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Cu^{2+}–heparin interaction studied by polarimetry

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Plots analogous to isothermal binding curves were derived from polarimetric studies of solutions of Na^+–heparin to which Cu^{2+} was added. The shape of the plots suggests that discrete Cu^{2+}–heparin complexes may form, the nature and stoichiometry of which depend upon the conditions under which the interaction occurs. Initially, complexes were formed with a stoichiometry of about 1 Cu^{2+} ion per heparin tetrasaccharide unit. Under these conditions, the complex may exist as a colloid-like phase that is not subject to simple solution equilibrium processes, and that is maintained by forces in addition to electrostatic ones. Further addition of Cu^{2+} resulted in the formation of a different complex with a stoichiometry of about 1 Cu^{2+} ion per disaccharide unit.

INTRODUCTION

Heparins are chemically complex strongly acidic polyelectrolytes that possess a range of metal cation-binding chemical groups. They are therefore candidates for various biological functions involving the binding and release of metal cations. Of these, Cu^{2+} has been reported to bind strongly to heparin, and a direct relationship between Cu^{2+}-binding capacity and anticoagulant activity in heparin has been reported (Stivala, 1977), although the presence of Cu^{2+} does not affect this activity (Stivala *et al.*, 1973). Heparin acquires angiogenic activity *in vivo* upon complex-formation with Cu^{2+} (Raju *et al.*, 1982; Alessandri *et al.*, 1983; Bergendal *et al.*, 1988), and it has been suggested that heparins may play a role in limiting free-radical damage by sequestering transition-metal ions such as those of copper (Ross *et al.*, 1991).

Some debate has arisen over whether the binding of small cations to heparin occurs at specific localized sites on the polymer, or in a delocalized non-specific manner (summarized in Nieduszynski, 1989). The latter possibility is often discussed in terms of the theories of Manning (1969*a*,*b*,*c*, 1977, 1978), which suggest that a critical charge density exists on a water-soluble polyelectrolyte, and that, when the net charge density (which includes the charge of the counter-cations) exceeds this, cations electrostatically 'condense' on the anionic sites in a delocalized non-specific fashion. The model is based on electrostatic energetic considerations, and does not deal with the possibility that non-electrostatic factors, including, for example, involvement of polymer-associated water molecules, play any role in the interaction.

The mechanisms by which Cu^{2+} interacts with heparin are particularly unclear. Stivala & Liberti (1967), in polarographic studies, showed that the Cu^{2+}-binding capacity of heparin increased with increasing M_r, and that Cu^{2+} binding occurred with a minimum stoichiometry of 0.5 Cu^{2+} ion per heparin disaccharide unit. They suggested the involvement of heparin carboxylate and *N*-sulphonate groups in Cu^{2+} binding, perhaps by chelation. Lages & Stivala (1973), using equilibrium dialysis, demonstrated that under certain conditions Cu^{2+} bound to heparin at two types of sites: at one, about 0.25 Cu^{2+} ion bound per disaccharide unit; at the other, about 1.5 Cu^{2+} ions bound per disaccharide unit. They postulated involvement of *N*-sulphonate groups in the former and carboxylate groups in the latter type of binding. C.d. and viscosity measurements by Chung & Ellerton (1976) showed that the mechanism by which Cu^{2+} interacted with heparin differed from that involved in the binding of other uni- and bi-valent metal cations studied. Cu^{2+} chelation, it was suggested, might tighten and stabilize a heparin helical structure by reducing its pitch; it was suggested that the carboxylate groups were involved in the binding. Herwats *et al.* (1977) studied competitive binding of Na^+ and Cu^{2+} to heparin by using ^{23}Na-n.m.r. spectroscopy; they calculated a Cu^{2+}–heparin association constant of about 1400 M^{-1}. In contrast, analysis of absorption spectra revealed association constants of approx. 10000 and 200 M^{-1} for the interactions of Cu^{2+} with heparin and de-*N*-sulphonated heparin respectively (Mukherjee *et al.*, 1978). Nevertheless, those authors considered interaction with the carboxylate groups to be of paramount importance. They suggested that chelation of Cu^{2+} might occur. Analysis of spectrophotometric results was also interpreted in terms of a Cu^{2+}–carboxylate interaction, one cation binding per disaccharide residue (Park & Mukherjee, 1980). Crescenzi *et al.* (1981*a*,*b*) measured the heat of interaction of Cu^{2+} with heparin; their results suggest that, under the conditions studied, binding approached completion at a [Cu^{2+}]/[heparin disaccharide unit] ratio of about 1:1. Ultrasonic relaxation studies of Cu^{2+}–heparin were tentatively interpreted in terms of either a continuous formation and dissociation of a Cu^{2+}–heparin complex or a Cu^{2+}-induced fluctuation in the conformation of the heparin (Stivala & Murray, 1981). Grushka & Cohen (1982) used gel-permeation chromatography to prepare Cu^{2+}–heparin complexes. Their work showed that Cu^{2+} bound at ratios of about 0.07 and 0.2 Cu^{2+} ion per disaccharide unit at pH 2.0 and pH 4.2 respectively. They suggested that carboxylate, *O*-sulphate and *N*-sulphonate groups were involved in metal ion binding. From analysis of the spectral behaviour of the Cu^{2+}–heparin complex, an association constant for the interaction at pH 2.0 of about 1050 M^{-1} was deduced. Panov & Ovsepyan (1984) interpreted i.r. spectra of Cu^{2+}–heparin in terms of a strong interaction between the cation and carboxylate groups. More recent i.r. spectroscopic analysis showed a cation-dependency of the absorbance due to the main carboxylate carbonyl symmetric stretching, suggesting direct involvement of the carboxylate groups in cation binding (Grant *et al.*, 1987). However, perturbation of the stretching energy of the carboxylate groups was greater for the Cu^{2+}–heparin complex than was predicted by an observed linear dependency of band frequency on the polarizing power of the cations. Cu^{2+} binding also affected the ionic *O*-sulphate

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asymmetric stretching frequency; this was not the case for most of the cations studied. In addition, Cu^{2+} binding had a greater effect on the H–O–H bending vibration frequency than that predicted from an observed linear relationship between this frequency and the unhydrated ionic radii of other bivalent cations (Grant *et al.*, 1987).

The present paper re-affirms the complexity of the Cu^{2+}–heparin interaction; it suggests the existence of multiple complexes, in at least one of which the bound copper is not able to exchange with free cation through simple thermodynamic equilibria.

EXPERIMENTAL

The source, preparation and properties of the heparin and of the chemically modified heparins used have been described previously (Grant *et al.*, 1987, 1989). Pools of heparin fragments were prepared with the use of heparinase I (EC 4.2.2.7) as described by McLean *et al.* (1984). In brief, heparin (2.0 g) was dissolved in phosphate buffer, pH 7.0, and incubated with heparinase I (145 units) at 25 °C for 3 h, after which the digest was placed on ice and the pH lowered to 4.6 with HCl. The saccharides were chromatographed on Bio-Gel P-2. The di-, tetra- and oligo-saccharides were individually pooled. The oligosaccharides were desalted on Sephadex G-25 and then chromatographed on a Bio-Gel P-6 (200–400 mesh) column (3.2 cm × 146 cm) with acetate buffer at a flow rate of 0.3 ml/min. Fractions were analysed for uronic acid content and for absorption at 235 nm. Appropriate fractions were pooled, desalted and characterized by ^{13}C-n.m.r. spectroscopy, by polarimetry and by h.p.l.c. analysis of their heparinase II (EC 4.2.2.7/8) degradation products. Although the heparin was supplied as an Na^+ form, spark-source m.s. revealed the following additional elemental content (p.p.m.): Ca, 25000; Si, 5900; K, 2000; Mg, 1300; Fe, 1100; Cu, 730; P, 440; Ti, 390; Ba, 140; Zn, 80; Sr, 65; Cr, 30; B, 25; Ga, 20; Co, 8. Polyanions were converted into Na^+ complexes by passage through the appropriate cation form of Amberlite IR-120 cation-exchange resin. Spark-source m.s. showed a greater than 99% efficiency of the cation-exchange process. An average heparin disaccharide unit was taken to be hexadecahydrated (Atkins *et al.*, 1974; Grant *et al.*, 1990) tetrasodium 2-*O*-sulphato-iduronosyl 6-*O*-sulphato-glucosamine 2-*N*-sulphamate.

Polarimetric measurements by the method described in Grant *et al.* (1992) were made with a Thorn Automation–NPL Automatic Polarimeter type 243. Addition of aqueous $CuSO_4$ solution to heparin elicited a decrease in optical rotation (Moffat *et al.*, 1984; Grant *et al.*, 1992). The change in optical rotation that occurred following cation addition was expressed as [optical rotation in the presence of cation minus optical rotation in the absence of cation]/[maximal optical rotation at high cation concentration minus optical rotation in the absence of cation]. At 'high' cation concentration the [cation]/[heparin disaccharide unit] ratio was 6:1. At this ratio no further change in the optical rotation value (corrected as described in Grant *et al.*, 1992) occurred on addition of more cation. Optical rotations were recorded with light of 546 nm wavelength in a pathlength of 0.61 dm at a temperature of 25 °C, and at a polymer concentration that, if not indicated otherwise in Fig. 1 legend, was 0.477 g/100 ml in water. Because the different degrees of hydration of heparin and of modified heparins (Grant *et al.*, 1992) contribute to polymer mass, but are unlikely to contribute directly to polymer optical activity, experimentally recorded optical rotations are reported directly, rather than as specific rotations.

RESULTS AND DISCUSSION

It is not clear why interaction of heparin with Cu^{2+} produces a decrease in optical rotation (Moffat *et al.*, 1984): it may reflect a particular polymer conformational/hydration change (Grant *et al.*, 1992): further, the copper chromophore may contribute to the effect. For Ca^{2+}–heparin binding, plots of isothermal saturation fractions occupied by Ca^{2+}, derived from equilibrium dialysis experiments, as a function of [total Ca^{2+}] closely coincide with equivalent plots derived from optical rotation changes observed under equivalent conditions of [heparin] and [NaCl] (Grant *et al.*, 1992). Moreover, the maximal change in optical rotation (obtained when high [bivalent cation] was added to solutions of Na^{2+}–heparin) is directly related to independently derived association constants for interactions between heparin and a number of cations, including Cu^{2+} (Grant *et al.*, 1992), and between de-*N*-sulphonated heparin and Cu^{2+} (D. Grant, W. F. Long, C. F. Moffatt & F. B. Williamson, unpublished work). These observations accord with the notion (Moffat *et al.*, 1984) that optical rotation measurements offer an indirect but simple fast non-destructive method of exploring heparin–cation binding.

Fig. 1 shows polarimetrically derived plots for Cu^{2+}–heparin interaction obtained with various concentrations of heparin to which $CuSO_4$ solution was added. Addition of $CuCl_2$ solution to heparin produced similar plots (D. Grant, W. F. Long, C. F. Moffatt & F. B. Williamson, unpublished work), but showed evidence of competition for binding of Cu^{2+} between Cl^- and heparin consistent with Cu^{2+}–Cl^- and Cu^{2+}–heparin association constants of 800 M^{-1} (Bjerrum, *et al.*, 1957) and 1200 M^{-1} (Herwats *et al.*, 1977) respectively. The plots obtained in the experiments with $CuSO_4$ suggest that the optical rotation changes near completion at a [Cu^{2+}]/[heparin disaccharide unit] ratio of about 1:1. An inflexion may occur at a ratio of about 0.5:1; certainly at lesser ratios a direct linear relationship appears to exist between the polarimetrically derived fraction and the [Cu^{2+}]/[heparin disaccharide unit] ratio. This, and the near coincidence of the linear plots obtained with different heparin concentrations, indicate that, under these conditions, Cu^{2+}–heparin interaction occurs by a mechanism that cannot be described adequately in terms of conventional solution-phase reversible-equilibrium thermodynamics. Such results would be consistent with a mechanism of Cu^{2+}–heparin binding that occurs by the kind of simple electrostatic mechanism suggested by Manning (1969*a*,*b*,*c*). However, the linear to non-linear transition occurs at a [Cu^{2+}]/[heparin disaccharide unit] ratio about half of that predicted by electrostatic theory. Elsewhere, an apparent polyanionic charge on heparin about half of that predicted by Manning (1969*a*,*b*,*c*) has been reported (Tivant *et al.*, 1983). Similar observations on Ca^{2+}–heparin binding to those reported here for Cu^{2+} binding have been interpreted in terms of the existence, at low [Ca^{2+}]/[heparin disaccharide unit] ratios, of a cation–polyanion complex that resembles a discrete hydrated-colloid mineral-like state that is not subject to simple aqueous solution equilibrium processes, and that is stabilized by forces additional to electrostatic ones. At higher [Ca^{2+}]/[heparin disaccharide unit] ratios destabilization of the phase, generating the non-linear portion of the plot, occurs (Grant *et al.*, 1992).

Polarimetrically derived plots arising from experiments in which cations are added to solutions of chemically modified heparins may help assessment of the importance of various heparin substituent groups to cation binding (Grant *et al.*, 1992). Table 1 shows maximal optical rotation changes seen when high [Cu^{2+}] was added to solutions of Na^+ forms of modified heparins. In most cases, modification decreased the extent of the optical rotation change, suggesting a lowering of the affinity of the polymer for Cu^{2+}. The [Cu^{2+}]/[heparin disaccharide unit] ratio at

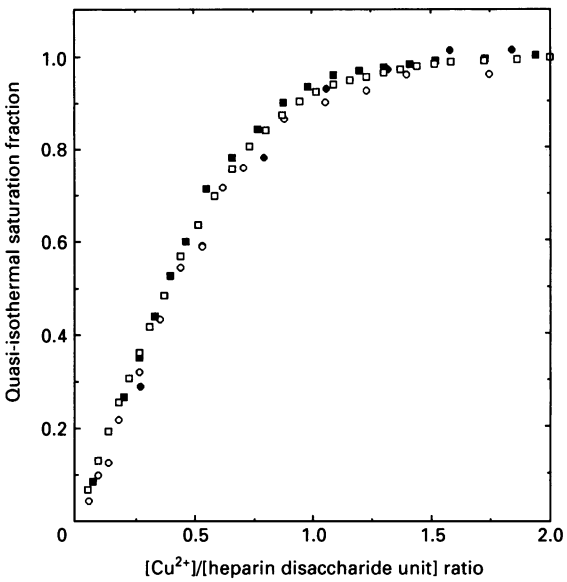

Fig. 1. Quasi-isothermal saturation fraction of binding sites on heparin occupied by Cu^{2+} as a function of [Cu^{2+}]/[heparin disaccharide unit] ratio

A binding site was taken to be an average heparin disaccharide unit (see the Experimental section). Quasi-isothermal saturation fractions were derived from polarimetric measurements as described in the Experimental section. Na^+–heparin concentrations in water were: ●, 2.38 mg/ml; ○, 4.77 mg/ml; ■, 9.56 mg/ml; □, 14.30 mg/ml.

which the linear to non-linear transition occurred in the polarimetrically derived plots for unmodified, de-*N*-sulphonated, de-*N*-sulphonated *N*-acetylated, de-*N*-sulphonated de-*O*-sulphonated *N*-acetylated, de-*N*-sulphonated de-*O*-sulphated re-*N*-sulphonated and carboxy-reduced heparins were 0.56 (at a fraction of 0.60), 0.38 (at a fraction of 0.52), 0.50 (at a fraction of 0.38), 0.41 (at a fraction of 0.70), 0.41 (at a fraction of 0.78) and 0.18 (at a fraction of 0.47) respectively. [The de-*N*-sulphonated de-*O*-sulphated *N*-acetylated heparin used was prepared by route (*b*) reported by Grant *et al.* (1989); when Cu^{2+} was added to the polymer prepared by route (*a*) of Grant *et al.* (1989), essentially similar results were obtained.] The values suggest that the particular Cu^{2+}–polymer complex inferred from the existence of the linear portion of the plots is stable only at [Cu^{2+}]/[disaccharide unit] ratios lower for the modified heparins than for the unmodified polymer. By using these values, it is possible to derive an approximate empirical relationship, in which the [Cu^{2+}]/[heparin disaccharide unit] ratio at the linear to non-linear transition point = (0.28 × carboxylate groups/disaccharide) + (0.15 × *N*-sulphonate groups/disaccharide) + (0.14 × *N*-acetyl groups/disaccharide) + (0.06 × *O*-sulphate groups/disaccharide). This relationship suggests that replacement of *N*-sulphonate groups by *N*-acetyl groups may not greatly affect the ability of heparin to bind Cu^{2+} by the process that gives rise to the linear portion of the plot. This is of interest because binding of Cu^{2+} to hyaluronate appears to involve interaction with glucuronate, carboxylate and *N*-acetyl groups (Kosmus & Schmut, 1985; Sterk *et al.*, 1985). Further, the effects of Cu^{2+} binding on the c.d. spectra of heparin and heparan sulphate (which contains more *N*-acetyl and fewer *N*-sulphonate groups than heparin) are similar (Mukherjee *et al.*, 1978).

Table 1 also shows that a decrease in the degree of polymerization of heparin decreased the extent of the optical rotation change seen in the presence of excess Cu^{2+}, suggesting a decreased affinity for Cu^{2+}. The [Cu^{2+}]/[heparin disaccharide unit] ratios at which the linear to non-linear transition in the plots occurred were, for starting heparin (average degree of polymerization: 35) 0.56 (at a fraction of 0.59), for decasaccharides 0.55 (at a fraction of 0.62), for a hexasaccharide/octasaccharide mix 0.43 (at a fraction of 0.58) and for tetrasaccharides 0.51 (at a fraction of 0.71). These values suggest that a decrease in degree of polymerization did not greatly affect the range of [Cu^{2+}]/[heparin disaccharide unit] ratios over which that heparin–Cu^{2+} interaction giving rise to the linear portion of the plots occurs.

The technique used in the present study has not been used in this context before, and, like most of the methods used previously, gives indirect insight into the binding process. Unlike previous work, this study involves complexes in which the cation content is rigorously defined: this is an important aspect, given the avidity with which many cations interact with heparin. Results reported here suggest that, when Cu^{2+} is added to Na^{2+}–heparin solution, initial binding occurs to produce a complex with a stoichiometry of 1 Cu^{2+} ion per heparin tetrasaccharide unit; further Cu^{2+} addition results in the formation of a complex in which the stoichiometry is 1 Cu^{2+} ion per heparin disaccharide unit. In this regard, the interactions of Cu^{2+} and Ca^{2+} (Grant *et al.*, 1992) with heparin appear to resemble one another. Differences between heparin interactions with Ca^{2+} and with Cu^{2+} include a direct relationship between polymer water content and apparent affinity for Ca^{2+} (Grant *et al.*, 1992) but not for Cu^{2+} (D. Grant, W. F. Long, C. F. Moffatt & F. B. Williamson, unpublished work), a direct relationship between heparin degree of polymerization and apparent affinity for Cu^{2+} (Table 1) but not for Ca^{2+} (D. Grant, W. F. Long, C. F. Moffatt & F. B.

Table 1. Maximal optical rotation changes occurring on addition of Cu^{2+} to Na^+ forms of modified heparins and heparin fragments

Optical rotations were measured as described in the Experimental section. 'High' [Cu^{2+}] refers to a [Cu^{2+}]/[disaccharide unit] ratio of 6:1.

Heparin	Optical rotation (°)		
	In the absence of Cu^{2+}	In the presence of high [Cu^{2+}]	Change
Unmodified	0.1548	0.1136	0.0412
De-*N*-sulphonated	0.2048	0.1955	0.0093
De-*N*-sulphonated, *N*-acetylated	0.1683	0.1548	0.0135
De-*N*-sulphonated, de-*O*-sulphated, *N*-acetylated	0.2760	0.2350	0.0410
De-*N*-sulphonated, de-*O*-sulphated, re-*N*-sulphonated	0.2441	0.1999	0.0442
Carboxy-reduced	0.1682	0.1480	0.0202
Decasaccharides	0.1557	0.1228	0.0329
Hexa-/octa-saccharides	0.1397	0.1125	0.0272
Tetrasaccharides	0.1232	0.1029	0.0203

Williamson, unpublished work) and a lesser importance of the nature of the amino sugar *N*-substituent for Cu^{2+} than for Ca^{2+} binding (Grant *et al.*, 1992).

As noted elsewhere (Grant *et al.*, 1992), future polarimetric measurement, with even more precisely structurally defined polymer preparations, may allow more detailed analysis of putative points of inflexion than can be undertaken following the use of techniques such as equilibrium dialysis, in which the limited number of samples taken restrict the precision of the binding plots constructed; such experiments may reveal further subtleties in cation–heparin interaction.

The Cu^{2+}–heparin interaction inferred from the existence of the linear portion of the polarimetrically derived plots may involve a colloid-like phase that does not form in accordance with simple solution-phase electrostatic theory. Incompletely reversible optical rotation changes that occur when one heparin counter-cation is exchanged for another (D. Grant, W. F. Long, C. F. Moffatt & F. B. Williamson, unpublished work) accord with this possibility. If particular cation–heparin interactions indeed result in the partition of cations between more than one phase, exchanges between which do not occur through simple reversible themodynamic equilibrium processes, then the kinetics of phase formation will be critical, and only very precise techniques, in which, for example, nucleation events are controlled, will allow reliable results to be achieved. An ability of cations and sulphated glycosaminoglycans to switch between metastable colloid-like phases may affect the biological potential of both partners in the complex.

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