Spectroscopic Studies on Cu^{2+} and Ca^{2+} Binding with Glycosaminoglycans*

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A general spectroscopic method is described for studies on the complex formation between metal ions and ligands, and is applied to Cu^{2+} and Ca^{2+} binding to glycosaminoglycans. The order of binding constants for both ions is heparin > dermatan sulfate > chondroitin sulfate. The electrostatic forces are shown to be the predominant factor in the interaction. The 2- to 3- fold higher affinity for Cu^{2+} than for Ca^{2+} is obtained for heparin and dermatan sulfate, but little difference for chondroitin sulfate. These results are explained as chelation of both carboxyl and sulfate groups to Cu^{2+} in former cases. The difference of binding constants among glycosaminoglycans is related to proposed various biological functions of the biopolymers.

Introduction

The glycosaminoglycans(GAG), a group of acidic polysaccharides consisting of disaccharide unit of uronic acid and N-substituted aminosugars, occur in many animal tissues and fluids and in some cells and cell membranes.[1] These biopolymers are well characterized by their molecular structure, and by anionic carboxyl and/or sulfate groups and their spatial distribution, which are believed to be the determining factors for the physicochemical properties of the molecules.

The binding or interaction of metal ions with glycosaminoglycans, mainly due to the anionic groups, has been intensively investigated in relation to structure[2-5] and to the biological functions of the polymers.[6-8] Various proposed biological functions of glycosaminoglycans were related to the interaction of the biopolymers with metal ions.[6-12]

However, studies on the interaction of Ca^{2+} as well as other ions with glycosaminoglycans encountered difficult analytical problems mainly due to the lack of convenient means to detect complexed species: most investigators used tedious and sometimes erroneous equilibrium dialysis methods followed by analysis of free metal ion concentration by either seletive ion electrode or other techniques.

We have previously shown that glycosaminoglycan-Cu^{2+} complexes exhibit a characteristic absorption band attributed to a charge transfer band from ligand to metal ion and the formation constants of Cu^{2+} complex with hyaluronic acid and heparin were determined spectrophotometrically.[4,5] The circular dichroic spectra of Cu^{2+} complexes of the reports suggested that both carboxyl and sulfate groups of heparin and dermatan sulfate might be involved in the chelation to Cu^{2+}, whereas only the carboxyl group of chondroitin sulfate was the site for the metal ion binding. Such difference in binding mode is not expected for Ca^{2+}, which exhibits little tendency for chelation in solution. Thus, a large difference in binding constants between Ca^{2+} and Cu^{2+} for haparin and dermatan sulfate can be observed, whereas the difference for chondroitin sulfate can be observed, whereas the difference for chondroitin sulfate is expected to be small. To investigate these possibilities and explore the nature of binding further, we devised a spectroscopic method to study Ca^{2+} binding through competitive reactions between the metal ions with glycosaminoglycans at different pH's and salt concentrations. The study yields binding constants of both Ca^{2+} and Cu^{2+} and information regarding the mechanism of binding. The large difference in binding constants among glycosaminoglycans have been related to possible biological function of the biopolymers. Conceptually, as the technique applied here can be used for binding studies of any ions for molecules containing the carboxyl group and other systems, the technique is described in general.

Materials and Methods

Sodium salts of heparin (163 USP units) and chondroitin sulfate (super special grade) was obtained from Seikagaku Kogyo Co. through Miles laboratories. All chemicals were used as received. Standard procedure was employed to prepare glycosaminoglycan solutions of desired pH values in 0.001 *M* cacodylate buffer.[5] Absorption spectra were taken at ambient temperatures, with cells of 0.876 cm light path length; the cells are partitioned into two equal compartments so that spectra could be taken before and after mixing reactants, which were at the same pH and salt concentration. Concentrations were calculated based on dry weight of samples using average dimeric formula weights of 600 for heparin and 504 for dermatan sulfate and chondroitin sulfate.

Results

Studies on Binding Yielding Spectral Change: Application to Cu^{2+}. When a complex or bound species shows significantly different spectrum from its components, the characterization and determination of binding constant of the complex is possi-

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ble from spectral data. For generality, consider binding of a metal ion M to ligand L giving complex ML_n, the absorbance change by complexing is given by;

$$\Delta A = (\varepsilon_{ML_n} - n\varepsilon_L - \varepsilon_M) \cdot [ML_n] \cdot l = \Delta\varepsilon_{ML_n} \cdot [ML_n] \cdot l \quad (1)$$

where l is the path length of a cell employed, ε's are extinction coefficients of species shown, and n is the stoichiometric ratio of the complex. If all binding sites (ligand L) of a polymer or ligands are equivalent and independent, the apparent binding constant of M to the ligand is expressed as

$$K_{ML_n} = [ML_n] \,/\, [L]^n \cdot [M] \quad (2)$$

Substituting equation 1 into 2 and rearranging the equation, one obtains

$$[M]_0 / \Delta A = 1 / \Delta\varepsilon_{ML_n} \cdot l + 1/\Delta\varepsilon_{ML_n} \cdot l \cdot K_{ML_n} \cdot [L]^n \quad (3)$$

where $[M]_0$ is total metal ion concentration. The plot of $[M]_0/\Delta A$ against $1/[L]^n$ should yield a straight line for a proper choice of stoichiometric coefficient n, and $\Delta\varepsilon_{ML_n}$ and K_{ML_n} are calculated from the plots. Such plot is possible by putting [L] into total ligand (disaccharide unit of glycosaminoglycans) concentration $[L]_0$ at condition $[L]_0 >> n[M]_0$.

Neither glycosaminoglycan nor Cu^{2+} displays an absorption maximum above 200 nm. However, the mixture shows a band with maximum near 237 nm in a difference spectrum obtained by a method described in the experimental section and in a spectrum run against water. In Figure 1, spectra of heparin, Cu^{2+}, and their mixture taken against water at pH 5 are shown. The difference spectrum of the complex is also included. The shape and position of the band are virtually unchanged with variation of solution composition for all glycosaminoglycans studied. The plots of Eq.(3) are shown in Figure 2 for $n = 1$ for Cu^{2+}-glycosaminoglycan systems; other values of n did not yield the expected straight line. These results indicate that one disaccharide unit of the glycosaminoglycans studied forms a complex with a copper ion. Once the n value is known from the plots as shown in Figure 2, K_{ML_n} values can be calculated for each experimental point. The $\Delta\varepsilon_{ML_n}$ and K_{ML_n} values are also calculated from the plots by using Eq. (3). The values obtained by these calculations are slightly lower, typically 10–20% near a 1:1 ratio of Cu^{2+} to polymer, than extrapolated values obtained from Figure 2. Decrease in charge density of polymer upon Cu^{2+} binding can explain the result. The $\Delta\varepsilon$ and K values averaged near 1:1 ratio, after correcting for the pH effect as discussed in the following paragraph, are listed in Table 1.

The ligand to Cu^{2+} in glycosaminoglycans is mainly ionized carboxylate group,[4,5] and the concentration of ligand is thus pH dependent. Since the concentration of ligand (glycosaminoglycan) is in high excess in comparison to Cu^{2+} and thus the fraction of the complex is small (in this case, the *a priori* assumption of equivalence and independence of ligand

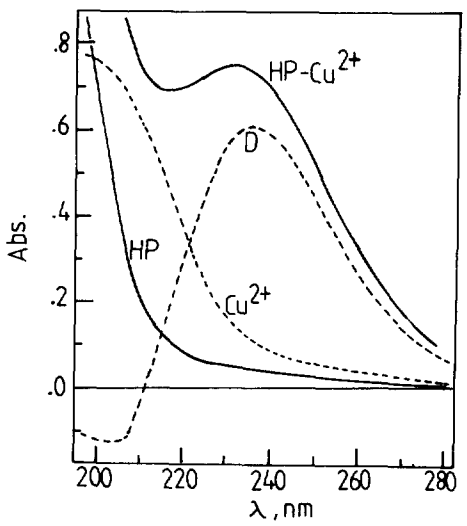

Figure 1. Absorption spectra of 8.33×10^{-1} *M* heparin (HP), 5×10^{-1} *M* Cu^{2+}, and mixture of heparin and Cu^{2+} at pH 5.0. D is the difference spectra between the mixture and unmixed components.

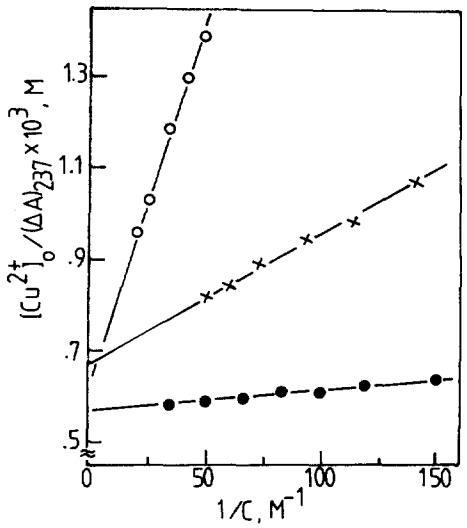

Figure 2. Plots of $[Cu^{2+}]_0(\Delta A)_{237\,nm}$ against inverse of glycosaminoglycans concentration: chondroitin sulfate(○); dermatan sulfate(x); heparin(●).

may also be met), the pH dependence of the apparent binding constant becomes, in general formula;

$$K_{ML_n} = (K_{ML_n})pH \cdot \left(\frac{k_a + H^+}{k_a}\right)^n \quad (4)$$

where $(K_{ML_n})pH$ is the apparent binding constant at the pH, and k_a denotes the dissociation constant of the ligand.

Studies on Binding Showing Little Spectral Change: Application to Ca^{2+}. Even if a complex does not show significant spectral change from its components, the method described in the previous section can be used to study the complex formation by competitive complexing. For example, the absorption spectra of glycosaminoglycans in the presence of Ca^{2+} differ insignificantly from those of glycosaminolvcans themselves. However, addition of Ca^{2+}-glycosaminoglycan mixture; a

gradual decrease in the magnitude of difference spectra with increasing Ca^{2+} concentration was observed, but no changes in band shape and position. This can be explained by competition between Ca^{2+} and Cu^{2+} to the common ligand, carboxylate, of the polymers. We show variations of ΔA with chondroitin sulfate concentration in $1\times10^{-3}M$ Cu^{2+} solution and also with Ca^{2+} in $1\times10^{-3}M$ Cu^{2+} chondroitin sulfate in Figure 3. The effect of NaCl on the latter system is also presented. At the same ionic strength, the effect of Ca^{2+} on ΔA is far greater than that of NaCl. This result clearly suggests that the absorption chage with Ca^{2+} is not due to change of ionic strength of the medium but rather is due to binding of Ca^{2+} to the same binding site as Cu^{2+}, replacing the bound Cu^{2+} by Ca^{2+}.

The set of curves as drawn in Figure 3 for each glycosaminoglycan was used to determine the Ca^{2+} binding constant of the glycosaminoglycans. For example, Figure 3 shows that ΔA at 237 nm changes from 0.592 to 0.470 when $1\times10^{-3}M$ Ca^{2+} is added to the mixture specified. The abosorption value of 0.470 corresponds to the absorption value of only $1.54\times10^{-3}M$ chondroitin sulfate. Therefore, the difference, 0.46×10^{-3} (2.00×10^{-3} - 1.54×10^{-3}), M of chondroitin sulfate can be assumed to be bound to Ca^{2+}. From the known value of $\Delta\varepsilon_{\text{Cu-heparin}}$ (from the Cu^{2+} binding study), the concentration of copper complex, and then that of free chondroitin sulfate, is calculated. The Ca^{2+} binding constant, are then calculated from these values, using Eq.(2). and is listed in Table 1 along with values for other systems. The Cu^{2+} binding constants calculated from each experimental point with Ca^{2+} up to $3\times10^{-3}M$ were in good agreement with values determined in the previous section.

Effects of Ionic Strength. From the nature of ionic interaction in complex formation between metal ion and glycosaminoglycans, strong ionic strength dependence in binding constant is expected. Such dependence is demonstrated in Figure 3.

The effects of NaCl on $5.0\times10^{-4}M$ Cu^{2+} and 6.67×10^{-4} heparin solution were studied at pH 6.1, and apparent binding constants using $\Delta\varepsilon$ obtained from zero salt are plotted against $\mu^{1/2}$ in Figure 4. The data fit well with an empirical equation, $\log K = 3.98 - 2.98\,\mu^{1/2}$, for the system. Similar ionic strength dependence was also observed with other glycosaminoglycans, as expected from the Debye-Hückel limiting law.

Discussion

The present study indicates that the ratio between disaccharide unit to metal ions is 1 for all glycosaminoglycans studied. The data also show that Cu^{2+} and Ca^{2+} bind preferably to the carboxyl group; if there is significant binding

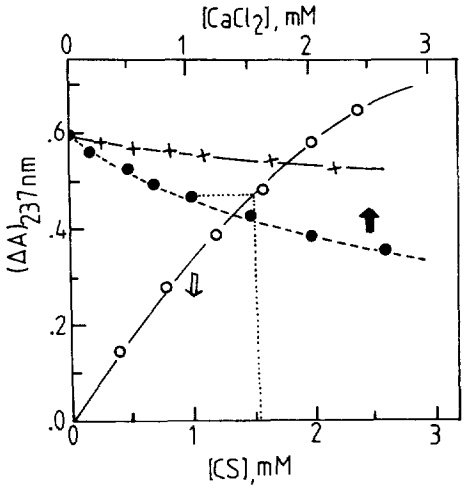

Figure 3. Variations of $(\Delta A)_{237\text{ nm}}$ of 1.0×10^{-3} M Cu^{2+} with chontroitin sulfate(0). Filled circles are those of 1.0×10^{-3} M Cu^{2+} and 2×10^{-3} M chondroitin mixtures against added $CaCl_2$ concentrations. x are values for these mixture with added Nacl concentration in same ionic strength scale as $CaCl_2$.

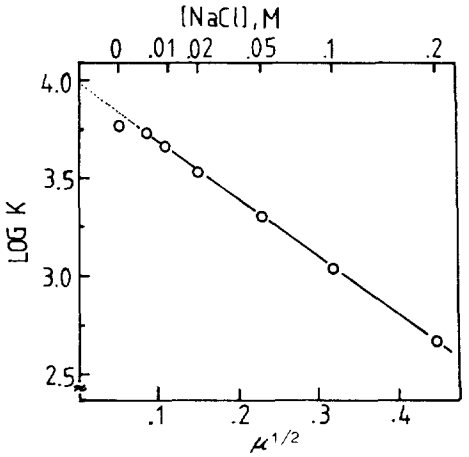

Figure 4. Log K of Cu-heparin plotted against square root of ionic strength of nonpolymer electrolytes at pH 6.1. Top scale is concentration of NaCl added to adjust the ionic strength.

TABLE 1: Binding Constants of Ca^{2+} and Cu^{2+} with Glycosaminoglycans and $\Delta\varepsilon$ of Cu^{2+} Complexes

	NaCl	Heparin			Chondroitin sulfate			Dermatan sulfate[a]		
pH	conc.	$(\Delta\varepsilon)_{237}$	$K_{Ca^{2+}}$	$K_{Ca^{2+}}$	$(\Delta\varepsilon)_{237}$	$K_{Cu^{2+}}$	$K_{Ca^{2+}}$	$(\Delta\varepsilon)_{237}$	$K_{Cu^{2+}}$	$K_{Ca^{2+}}$
5.0	0.0 M	2040	12500	3600	1400	680	670			
	0.1 M	2240	980	(340)[b]	1080	73	(55)[b]			
6.1	0.1 M	2100	900	340	1800	41	55	1710	230	120

[a]Limited quantity did not allow us to measure at other conditions. [b]Assumed to be same as pH 6.1.

of the metal ions to sulfate, the apparent binding constants calculated by the present method should vary markedly with the metal ion concentration; which we failed to observe. These results are in good agreement with our previous suggestions.

Generally, the stability of a complex formed from charged species is determined by the hydration energy of the ions, the electrostatic force, and the energy of covalent bond formation. The large difference in binding constant between heparin and dermatan sulfate for metal ions studied reflects mainly the electrostatic interaction, which is related in large part to the negative charges, whereas dermatan sulfate has 2 per disaccharide unit. However, the number of charges per disaccharide unit cannot explain the large difference in binding constants between dermatan sulfate and chondroitin sulfate, which have the same charge. Dermatan sulfate has glucuronic acid of pK_a 3.8 (the pK_a of iduronic acid in heparin is 5.3).[13] Despite the same charge, the spatial orientation of the carboxyl group in dermatan sulfate differs from that in chondroitin sulfate, and they interact with cations in different degree of electrostatic force, giving higher binding for H^+ (higher pK_a) and for metal ions. It has been suggested that the electrostatic force-dependent part in the complex formation (binding) constant can be replaced by the ionization constant of the polyacid.[14,15] Our data qualitively validate such suggestion, when we compare the magnitude of the binding constants for Ca^{2+} and pK_a of the carboxyl group. The order of Ca^{2+} and Cu^{2+} binding constants among glycosaminoglycans studied, heparin > dermatan sulfate > chondroitin sulfate, agrees well with reported data on $Co(NH_3)_6^{3+}$,[16,17] and Ca^{2+} [18] binding. It is interesting to compare this result with the observation of higher affinity of iduronic acid-containing polymers than glucuronic acid-containing ones for the interaction with macromolecules such as cationic polypeptides[19] and lipoproteins.[20] The parallelism in binding behavior of glycosaminoglycans with cationic macromolecules and metal ions suggests that similar electrostatic factors may predominate for both types of interaction.

For chondroitin sulfate, the binding constants of Cu^{2+} and Ca^{2+} do not differ very much, but much higher binding constants for Cu^{2+} with heparin and dermatan sulfate than those for Ca^{2+} were obtained. The proposed involvement of sulfate in chelation of heparin and dermatan sulfate, not in chondroitin sulfate, with Cu^{2+} providing extra stability of complexes,[5] can explain the results.

The large increase in $\Delta\varepsilon$ of the Cu^{2+} complex with pH change from 5.0 to 6.1 is an indication of the difference in the nature of the complexes between these two pH's. Cu^{2+} starts to be monohydroxylated when the pH of solution is raised from 5.0. The complexes between monohydroxylated Cu^{2+} ($Cu(OH)^+$) and glycosaminoglycans may give large molar absorptivity change, $\Delta\varepsilon$, of the complex formation, but show less stability because of reduced charge on the copper ion. Such effects of hydroxylation of copper ion on the binding property toward heparin are less apparent, in comparison with the case of chondroitin sulfate, because of the involvement of the sulfate groups in chelation. The chelation of both carboxyl and sulfate groups of heparin to $Cu(OH)^+$ is unlikely because of electrostatic and stereochemical factors, and thus the concentration of $Cu(OH)^+$-heparin complex is expected to be small unless the pH of the solution is significantly higher than 6. However, the decreased apparent K Cu-heparin with increased pH reflects the decreased concnetration of Cu^{2+}.

Our data on the Ca^{2+} binding constant of chondroitin sulfate agree well with the report of MacGregor and Boweness.[7] Buddecke and Dezeniek[18] reported the value as 16.3 $mole^{-1}$ in 0.15 M NaCl, which is considerably smaller than our result of 55, and MacGregor and Bowness' 65 $mole^{-1}$ in 0.1 M NaCl, even considering the difference in ionic strength.

Naturally occuring aggregated cartilage proteoglycans contain approximately one calcium ion per two disacchride units of chondroitin sulfate. The presence of such high concentration of calcium in the tissue cannot be explained by binding constants of the metal ion with free chondroitin sulfate. MacGregor and Bowness[7] reported significantly higher binding constants for aggregated puppy rib proteoglycans than for chondroitin sulfate. In an aggregated proteoglycan, Ca^{2+} may bind to two anionic groups of different polysaccharide chains, giving higher observed binding constants. Another possible explanation is condensation of divalent ion onto a polyelectrolyte as theoretically predicted by Manning.[21]

Our results imply that approximately 40% of heparin is bound to Ca^{2+}, whereas the ratio is only 6% for chondroitin sulfate, in 0.1 M NaCl solution in the presence of 1×10^{-3} M free Ca^{2+}. This example can explain a good correlation between the Ca^{2+} content of cells and cell coat glycosaminoglycans, which showed less Ca^{2+} and sulfated glycosaminoglycans in growing 3T3 cells than in normal cells.[10] Vannucchi *et al.*[10] suggested that N-sulfated glycosaminoglycans of the cell coat could exert their negative effect on growth *via* their strong sorption of Ca^{2+} ions. This is also in good agreement with reports of decreased amounts of dermatan sulfate and heparan sulfate and increased chondroitin sulfate content of neonate and tumoral tissues, in comparison ot normal adult tissue, and with the suggestion of the possible role of sulfated glycosaminoglycans in cell recognition and adhesiveness.[22,23] Because of the large difference in the affinity of Ca^{2+} to different glycosaminoglycans, changes in the composition of cell-surface glycosaminoglycans or in the environments of the polymers will undoubtedly vary the content of Ca^{2+} in the cell surface and thus in cytoplasm, leading to different cell behaviors including growth.

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The Charge Transfer Complexes of Monoalkylbenzene with Iodine in Carbon Tetrachloride (II)

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Ultraviolet spectrophotometric investigations were carried out on monoalkylbenzene-iodine systems in carbon tetrachloride. The results reveal the formation of one-to-one molecular complexes. On the basis of the equilibrium constants for these complexes of representative monosubstituted benzenes, the following order of increasing stability is obtained: *i*-propyl-<*i*-butyl-<*t*-butyl-<*n*-butyl-benzene. The values of ΔH, ΔG and ΔS for the interaction of a number of monoalkyl substituted benzenes with iodine have been determined. In general, it can be said that as ΔH becomes increasingly negative, corresponding decreases in the ΔG and the ΔS values are observed, and these variations are linear. The thermodynamic constants become increasingly negative with increasing monoalkyl substitution of the aromatic donor nucleus. The complex bond is therefore weak, and its formation is accompanied by relatively small entropy changes. Thus, analysis of these findings is discussed.

Introduction

After Benesi and Hildebrand[1] first demonstrated the formation of one to one molecular complex between benzene and iodine in carbon tetrachloride solution, studies were extended to other system by a number of workers[2].

In a previous study[2] of this series[1], it was observed that the solutions of monoalkylbenzene (benzene, methyl-, ethyl-, *n*-propyl-benzene) with Iodine in carbon tetrachloride showed the presence of absorption maxima in the vicinity of 300 m μ, where none of the component materials had strong absorption. This phenomenon was attrituted to the formation of one to one molecular complexes in solution. From spectrophotometric data, we obtained the equilibrium constants for the complex formation and the molar absorptivities of the complexes at their absorption maxima. The equilibrium constants at 25 °C for the monoalkylbenzene complexes were found to increase in the order, benezene<methyl-<ethyl-<*n*-propyl-benzene.

The present study involved the spectrophotometric investigations at 25, 40, 60 °C on the systems of monoalkylbenzene (*i*-propyl-, *n*-butyl, *i*-butyl-, *t*-butyl-benzene) with iodine in carbon tetrachloride. Carrying out ultraviolet spectrophotometric studies to measure the temperature dependence of complex formation, Keefer and Andrews[3] determined ΔH, ΔG and ΔS for carbon tetrachloride solutions of iodine with