Zn²⁺–heparin interaction studied by potentiometric titration

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Measurement of the decrease in pH that accompanies the addition of Zn²⁺ to heparin in solution provided an indirect method of examining cation–polyanion interaction. Construction of plots analogous to isothermal saturation binding plots revealed the existence, for defined conditions of interaction, of a [heparin]-independent direct proportionality between the fraction of the maximal pH change occurring and the [Zn²⁺]/[heparin disaccharide] ratio. This accords with results from polarimetric examination of Ca²⁺– and Cu²⁺–heparin interactions. It suggests that, under the conditions used, cation–heparin interaction may result in the formation of a complex that exists in a colloid-like phase, between which and the aqueous phase, exchange of cations does not follow simple solution-phase reversible equilibrium thermodynamic behaviour. The results suggest that the putative Zn²⁺-containing complex is less stable in the presence of NaCl than is the corresponding Ca²⁺-containing complex. Addition of Zn²⁺ to low concentrations of heparins is accompanied by the usual decrease in pH, followed by a removal of H⁺ from solution as the [Zn²⁺]/[heparin disaccharide] ratio increases, suggesting dissolution of the putative complex. This reversal of the initial pH change was not seen for most other cation–heparin interactions under the conditions studied.

INTRODUCTION

Zinc is present in all living organisms and has a greater variety of biochemical functions than any other trace element. It is an essential component of nearly 300 enzymes involved in many different aspects of metabolism. Recently, the role of Zn²⁺ in the activities of nucleic acid-binding and gene-regulatory proteins has received much attention. In addition, zinc stabilizes the hydrated structures of proteins and nucleic acids, preserves the integrity of subcellular organelles, participates in transport processes, and has important roles in several viral and immune phenomena. Its nutritional essentiality has focused attention on the pathology and clinical consequences of both the deficiency and toxicity of the metal. Major reasons for the biological versatility of zinc complexes is the variable degree of Lewis acidity of this metal and its accommodating flexibility of co-ordination geometry (Bryce-Smith, 1989; Berg, 1990; Vallee & Auld, 1990); a consequence of these properties is that zinc–ligand interaction may result in alterations in the biochemical properties of both components of the interaction.

Heparins and heparan sulphates are glycosaminoglycans composed of repeating disaccharide units of substituted D-glucosamine and uronic acid residues (Gallagher & Walker, 1985). Heparins are widely used pharmacological anticoagulants, are components of mast cells, and are of uncertain physiological function. Heparan sulphates contain less sulphate groups and iduronic acid residues and more acetyl groups and glucuronic acid residues than heparins, are present in mammalian pericellular matrices and nuclear membranes, and apparently play roles in cell adhesion and in the determination of the proliferative state of cells. Such polymers possess substituent groups so stereochemically disposed as to permit complex co-ordination and chelation of a range of inorganic and organic cations, and several cation–heparin interactions putatively affecting the activities of either or both of the participants have been described (Nieduszynski, 1989).

Glycosaminoglycan–zinc interaction may underlie the binding of ⁶⁵Zn²⁺ to formalin-fixed sections of human prostatic tissues (Gyorkey & Sato, 1968; Sato & Gyorkey, 1969), the antiviral efficacy of heparin–zinc salt mixtures (Lukas, 1979; Takahashi *et al.*, 1986), an enhanced recovery of heparin from tissue treated

with zinc acetate (Mihailescu & Nitelea, 1983), a displacement from heparan sulphate *in vitro* of ⁴⁵Ca by incubation with Zn²⁺ (Kojima *et al.*, 1983), a decrease in ⁶⁷Ga uptake in zinc-treated rats (Kojima *et al.*, 1983, 1984), the nuclear decondensation of mammalian spermatozoon nuclei by heparin (Reyes *et al.*, 1983), and a competitive binding assay for the measurement of heparan sulphate present in tissue digests (Wu & Cohen, 1984). These observations hint at possibly important roles for zinc–heparin/heparan sulphate interactions *in vivo*.

Zinc is present in pharmaceutical heparin (Gervin *et al.*, 1983; Grant *et al.*, 1987), and gel-filtration-chromatography experiments showed that Zn²⁺ bound to heparin more readily than to other glycosaminoglycans (Sato & Gyorkey, 1976; Parrish & Fair, 1981; Grushka & Cohen, 1982). Potentiometry indicated the particular stability of complexes formed between heparin and Zn²⁺ (Burger *et al.*, 1984), and the spectrophotometric experiments of Mattai & Kwak (1988) suggested that Zn²⁺ binding to heparin (like that of Ca²⁺) was stronger and more specific than would be expected if the interaction were simply electrostatic. A temperature-dependency of the binding, measured by equilibrium dialysis, of ⁶⁵Zn²⁺ to heparin indicated that such binding derived principally from an entropic driving force perhaps arising from a perturbation of the environment of water molecules associated with the polymer (Woodhead *et al.*, 1986); in other words, the interaction appeared to involve an energetically favourable change in the hydration of the interacting species. The near-i.r. spectra of metal-ion–heparin complexes (including that containing Zn²⁺) depends on the nature of the cation, and this certainly suggests that such perturbations in hydration accompany cation–heparin interactions (Grant *et al.*, 1987). Another i.r. spectroscopic study showed that the spectrum of Zn²⁺–heparin contained unusually complex overlapping absorptions due to the carboxylate groups of the polymer (Panov & Ovsepyan, 1984). N.m.r. spectroscopy of complexes formed between Zn²⁺ and model monosaccharides suggested that metal binding induces conformational changes in the iduronate ring (Whitfield & Sarkar, 1991).

Recently, results of polarimetric measurements and potentiometric titrations indicated that, under the conditions used, cations such as Ca²⁺ and Cu²⁺ interacted with heparin by mechanisms that were not identical (Grant *et al.*, 1992a,b), and

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re-affirmed the notion (Mattai & Kwak, 1988) that such binding cannot be described adequately in terms of simple electrostatic concepts. Further, cation–polyanion interaction appeared not to be explicable in terms of simple equilibrium phenomena. Rather, interaction was considered to involve partition of cations between more than one phase, exchange between which did not occur through reversible thermodynamic equilibrium processes. In such a situation the kinetics of the molecular events leading to complex formation and hence the precise conditions under which the interacting molecules are presented to one another, must be critical to the nature of the interaction (Grant *et al.*, 1992a,b,c).

In the present paper, potentiometric titration is used to examine the interaction of Zn^{2+} with heparin. The study affirms the complex nature of bivalent-cation–heparin interaction, and reveals an aspect of Zn^{2+} –heparin interaction not observed during the binding to heparin of most of the other cations so far studied under these conditions.

EXPERIMENTAL

The heparin used was from Evans Pharmaceuticals (Grant *et al.*, 1992c). Other materials were of AnalaR grade. Solutions were prepared with distilled water which was boiled to remove CO_2 and cooled under O_2 -free N_2 . An average heparin disaccharide unit was taken to be hexadecahydrated tetrasodium 2-*O*-sulphatiduronosyl 6-*O*-sulphatoglucosamine-2-*N*-sulphate. It is acknowledged that polysaccharide heterogeneity means that this assumed structure and hydration allow only 'conventional' M_r values, of average disaccharide units, to be deduced. M_r values so deduced were used to calculate the ratios plotted as ordinates in Figs. 1–5 (below), and throughout the paper, heparin concentrations are expressed with respect to this average disaccharide unit.

Potentiometric titrations were carried out using the following equipment from Radiometer (Copenhagen): Research pH Meter (PHM 84), Autoburette (ABU 80), Servograph Recorder (REC 80) and mechanically stirring Titration Assembly (TTA 80), the water-jacket of which was kept at 37 °C using a Haake (Berlin) circulating water thermostat (F 4391). Salt solution was added at a rate of 0.1 ml/min to 15 cm³ of heparin solution (15 ml of a 5.6 mM solution, unless otherwise stated in Fig. legends.) The change in pH which occurred after cation addition was expressed as (pH in the presence of cation – pH in absence of cation)/(minimal pH at 'high' cation concentration – pH in absence of cation). At 'high' cation concentration, the [cation]/[heparin disaccharide] ratio was 3 and 12 in the absence and presence respectively of NaCl. Under these conditions, no further decrease in pH occurred on addition of more cation. The initial pH, before salt addition, was 6.9, and the maximal pH change occurring under standard conditions, as used, e.g. in Fig. 2 (below), was –1.2 pH unit. All experiments, except those described in Figs. 1 and 3 below, were carried out in the absence of additional electrolyte.

Polarimetric measurements by the method described by Grant *et al.* (1992a) were made using a Thorn Automation-NPL Automatic Polarimeter type 243. Addition of aq. ZnCl_2 solution to heparin elicited an increase in optical rotation (Moffat *et al.*, 1984; Grant *et al.*, 1992a). The change in optical rotation which occurred after cation addition was expressed as (optical rotation in presence of cation – optical rotation in the absence of cation)/(maximal optical rotation at 'high' cation concentration – optical rotation in absence of cation). At 'high' cation concentration, the [cation]/[heparin disaccharide] ratio was 3. At this ratio no further change in the optical-rotation value (corrected as described in Grant *et al.*, 1992a) occurred on addition of more cation.

The changes in pH and in optical rotation observed on cation–heparin interaction were expressed, as described in the preceding two paragraphs respectively, as arithmetic ratios of the maximal changes occurring in these properties when metal ion–heparin interaction occurred, in order that values analogous to isothermal saturation fractions of polymer-binding sites occupied by the cation could be calculated. Graphs analogous to conventionally derived adsorption isotherms could then be plotted.

RESULTS AND DISCUSSION

Fig. 1 shows (open symbols) the binding of Zn^{2+} to heparin as measured by equilibrium dialysis; the results are taken from Woodhead *et al.* (1986) and are conventionally presented as isothermal saturation fractions plotted against the ratio $[\text{Zn}^{2+}]/[\text{heparin disaccharide}]$. In this early study the ratio at which the saturation of Zn^{2+} -binding sites occurred was probably somewhat underestimated. Fig. 1 also shows (closed symbols) an experiment in which Zn^{2+} –heparin interaction occurred under identical conditions but was examined by potentiometric titration. To allow comparison with the equilibrium-dialysis study, the change in pH that accompanied the cation–heparin interaction was plotted as a fraction equivalent to a conventionally derived isothermal saturation fraction (see Experimental section).

Recently it has been shown that plots analogous to, and closely resembling, isothermal binding curves may be obtained by measuring the change in optical rotation that accompanies addition of a bivalent cation to heparin (Grant *et al.*, 1992a,b). Fig. 2 shows the binding of Zn^{2+} to heparin as measured by polarimetry (open symbols) and potentiometric titration (closed symbols). The results in Figs. 1 and 2 reinforce the notion that potentiometric titration affords a rapid and simple method by which cation–heparin interaction may be indirectly examined (Grant *et al.*, 1992c).

The potentiometrically derived plots in Figs. 1 and 2 are not identical, because they represent Zn^{2+} –heparin interaction occurring in the presence and absence respectively of 0.15 M-NaCl. In particular, the plot in Fig. 2 is linear up to a $[\text{Zn}^{2+}]/[\text{heparin disaccharide}]$ ratio of about 1. Linearity in analogous plots derived from polarimetric examination of Ca^{2+} and Cu^{2+} inter-

Fig. 1. Isothermal saturation fractions of binding sites on heparin occupied by Zn^{2+} and equivalent fractions derived from potentiometric measurements, as functions of $[\text{Zn}^{2+}]/[\text{heparin disaccharide}]$

A binding site was taken to be an average heparin disaccharide unit (see the Experimental section). Isothermal saturation fractions obtained by equilibrium dialysis (○) and equivalent fractions derived from potentiometric measurements (●) were derived as described in the Experimental section. In both experiments, the $[\text{NaCl}]$ was 0.15 M.

Fig. 2. Fractions equivalent to isothermal saturation fractions of binding sites on heparin occupied by Zn²⁺, derived from polarimetric and potentiometric measurements, as functions of [Zn²⁺]/[heparin disaccharide]

A binding site was taken to be an average heparin disaccharide unit (see the Experimental section). Fractions equivalent to isothermal saturation fractions and derived from polarimetric (○) or potentiometric measurements (●) were obtained, in the absence of NaCl, as described in the Experimental section.

action with heparin, and occurring at low [cation]/[heparin disaccharide] ratios, have been observed (Grant *et al.*, 1992a,b). The linearity suggests that the interactive process, presumed to be the formation of a species of cation-heparin complex, which gives rise to this part of the plot, occurs by a mechanism that does not conform to simple single solution-phase reversible equilibrium thermodynamics. Any process which did so conform would result in a hyperbolic plot. The plot obtained suggests that, at higher [cation]/[heparin disaccharide] ratios, further cation interaction occurs. In the case of the Zn²⁺-heparin

interaction this appears, under the conditions explored in Fig. 2, to approach completion at a [cation]/[heparin disaccharide] ratio of about 3. It has been suggested that such observations may reveal the production, at low [cation]/[heparin disaccharide] ratios, of a cation-polyanion complex that resembles a discrete hydrated colloidal mineral-like state that is, therefore, not subject to simple aqueous solution equilibrium processes and that is stabilized by forces additional to electrostatic ones. At higher [cation]/[heparin disaccharide] ratios, destabilization of the phase, generating the non-linear portion of the plot, may occur (Grant *et al.*, 1992a,b). That a particular cation may interact with a polyanion such as heparin to produce multiple discrete complexes, the nature and proportion of which depend upon the conditions of interaction, is suggested by the work of Burger *et al.* (1984).

In contrast, the curved nature of the potentiometrically derived plot in Fig. 1 suggests that, in the presence of 0.15 M-NaCl, interaction of Zn²⁺ with heparin at low [Zn²⁺]/[heparin disaccharide] ratios results in the formation of a complex, between which and the solution interchange of Zn²⁺ occurs by a conventional reversible equilibrium process.

Fig. 3 (closed symbols) shows potentiometrically derived plots of Zn²⁺-heparin interaction occurring in the presence or absence of NaCl; Fig. 3 (open symbols) shows similar plots for Ca²⁺-heparin interaction. The production of linear plots for Zn²⁺-heparin interaction occurring in the absence of NaCl, and the production of such plots for Ca²⁺-heparin interaction occurring in the presence of NaCl, suggest that a putative Ca²⁺-heparin interaction not conforming to conventional solution-phase thermodynamics is more stable in the presence of NaCl than is an analogous Zn²⁺-heparin interaction.

Fig. 4 shows the near identity, at low [Zn²⁺]/[heparin disaccharide] ratios, of potentiometrically derived plots obtained from experiments in which Zn²⁺ and heparin were mixed under conditions of widely differing [heparin]. Such similarity is also seen in polarimetrically derived plots in which Ca²⁺- and Cu²⁺-heparin interactions are explored (Grant *et al.*, 1992a,b), and accords with the idea that processes not conforming to

Fig. 3. Fractions equivalent to isothermal saturation fractions of binding sites on heparin occupied by cation, derived from potentiometric measurements, as functions of [cation]/[heparin disaccharide]: effect of the presence of [NaCl]

CaCl₂ (○) or ZnSO₄ (●) was added to heparin (20 ml of a 0.56 mM solution) in the presence of the following concentrations (M) of NaCl: (a) 0; (b) 0.15; (c) 0.20. Other details are as given in the legend to Fig. 2.

Fig. 4. Fractions equivalent to isothermal saturation fractions of binding sites on heparin occupied by Zn^{2+} , derived from potentiometric measurements, as functions of $[\text{Zn}^{2+}]/[\text{heparin disaccharide}]$: effect of variation in [heparin]

ZnSO_4 was added to 20 ml of a solution containing the following concentrations of heparin (mM) in the absence of NaCl: (Δ) 0.011; ($\blacksquare$) 0.042; ($\square$) 0.15; ($\bullet$) 0.29; ($\circ$) 0.59. Other details are as given in the legend to Fig. 2.

Fig. 5. Fractions equivalent to isothermal saturation fractions of binding sites on heparin occupied by cations, derived from potentiometric measurements, as functions of $[\text{cation}]/[\text{heparin disaccharide}]$: effect of adding cation to low [heparin]

The following salts were added to 20 ml of a solution of heparin (0.56 mM) in the absence of NaCl: ($\circ$) Al_2SO_4 ; ($\bullet$) CaCl_2 ; ($\square$) CuSO_4 ; (Δ) $\text{FeSO}_4 \cdot (\text{NH}_4)_2\text{SO}_4$; ($\blacksquare$) ZnSO_4 . Maximal pH changes recorded were -3.1 , -0.65 , -1.9 , -2.4 and -1.2 pH units respectively. Other details are as given in the legend to Fig. 2.

reversible equilibrium thermodynamics are occurring. Any process which did so conform would result in different plots being obtained at different [heparin] values.

Further, Fig. 4 shows that the initial Zn^{2+} addition to low concentrations of heparin is accompanied by the usual decrease in pH, but that, as the $[\text{Zn}^{2+}]/[\text{heparin disaccharide}]$ ratio is increased by further Zn^{2+} addition, this pH change is reversed. The source of the protons released during metal cation binding to heparin is not known, but may involve direct or indirect replacement of covalently bound protons by metal cations (Grant *et al.*, 1988); some of these protons may be present in hydrogen-bonded structures, including those occurring in heparin-associated water molecules (Grant *et al.*, 1992c). The results suggest that, under the conditions examined, formation of a putative colloid-like Zn^{2+} -heparin complex with an accompanying release of protons, may be followed, as the $[\text{Zn}^{2+}]/[\text{heparin disaccharide}]$ ratio is increased, by dissolution of the complex accompanied by proton removal from solution.

Fig. 5 shows that, under conditions in which the proton release after addition of Zn^{2+} or Ca^{2+} ions to heparin is reversed at high [cation]/[heparin disaccharide] ratios, addition of Cu^{2+} , Fe^{3+} or Al^{3+} to the same high ratios does not result in a reversal of proton release. A proton-pumping or transport system based on the formation and dissolution of particular bivalent-cation-glycosaminoglycan complexes is an intriguing possibility.

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REFERENCES

- Berg, J. M. (1990) *J. Biol. Chem.* **265**, 6513–6516
- Bryce-Smith, D. (1989). *Chem. Br.* **25**, 783–786
- Burger, K., Gaizer, M., Pekli, M., Takacs Nagy, G. & Siemroth, J. (1984) *Inorg. Chim. Acta* **92**, 173–176
- Gallagher, J. T. & Walker, A. (1985) *Biochem. J.* **230**, 665–674
- Gervin, C. A., Gervin, A. S., Nichols, W. & Corrigan, J. J. (1983) *Life Sci.* **33**, 2643–2649
- Grant, D., Long, W. F. & Williamson, F. B. (1987) *Biochem. J.* **244**, 143–149
- Grant, D., Long, W. F. & Williamson, F. B. (1988) *Biochem. Soc. Trans.* **16**, 1028–1029
- Grant, D., Long, W. F., Moffat, C. F. & Williamson, F. B. (1992a) *Biochem. J.* **282**, 601–604
- Grant, D., Long, W. F., Moffat, C. F. & Williamson, F. B. (1992b) *Biochem. J.* **283**, 243–246
- Grant, D., Long, W. F. & Williamson, F. B. (1992c) *Biochem. J.* **283**, 243–246
- Grushka, E. & Cohen, A. S. (1982) *Anal. Lett.* **15**, 1277–1288
- Gyorkey, F. & Sato, C. S. (1968) *J. Mol. Pathol.* **8**, 216–224
- Kojima, S., Hama, Y., Sasaki, T. & Kubodera, A. (1983) *Eur. J. Nucl. Med.* **8**, 52–59
- Kojima, S., Sasaki, T. & Kubodera, A. (1984) *Eur. J. Nucl. Med.* **9**, 33–38

- Lukas, B. (1979) European Patent A 61K33/30
- Mattai, J. & Kwak, J. C. T. (1988) *Biophys. Chem.* **31**, 295–299
- Mihailescu, G. & Nitelea, I. (1983) Romanian Patent RO 76.508
- Moffat, C. F., Grant, D., Long, W. F. & Williamson, F. B. (1984) *Biochem. Soc. Trans.* **12**, 301
- Nieduszynski, I. (1989) in *Heparin: Chemical and Biological Properties, Clinical Applications* (Lane, D. A. & Lindahl, U., eds.), p. 51–63, Edward Arnold, London, Melbourne and Auckland
- Panov, V. P. & Ovsepyan, A. M. (1984) *Vysokomol. Soedin. Ser. A* **26**, 1963–1970
- Parrish, R. F. & Fair, W. R. (1981) *Biochem. J.* **193**, 407–410
- Reyes, R., Magdaleno, V. M., Hernandez, O., Rosado, A. & Delgado, N. M. (1983) *Arch. Androl.* **10**, 155–160
- Sato, C. S. & Gyorkey, F. (1969) *Cancer Res.* **29**, 399–402
- Sato, C. S. & Gyorkey, F. (1976) *J. Biochem. (Tokyo)* **80**, 883–886
- Takahashi, M., Yamagami, S. & Kawato, A. (1986) Japanese Patent JP 61, 106, 514
- Vallee, B. L. & Auld, D. S. (1990) *Biochemistry* **29**, 5647–5659
- Whitfield, D. M. & Sarkar, B. (1991) *J. Inorg. Biochem.* **41**, 157–170
- Woodhead, N. E., Long, W. F. & Williamson, F. B. (1986) *Biochem. J.* **237**, 281–284
- Wu, V.-Y. & Cohen, M. P. (1984) *Anal. Biochem.* **139**, 218–223

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