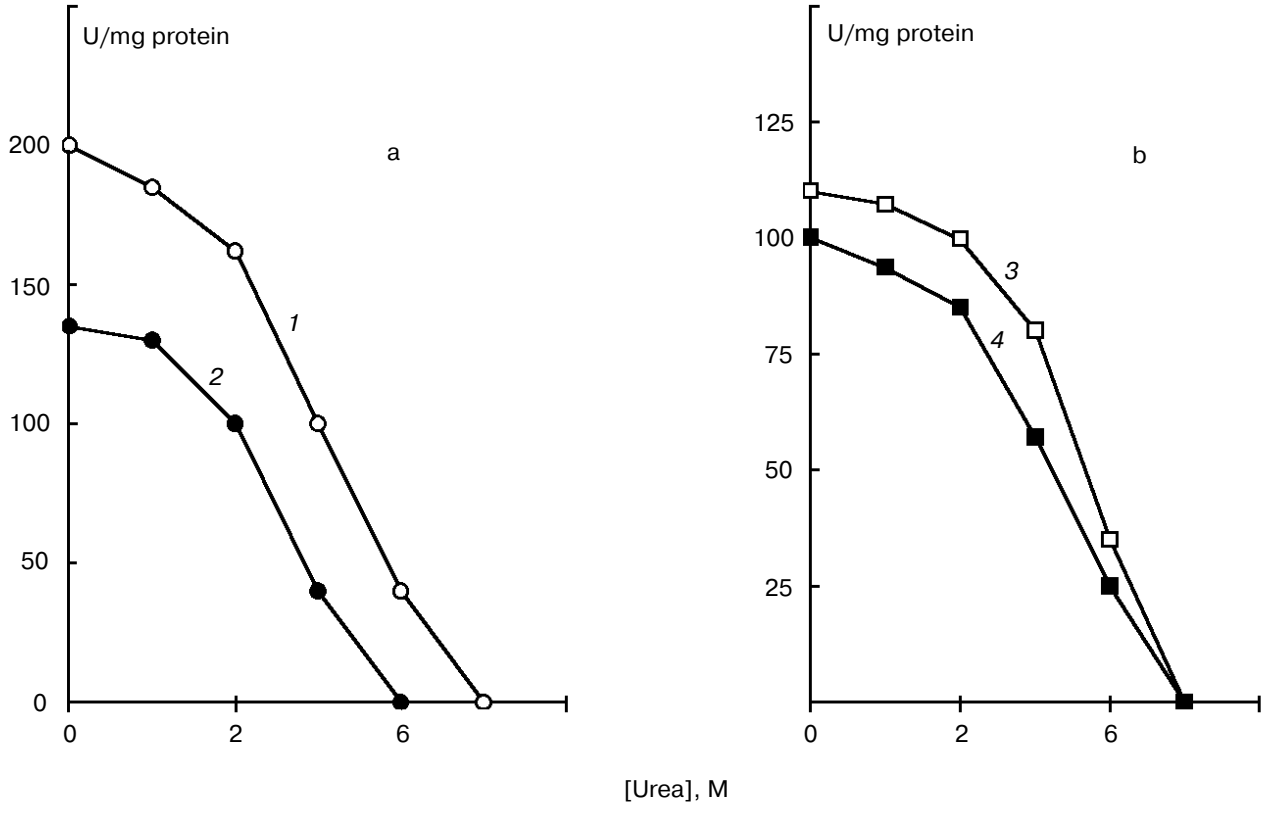

Fig. 7. Inactivation of native (a) and dextran-modified (b) hyaluronidase in the absence (*1*, *3*) and in the presence (*2*, *4*) of heparin.

lar mass of 60-68 kD (Figs. 1 and 2, table). According to literature data, the molecular mass of hyaluronidase varies in the range 60-90 kD [9]. The enzyme has pH optimum at 5.0-6.0 (Fig. 3). This is consistent with previously reported values for the pH optimum for the testicular enzyme at pH 5.0 [14] and 6.0 [15]. Optimal ionic strength promoting manifestation of the highest hyaluronidase activity is achieved at 0.15 M NaCl (Fig. 4). Some evidence exists that the hydrolytic activity of this enzyme depends on the presence of sodium chloride [14]. Heparin inhibits hyaluronidase activity of the native enzyme by 1.5- and 1.25-fold at pH 5.5 and 7.5, respectively (Fig. 4c). At these pH values the dependence of catalytic activity of native and modified hyaluronidase on ionic strength (assayed in the presence and in the absence of heparin) is also symbatic. This suggests that electrostatic interactions do not dominate in the enzyme– inhibitor interaction (Fig. 4c). Electrostatic forces influence surface chromophores of the hyaluronidase molecule, and this results in a decrease in the intensity of intrinsic protein fluorescence with increasing sodium chloride concentration in the medium (Fig. 6, curve *1*). The presence of heparin in the system attenuates this effect (Fig. 6, curve 2) suggesting the development of interactions other than electrostatic ones. The experiments with denaturing agents seem to support this point (Fig. 7). Treatment with urea or guanidine compounds denatures globular proteins due to destruction of hydrophobic interactions [16]. Indeed, increasing urea concentration, which causes unfolding of proteins, is accompanied by inactivation of hyaluronidase (Fig. 7a). Heparin potentiates the denaturing effect possibly due to additional destructive effect on hydrophobic interactions and hydrogen bonds of the native structure.

Dextran-modified hyaluronidase. Since we found previously that increasing modification of hyaluronidase is accompanied by pronounced decrease in its sensitivity to inhibition by heparin [8], the enzyme with the highest modification degree was used in the present study. Covalent modification significantly increased the apparent molecular mass of the resulting hyaluronidase–dextran conjugate to 100-140 kD (Figs. 1 and 2). In spite of some inactivation, the conjugated enzyme retained high catalytic activity (table). Modification extended the pH optimum to the range 4.5-6.5 (Fig. 3); the optimal sodium chloride concentration for hyaluronidase activity also became wider (0.1-0.3 M, Fig. 5). The latter indicates attenuation of electrostatic effect on the hyaluronidase activity of the modified preparation. At pH 7.5 this effect became almost negligible (Fig. 5c). Modification of hyaluronidase with neutral dextran eliminates the positive change of its surface amino groups. This was accompanied by a decrease of the modified enzyme inhibition by the polyanion heparin: at pH 5.5, it reduced hyaluronidase activity by 1.2-fold, and at pH 7.5 the inhibition was almost negligible (Fig. 5c). The symbatic mode suggests lack of significant electrostatic effect on the inhibition process. The modification of the enzyme with dextran reduced the effect of electrostatic interactions (Fig. 6, curves *1* and *3*). In the presence of heparin it was also very weak (Fig. 6, curves *2* and *4*). Previously we demonstrated [8] that modification introduced similar limiting changes into the interaction of the enzyme with heparin and also in modification-induced conformational changes of the enzyme molecule. However, the nature of such changes remained unclear. The data suggest hydrophobic and steric effects. The effect of denaturing agents used in the present study revealed the modification-induced augmentation of hydrophobic interactions (Fig. 7, curves *1* and *3*). The destabilizing effect of heparin does not contradict an alteration by heparin of the network of hydrophobic interactions and hydrogen bonds in the modified enzyme. The chemically modified hyaluronidase was more stable to denaturing agents and their combination with heparin than the native hyaluronidase. Interestingly, difference in endo- and exo-depolymerizing activities of carbohydrases is explained by the topography of their active sites [17]. Endo-specificity is determined by an extended cleft of the active site in which linear oligosaccharide substrates are randomly (i.e., statistically) subjected to catalytic attack along the whole substrate chain. Exo-specificity is mainly associated with active site structure of a closed tunnel in which the substrate molecule sequentially moves from the non-reducing end. Statistical endoglycosidase activity of hyaluronidase [9] implies the existence of the active site as an open extended cleft. It is possible that under high modification degree the dextran chain repeats fold in the active site region and induces additional hydrophobic interactions and steric hindrances that counteract denaturing agents and inhibitors. Thus, possible formation of modifier-induced shape of the active site (as extended open cleft) promotes endoglycosidase activity of the biocatalyst (by reducing the depressive effect of Coulomb forces and heparin inhibition, Fig. 5) and increase of its stability (Fig. 7).

Targeted alteration of the properties of hyaluronidase by chemical modification. Native testicular hyaluronidase is an acidic endoglycosidase; its catalytic activity depends on electrostatic interactions. Increase of ionic strength above 0.1-0.2 M NaCl initiates the transfer chromophore groups of hyaluronidase to a more polar environment, and this results in a decrease in enzymatic activity. The effect of heparin on hyaluronidase is obviously realized via hydrophobic interactions and rearrangement of hydrogen bonds accompanied by enzyme inhibition and attenuation of the role of electrostatic forces.

Chemical conjugation of hyaluronidase with dextran increased hydrophobic interaction in protein glob-

ule. Such strengthening of this structure reduced sensitivity to heparin, which was almost insensitive to the ionic strength of incubation medium (Fig. 5c). Thus, dextran-modified hyaluronidase was insignificantly inhibited by heparin at pH 7.5. It was previously reported that increase in glycozylation of antithrombin reduced its affinity for heparin [18]. This suggested the existence of a heparin-binding site on protein molecule that can be sterically controlled by oligosaccharide chains. Perhaps contribution of a steric factor should not be ignored during hyaluronidase modification. Marked reduction of the sensitivity of hyaluronidase to inhibition by heparin during modification of buried amino groups [8] suggests the existence of a heparin-binding site on the hyaluronidase molecule that is deformed in the process of deep modification of the biocatalyst. Study of localization of this site by means of such modification and evaluation of structure/activity ratio may suggest approaches for regulation of enzyme functioning in biological systems. Subsequent studies will clarify whether such changes in the properties of hyaluronidase (reduction of inhibition, increase of molecular size, stabilization) are sufficient for manifestation of therapeutic treatment of acute cardiovascular lesions or whether it is possible to obtain modified hyaluronidase with similar properties but with higher catalytic activity.

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