Biophysical Chemistry, 31 (1988) 295–299
Elsevier

BPC 01303

Quantitative similarity of zinc and calcium binding to heparin in excess salt solution

J. Mattai and Jan C.T. Kwak

Department of Chemistry, Dalhousie University, Halifax, Nova Scotia B3H 4J3, Canada

Received 5 June 1988
Revised manuscript received 18 May 1988
Accepted 14 June 1988

Zn^{2+}; Ca^{2+}; Heparin binding; Salt effect; Scatchard plot; Binding constant

Zn^{2+} binding to the anticoagulant heparin was examined using a dye spectrophotometric method, with added NaCl concentrations of 0.005, 0.0075, 0.01, 0.02 and 0.04 mol/l. The results are shown as Scatchard plots and demonstrate the entropy-driven anticooperativity of Zn^{2+} binding to heparin. From these Scatchard plots, intrinsic binding constants are determined and are compared to our earlier data for Mg^{2+} and Ca^{2+} binding to heparin at similar ionic strengths (J.Mattai and J.C.T. Kwak, Biochim. Biophys. Acta 677 (1981) 303), and to Manning's two-variable theory (G.S. Manning, Q. Rev. Biophys. 2 (1978) 179) for a generalized system of polyelectrolyte + divalent cations + univalent cations. While Mg^{2+} binding to heparin is purely electrostatic (delocalized or territorial), Zn^{2+} and Ca^{2+} binding is much stronger and more specific. Binding constants for these two cations are identical, suggesting similar mechanisms for Zn^{2+} and Ca^{2+} binding to heparin.

1. Introduction

The anticoagulant heparin is an anionic polyelectrolyte with a tetrasaccharide as its basic structural unit [1]. It is considered as a helix of 1 → 4-linked α-D-glucosamine, β-D-glucuronate and iduronate with the ionic charges derived from *O*-sulfate, *N*-sulfate and carboxylate groups in the ratio of 3:2:2 per tetrasaccharide unit [1]. The presence of a high density of anionic charges on the polyion backbone would indicate that heparin-cation interactions, in vivo, are biologically important. For example, Ca^{2+} affects the action of heparin on the rate of thrombin formation [2]. A number of studies have examined heparin-metal ion interactions in vitro, using a variety of techniques [3–15]. In our laboratory, we have quantitatively examined the binding of Mg^{2+} and Ca^{2+} to heparin, using a dye spectrophotometric method [16], in the mixed counterion systems: heparin + MCl_2 + NaCl ($M = Mg^{2+}$ or Ca^{2+}) [8]. Binding isotherms and Scatchard plots [17] were compared to theoretical predictions derived from Manning's two-variable theory [18] for a mixed counterion system of polyelectrolyte + $M'Cl_2$ + $M''Cl$. The theory is based on the counterion condensation relations [19] and a free energy minimization procedure [18], and implicit in the derivation of the theory is the assumption that the divalent cationic species is territorially bound [20] to the polyanion, i.e., no specific interactions are involved. Such territorial binding was found for Mg^{2+} with heparin, in agreement with earlier ion-exchange studies by Dunstone [3], while a strong and specific interaction of Ca^{2+} with heparin was observed, also previously noted by other workers [7,10].

Two reports [5,9] have demonstrated the preferential binding of Zn^{2+} to heparin over other glycosaminoglycans, suggesting that the Zn^{2+}-heparin

Correspondence (present) address: J. Mattai, Research and Development Division, Colgate Palmolive Co., 909 River Road, Piscataway, NJ 08854, U.S.A.

interaction is also not of a purely electrostatic nature. Woodhead et al. [15] studied Zn^{2+} binding by heparin using an equilibrium dialysis technique. These authors interpret their results in terms of two binding regions, one of high affinity and the other of lower affinity, with both binding processes being entropy-driven. In the present communication, we have further examined the Zn^{2+}-heparin binding process in the presence of excess univalent salt and at relatively low heparin concentration. Free zinc activities were measured using a previously described spectrophotometric method with the dye tetramethylmurexide [21]. In this method, a relatively weakly binding dye serves as an 'activity probe' for the metal ion. By maintaining a low dye concentration relative to the total metal ion concentration, the metal ion-polyion equilibrium is not affected, and metal ion activities and free metal ion concentrations can be obtained. The results, presented as Scatchard plots [17] and in the form of binding constants at several ionic strengths, are compared to the two-variable theory of Manning [18] and to our earlier studies of Mg^{2+} and Ca^{2} binding to heparin [8]. The quantitative similarity, as well as the specificity, of Zn^{2+} and Ca^{2+} binding to heparin is demonstrated.

2. Experimental

Bovine lung sodium heparinate was a gift of the Upjohn Co. of Canada. Determination of its concentration and calculation of the charge density parameter, ξ, required for application of the two-variable theory [18], have been previously described [8]. Determinations of Zn^{2+} activity using tetramethylmurexide were performed as described previously [21].

3. Results and discussion

Single ion activities of Zn^{2+} were measured at total ionic strengths of 0.005, 0.0075, 0.01, 0.02 and 0.04 mol/l in the system heparin + $ZnCl_2$ + NaCl. The heparin concentration was 0.001 equiv./l (i.e., mol ionic groups/l) at all ionic strengths. Single ion activities of Zn^{2+} were determined over the range of C_2/C_p values, 0.02–1.0 (C_2 = total [Zn^{2+}] in mol/l; C_p = polyion concentration). From the single ion activities and the heparin concentration, the number of divalent metal ions bound per polyion fixed charge θ_2 ($\theta_2 = C_{2b}/C_p$; C_{2b} = concentration of bound divalent metal ions) and K_2 ($K_2 = \theta_2/C_{2f}$; C_{2f} = free divalent metal ion concentration) were determined as described previously [22]. From these data Scatchard plots are calculated for each ionic strength. The results are shown in fig. 1. The solid lines shown in fig. 1 are the theoretical predictions at various ionic strengths, derived from Manning's two-variable theory (ref. 18, eqs. 53 and 54) using a calculated value of 2.43 for the charge density parameter of heparin [8].

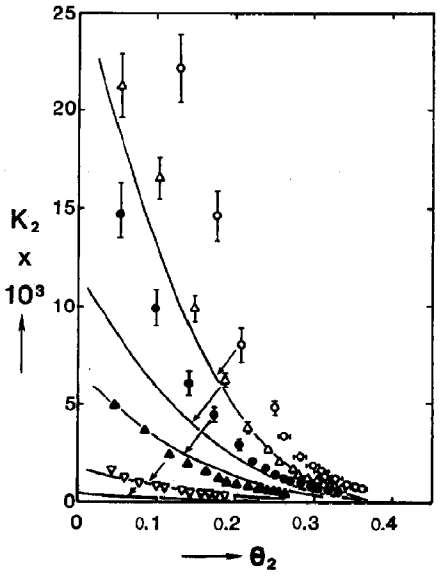

Fig. 1. Scatchard curves K_2 (1/mol) vs. θ_2 in the system $ZnCl_2$ + NaCl + heparin. C_p = 0.001 equiv./l at several ionic strengths, I. (○) I = 0.005, (△) I = 0.0075, (●) I = 0.010, (▲) I = 0.020, (▽) I = 0.040 mol/l. Theoretical curves: eqs. 53 and 54 of Manning's two-variable theory [18].

The Scatchard plots show anticooperativity and decreased binding of Zn^{2+} to heparin as the ionic strength increases, also predicted by the two-variable theory [18] and observed in other systems [8,21–23]. This anticooperative effect is more noticeable at lower ionic strengths. Accord-

ing to the theory, a condensation volume, V_p, exists around a polyion in which in the absence of M^{2+} only univalent cations, M^+, are present. These cations are considered territorially bound [20] but they have freedom of motion within V_p. As a consequence, there is a large concentration difference for the M^+ cations between V_p and the bulk solution. On addition of M^{2+}, M^+ are displaced from V_p to the bulk solution. The release of M^+ cations, with a subsequent gain in entropy, is the driving force of M^{2+} interaction with polyelectrolytes in the presence of added univalent salts. As more M^{2+} ions are bound, the concentration of M^+ in V_p decreases and the corresponding gain in entropy upon release to the bulk is reduced. Therefore, K_2 decreases with θ_2. The greater anticooperativity at lower ionic strengths is related to the higher initial concentration gradient of M^+ between V_p and the bulk solution.

In contrast to the present work, equilibrium dialysis measurements of the Zn^{2+}-heparin interaction [15] gave Scatchard plots which were analysed by assuming regions of low and high affinity in the interaction. Both types of interaction were determined to be entropy-driven, but the entropy change was attributed to the dehydration of the sulfate ester groups, with subsequent release of bound water. Our results, taken together with theoretical predictions [18], suggest that the release of Na^+ from a condensation volume around the heparin polyion to the bulk solution is the dominant entropic driving force for Zn^{2+} binding, with possible additional contributions from dehydration and volume change effects.

While the anticooperativity of Zn^{2+} binding to heparin is theoretically predicted, there is a large deviation between the experimental and theoretical values for K_2. At all ionic strengths, the experimental values are more than twice the predicted values for any given θ_2 value. In order to determine the extent of this deviation from the two-variable predictions, we have followed a previous procedure [8,18] to determine K_2 in the limit as $\theta_2 \to 0$, by extrapolation of the experimental data to $\theta_2 = 0$. These K_2 values, at different ionic strengths, are called 'intrinsic binding constants' [18], $K^0_{M^{2+}}$. The theoretical $K^0_{M^{2+}}$ values can again be calculated from the two-variable theory (ref. 18, eqs. 29).

In table 1, log $K^0_{M^{2+}}$ values for Zn^{2+}, Ca^{2+} and Mg^{2+} at a number of ionic strengths are presented, together with the values predicted from the two-variable theory [18]. Values for Ca^{2+} and Mg^{2+} are taken from our earlier investigation of the mixed counterion systems heparin + $CaCl_2$ + NaCl and heparin + $MgCl_2$ + NaCl [8]. Quite noticeable is the remarkable agreement of log $K^0_{Mg^{2+}}$ at all ionic strengths with the predicted values. This excellent correlation indicates that magnesium binding to heparin is delocalized or 'territorial' [20] in agreement with the result of earlier studies by Dunstone [3]. Nonspecific binding of Mg^{2+} to dextran sulfate [22] and polystyrenesulfonate [23] was also noted previously.

Within the limits of error, log $K^0_{Zn^{2+}}$ is found to be identical to log $K^0_{Ca^{2+}}$ at all ionic strengths, Zn^{2+} and Ca^{2+} being bound to the same extent by heparin. A comparison of the binding constants of

Table 1

Comparison of theoretical and experimental values of K_2^0

Ionic strength (mol/l)	log $K^0_{M^{2+}}$ (theoretical)	log $K^0_{Mg^{2+}}$ (experimental) [a] (sodium heparinate)	log $K^0_{Ca^{2+}}$ (experimental) [a] (sodium heparinate)	log $K^0_{Zn^{2+}}$ (experimental) (sodium heparinate)
0.04	2.61	2.77 ± 0.05	3.20 ± 0.05	3.25 ± 0.05
0.02	3.21	3.30 ± 0.05	3.85 ± 0.05	3.84 ± 0.05
0.01	3.81	3.83 ± 0.05	4.29 ± 0.05	4.30 ± 0.05
0.0075	4.06	4.12 ± 0.05	4.48 ± 0.05	4.53 ± 0.05
0.005	4.41	4.45 ± 0.05		

[a] Data from ref. 8.

these metal ions with those of Mg^{2+} shows that Zn^{2+} and Ca^{2+} are bound to a much greater extent than Mg^{2+}. There is also a large deviation, approx. 0.5 (log $K^0_{M^{2+}}$) units, of the binding constants of Zn^{2+} and Ca^{2+} from the predicted values at all ionic strengths, suggesting more specific and stronger interactions of these metal ions with heparin. In contrast, both Ca^{2+} and Zn^{2+} were shown to bind in a delocalized manner to dextran sulfate [21,22], although Ca^{2+} had a slightly greater affinity than Zn^{2+}. The stronger affinity of Zn^{2+} and Ca^{2+} for heparin is consistent with similar observations made using other techniques [9].

The nature of the binding sites for Zn^{2+} or Ca^{2+} cannot be determined from our or any other thermodynamic measurements. The much studied Ca^{2+}-heparin system was investigated by Boyd et al. [7] using circular dichroism, optical rotation and ^{13}C-NMR. Significant conformational changes were observed with Ca^{2+} binding to heparin, relative to Mg^{2+}, while the NMR data showed the specific chelation of Ca^{2+} with two iduronate carboxyl groups. Liang et al., [10] also demonstrated the chelation of Ca^{2+} with heparin using circular dichroism and ^{1}H-NMR, but experiments with *N*-sulfate hydrolysis of heparin also implicated the sulfoamino groups in the binding process. It was suggested that Ca^{2+} chelates to heparin's carboxyl groups, and that the resultant complex is stabilized by the sulfoamino groups from neighbouring sugar residues through a weak electrostatic interaction. The quantitative similarity of Zn^{2+} and Ca^{2+} binding to heparin would strongly suggest that an identical mechanism also exists for Zn^{2+} binding. A similar mechanism for Ca^{2+} and Zn^{2+} binding to heparin is also suggested by optical rotation measurements by Moffatt et al. [13]; Ca^{2+} and Zn^{2+} (as well as Ba^{2+}) produce maximum optical changes when these metal ions are added to sodium heparinate, while K^+ and Li^+ produce little change and Cu^{2+} induces a large decrease.

4. Conclusion

Quantitative data on binding constants at several ionic strengths were obtained in the present study for the Zn^{2+}-heparin interaction and the results, when compared to our earlier data with Ca^{2+} and Mg^{2+} [8], show the order of divalent metal ion binding to be: $Zn^{2+} = Ca^{2+} > Mg^{2+}$. The experimental binding constants, when compared to Manning's two-variable theory [18], show the electrostatic nature of the Mg^{2+}-heparin interaction, while additional specific interactions are suggested for Zn^{2+} and Ca^{2+} binding. The validity of this finding can be tested using other experimental techniques for studying the Zn^{2+}-heparin interaction such as optical rotation, circular dichroism and ^{1}H- and ^{13}C-NMR, analogous to those used for investigating Ca^{2+}-heparin binding.

Acknowledgement

This research was supported by the Natural Sciences and Engineering Research Council of Canada (N.S.E.R.C.).

References

1 M.E. Silva and C.P. Dietrich, J. Biol. Chem. 250 (1975) 6941.
2 G. Murano, Semin. Thromb. Hemostasis. 6 (1980) 140.
3 J.R. Dunstone, Biochem. J. 85 (1962) 336.
4 B. Lages and S.S. Stivala, Biopolymers 12 (1973) 127.
5 C.S. Sato and F. Gyorkey, J. Biochem. 80 (1976) 883.
6 D.J. Wedlock, G.P. Diakun, H.E. Edwards, G.O. Phillips and J.C. Allen, Biochim. Biophys. Acta 629 (1980) 530.
7 J. Boyd, F.B. Williamson and P. Gettins, J. Mol. Biol. 137 (1980) 175.
8 J. Mattai and J.C.T. Kwak, Biochim. Biophys. Acta 677 (1981) 303.
9 R.F. Parrish and W.R. Fair, Biochem. J. 193 (1981) 407.
10 J.N. Liang, B. Chakrabarti, L. Ayotte and A.S. Perlin, Carbohydr. Res. 106 (1982) 101.
11 D. Grant, W.F. Long and F.B. Williamson, Biochem. Soc. Trans. 11 (1983) 96.
12 N.E. Woodhead, W.F. Long and F.B. Williamson, Biochem. Soc. Trans. 11 (1983) 96.
13 C.F. Moffat, D. Grant, W.F. Long and F.B. Williamson, Biochem. Soc. Trans. 12 (1984) 301.
14 D. Grant, C.F. Moffat, W.F. Long and F.B. Williamson, Biochem. Soc. Trans. 12 (1984) 302.
15 N.E. Woodhead, W. F. Long and F.B. Williamson, Biochem. J. 237 (1986) 281.

16 J.C.T. Kwak and Y.M. Joshi, Biophys. Chem. 13 (1981) 55.
17 G. Scatchard, Ann. N.Y. Acad. Sci. 51 (1949) 660.
18 G.S. Manning, Q. Rev. Biophys. 2 (1978) 179.
19 G.S. Manning, J. Chem. Phys. 51 (1969) 924.
20 G.S. Manning, Acc. Chem. Res. 12 (1979) 443.
21 J. Mattai and J.C.T. Kwak, Biophys. Chem. 14 (1981) 55.
22 Y.M. Joshi and J.C.T. Kwak, Biophys. Chem. 13 (1981) 65.
23 J. Mattai and J.C.T. Kwak, Macromolecules 19, (1986) 1663.
24 P.C. Karenzi, B. Meurer, P. Spegt and G. Weill, Biophys. Chem. 9 (1979) 181.