Selective binding of zinc ions to heparin rather than to other glycosaminoglycans

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The relative binding affinity of Zn^{2+} to several glycosaminoglycans was determined by gel-filtration chromatography. Binding was observed only between Zn^{2+} and heparin. No binding was observed between Zn^{2+} and chondroitin 4-sulphate, chondroitin 6-sulphate, dermatan sulphate or hyaluronic acid. All of the glycosaminoglycans contained carboxy groups, but only heparin bound Zn^{2+} . This observation suggests that, contrary to a previously proposed hypothesis, simple electrostatic interactions between the negatively charged carboxy groups of the glycosaminoglycans and the positively charged Zn^{2+} cannot explain the observed binding.

Experimental evidence has shown that the normal prostate gland contains one of the highest concentrations of total zinc found in any organ or tissue in the human body (Mawson & Fischer, 1953). In addition, good correlations between the total zinc concentration of the prostate or its secretions and the physiological state of the prostate have been reported [i.e. patients with prostatic carcinoma (Mawson & Fischer, 1952) and chronic bacterial prostatitis (Fair *et al.*, 1976) demonstrate decreased contents of prostatic total zinc relative to that found in non-diseased prostatic tissue]. Although it has been established that Zn^{2+} complexed with a wide variety of natural and synthetic materials (Hughes, 1975), only limited experimental results have been published about the chemical status of zinc in the prostate. A portion of the prostatic total zinc is known to be present as a coenzyme or cofactor in prostatic proteins (Vallee & Wacker, 1970) (e.g. carbonic anhydrase). A Zn^{2+} -binding protein has been purified and partially characterized with respect to its molecular weight and amino acid composition (Reed & Stitch, 1973; Heathcote & Washington, 1973), and expressed prostatic secretions contain complexes between Zn^{2+} and small peptides (Fair *et al.*, 1976; Johnson *et al.*, 1969). Also, glycosaminoglycans (acid mucopolysaccharides) have been proposed as binding agents *in vivo* for prostatic Zn^{2+} (Sato & Györkey, 1969). However, it was not reported whether Zn^{2+} binding was a general property of all glycosaminoglycans, or if a

specific glycosaminoglycan was responsible. In an attempt to clarify this point, a series of binding experiments *in vitro* between Zn^{2+} and several glycosaminoglycans were performed. In the present paper, experimental evidence is presented that demonstrates that Zn^{2+} binding to glycosaminoglycans is not a general phenomenon, but is restricted to heparin.

Experimental

Materials

Chondroitin 4-sulphate, chondroitin 6-sulphate, dermatan sulphate, heparin and hyaluronic acid were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Pepsin was a product of Calbiochem (San Diego, CA, U.S.A.).

Analytical methods

Glycosaminoglycan concentrations were determined by the method of Bitter & Muir (1962). Zinc concentrations were determined by atomic absorption spectroscopy, with a Perkin–Elmer model 107 instrument.

Gel chromatography

Sephadex G-25 was swollen overnight at ambient temperature in a solution of 50 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/NaOH buffer, pH 7.0. A small column (1.5 cm $\times$ 48 cm) containing the swollen gel was prepared and equilibrated with the same buffer. A 1.0 ml sample of the appropriate reaction mixture was applied to the column, and the column was

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developed with the 50 mM-Hepes/NaOH buffer, pH 7.0. Fractions (35 drops each) were collected at a rate of 1 drop/18 s. Fractions were analysed for zinc and/or glycosaminoglycan by the methods indicated above. Exact composition of the samples applied to the column is given in the appropriate Table or Figure legend. After each experiment, the Sephadex G-25 column was washed with 200 ml of 50 mM-Hepes/NaOH buffer, pH 7.0, before application of the next sample.

Results

Zn^{2+} binding to a series of commercially available glycosaminoglycans was determined by gel-filtration chromatography. The theory of gel chromatography with respect to both high-affinity systems and systems of intermediate affinity has been reviewed by Ackers (1973). In brief, gel chromatography can be thought of as a continuous washing process, since the material passing down the column continually encounters fresh buffer. If a high-affinity glycosaminoglycan- Zn^{2+} complex were formed, it would be eluted in the void volume of the column, owing to the large size of the glycosaminoglycans. Any loosely bound Zn^{2+} that dissociated from the complex would be retained within the gel matrix and effectively removed from further interaction with the glycosaminoglycans. Thus the column technique is a very adequate model system

to compare the relative binding affinity of Zn^{2+} for the various glycosaminoglycans. Figs. 1 and 2 show the respective elution profiles for heparin and for Zn^{2+} obtained after gel chromatography over Sephadex G-25. However, when Zn^{2+} and heparin were incubated together and then subjected to gel chro-

Fig. 2. Elution profile of Zn^{2+} from Sephadex G-25. A 1.0 ml sample consisting of 50 mM-Hepes/NaOH buffer, pH 7.0, containing 2.5 mM-zinc acetate was applied to the column, and 35-drop fractions were collected. A 25 μl sample was removed, added to 1.00 ml of buffer, and the zinc content was measured as indicated in the Experimental section.

Fig. 1. Elution profile of heparin from Sephadex G-25. The column was equilibrated with 50 mM-Hepes/NaOH buffer, pH 7.0. A 1.0 ml sample consisting of 5 mg of heparin, dissolved in 50 mM-Hepes/NaOH buffer, pH 7.0, was applied to the column, and 35-drop fractions were collected. A 100 μl sample was removed from each fraction and assayed for glycosaminoglycan content as indicated in the Experimental section.

Fig. 3. Elution profiles for Zn^{2+} -heparin from Sephadex G-25.

A 1.0 ml reaction mixture consisting of 50 mM-Hepes/NaOH buffer, pH 7.0, containing 2.5 mM-zinc acetate and 5 mg of heparin was incubated for 10 min at ambient temperature and applied to the column, and 35-drop fractions were collected. A 100 μl sample was removed from each fraction and assayed for heparin (●) as indicated in the Experimental section. A second sample (25 μl) was added to 1.00 ml of 50 mM-Hepes/NaOH buffer, pH 7.0, and assayed for zinc (▲) as indicated in the Experimental section.

Table 1. *Binding of Zn^{2+} and glycosaminoglycans*

Glycosaminoglycan	Composition*	Present in prostate†	Binds zinc‡
Chondroitin 4-sulphate	<i>N</i> -Acetyl-D-galactosamine 4-sulphate, glucuronic acid	Yes	No
Chondroitin 6-sulphate	<i>N</i> -Acetyl-D-galactosamine 6-sulphate, glucuronic acid	Yes	No
Dermatan sulphate	<i>N</i> -Acetyl-D-galactosamine 4-sulphate, iduronic acid	Yes	No
Hyaluronic acid	<i>N</i> -Acetyl-D-glucosamine, glucuronic acid	Yes	No
Heparin	Glucosamine 6-sulphate, glucuronic acid 2-sulphate, iduronic acid	Unknown	Yes

* Whistler & Rowell (1966).

† Sato & Györkey (1972).

‡ Experimental conditions identical with those for Fig. 3.

matography, a new peak of zinc was observed (Fig. 3). This new peak of zinc corresponded to the heparin peak, indicating binding between heparin and Zn^{2+} . Binding to a protein contaminant in the heparin preparation was eliminated by the observation that pretreatment of the heparin with pepsin, a broad-specificity proteinase, did not eliminate the binding of Zn^{2+} and heparin. Control experiments indicated that pepsin did not bind Zn^{2+} .

To determine whether Zn^{2+} binding was a specific property of heparin or a general glycosaminoglycan effect, a series of glycosaminoglycans, previously shown to be present in human prostatic tissue (Sato & Györkey, 1972), was tested for Zn^{2+} binding under conditions identical with those previously utilized for heparin. The results presented in Table 1 showed that none of the other glycosaminoglycans exhibited any Zn^{2+} binding. It should be pointed out that it was necessary to dilute the heparin-containing fractions 41-fold in order to obtain satisfactory absorbance readings for the zinc determinations. However, when zinc concentrations were determined in the column fractions containing the other glycosaminoglycans, no dilution was made, and no zinc was detected in any fractions that contained glycosaminoglycan. This reinforces the selective nature of the Zn^{2+} binding by heparin relative to glycosaminoglycans previously demonstrated to be present in the prostate.

Discussion

The studies by Sato & Györkey (1969) and Györkey & Sato (1968), established that $^{65}Zn^{2+}$ would bind to formalin-fixed sections of human prostatic tissue, and suggested that the carboxy groups of glycosaminoglycans were responsible for the binding of Zn^{2+} . This hypothesis was based on two experimental observations. When the formalin-fixed sections of human prostatic tissue were treated

with methanol/HCl, under conditions known to esterify carboxy groups, Zn^{2+} binding to formalin-fixed tissue was abolished (Sato & Györkey, 1969). In addition, when the formalin-fixed tissue was stained for glycosaminoglycan with Alcian Blue, the Zn^{2+} binding was found to be localized in the glycosaminoglycan-staining area (Györkey & Sato, 1968). It was later reported that dermatan sulphate and hyaluronic acid accounted for 62% and 25% of the isolatable glycosaminoglycans from human prostate. The remainder of the isolatable glycosaminoglycans were reported to be chondroitin 4-sulphate and chondroitin 6-sulphate in amounts of 11% and 2% respectively (Sato & Györkey, 1972). However, the results shown in Table 1 clearly demonstrate that Zn^{2+} did not bind to any of the glycosaminoglycans previously shown to be present in the human prostate. Zn^{2+} bound only to heparin, thus indicating that Zn^{2+} binding is not a general property of glycosaminoglycans.

Although heparin binds Zn^{2+} in the test system *in vitro* utilized, there is no report of the presence of heparin in the human prostate. Therefore it would be premature to postulate that heparin is a prostatic Zn^{2+} -binding ligand. However, it is clear from the results presented in Table 1 and the structures of the various glycosaminoglycans that binding between Zn^{2+} and heparin cannot be the result of simple electrostatic interactions between the positively charged Zn^{2+} and the negatively charged carboxy groups of the heparin. At least one additional, as yet unidentified, ligand must be present for binding to occur.

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