Acceleration of Ionic Reactions by Naturally Occurring Glycosaminoglycans. II.*

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Abstract

The reactions of halopentammine cobalt(III) complexes with both Hg(II) (aquation reaction) and Fe(II) (electron transfer reaction) have been studied in the presence of chondroitin-4-sulphate and heparin. The rates of both reactions are increased in the presence of the glycosaminoglycan polyelectrolytes, but by different mechanisms. Inhibition of the Hg(II) reaction at lower polymer-to-reagent concentration ratios is consistent with specific polymer–Hg interactions which render a population of 'bound' ions unreactive. However, the activation energy of the Hg-induced aquation is lowered in the presence of the polyanion, consistent with stabilization of the expected trivalent cationic intermediate by electrostatic effects. Heparin, with the higher charge density, is especially effective in this regard. In contrast, the reduction of the Co(III) complexes by Fe(II) experiences an increased activation energy in the presence of polymer. The observed acceleration of this reaction may then be due largely to local concentration effects in the polymer domain, as implied by a two-phase model. Investigation of the mechanisms of these rate accelerations will therefore provide more detailed information on the electrostatic fields and specific metal ion interactions of these important biological polymers.

Introduction

The glycosaminoglycans (GAGs)§ are a group of carboxyl- and (or) sulphate-containing polysaccharides with widespread biological occurrence and a diversity of proposed functions [1, 2]. Those considered here are made up of repeating disaccharide subunits, each consisting of a hexosamine and uronic acid residue. Because of their anionic polyelectrolyte nature, it is appropriate that much attention has been given to their interactions with metal ions. Such interactions have been demonstrated to alter the solution properties [3–9] and dynamic conformation [3, 10–13] of the GAGs, and through such effects have been thought to participate in [1, 14–19], and perhaps modulate [20–22], their biological activities. Some interactions may be due to specific binding properties unique to certain ions, while others may depend only on the net structural charge density of the polymer and the valence of the counter-ion (*i.e.* Manning counter-ion condensation behaviour [1, 5, 6, 18, 23, 24]).

Mindful of earlier work by Booij [25] with chondroitin sulphates, we recently investigated the acceleration of the Hg(II)-induced aquation of $Br(NH_3)_5Co(III)^{2+}$ by a number of GAGs [26], with the aim of relating effective solution charge density to a measurable kinetic parameter. Our results indicated that all sulphated GAGs gave maximal rate accelerations consistent with similar effective surface potentials, under similar conditions. This behaviour was not true catalysis, and was not consistent with a two-phase model of rate acceleration, but could be explained as resulting from charge compensation of the polymer according to Manning's model of counter-ion condensation [26]. Left unexplained is the mechanism by which the partially charge-compensated polymers affect the reaction rate, *i.e.* the thermodynamic behaviour of the reactant ions in the polymer domain. In order to investigate further the participation of GAGs in this mechanism, we have extended our earlier study to include reduction of the Co(III) complex by Fe(II) as an example of a reaction with a different charge distribution in the transition state. Here we report the effects of sulphated chondroitin and heparin on this reaction, and on the activation energies of both electron transfer and aquation reactions.

Experimental

Chondroitin sulphate type A (ChS(A); chondroitin-4-sulphate) from whale cartilage and heparin (pig intestinal mucosa, grade I) were obtained from

*For Part I, see ref. 26.

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§Abbreviations: ChS(A) = chondroitin sulphate type A (chondroitin-4-sulphate), GAG(s) = glycosaminoglycan(s).

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Sigma Chemical Co. (St.Louis, Mo.), as the sodium salts, and were purified as described previously [26]. Structural charge densities of 0.94 and 1.49 respectively were calculated under conditions of carboxylate protonation [26]. $Hg(NO_3)_2$ (crystalline) was from Matheson, Coleman and Bell (Norwood, Ohio), and $Fe(ClO_4)_2 \cdot 6H_2O$ (99+%) from Johnson Matthey (Toronto, Ont.). $Br(NH_3)_5Co(III) \cdot Br_2$ ($\epsilon_{254} = 1.72 \times 10^4$ dm^3 mol^{-1} cm^{-1}) was synthesized by the method of Diehl *et al.*, and purified, as previously described [27]. $Cl(NH_3)_5Co(III) \cdot Cl_2$ ($\epsilon_{227} = 1.77 \times 10^4$ dm^3 mol^{-1} cm^{-1}, Co = 23.5%) was obtained from Johnson Matthey and used without further purification.

Water of 18 MΩcm (Milli Q system, Millipore) was used throughout. Stock solutions (1 mM) of the cobalt complexes were prepared fresh weekly and stored in the dark. Dilutions were prepared daily as required, and their concentrations checked by absorbance. Solutions of Fe(II) were prepared by addition of ferrous perchlorate to an empty volumetric flask in series with a water reservoir. The water was boiled vigorously as oxygen-free N_2 was aspirated through the system, then cooled under reduced pressure of N_2. After filling the volumetric flask, N_2-purging was continued overnight. For use, aliquots of this stock were transferred by positive N_2 displacement through needles into flushed, septum-capped tubes. Standardization of the Fe(II) stock against ceric sulphate (0.1000 ± 0.0005 N; Fisher Scientific, Fairlawn, N.J.) using phenanthroline as an indicator [28], before and after a series of experiments, revealed no discernible oxidation during this time.

All reactions were followed at the absorption maximum of the cobalt complex (254 nm for the Br salt, 227 nm for the Cl) with a Perkin-Elmer Lambda 4C spectrometer thermostatted at 25.0 ± 0.1 °C, except for the temperature variation experiments, and the data were analyzed in digital form using a PE 7700 computer. Rate constants, k_2, were calculated according to $-d[Co]/dt = k_2[M][Co]$, where [Co] is the concentration of the cobalt complex and [M] is the concentration of Hg(II) or Fe(II). Rate constants in the absence of added polymer are designated $k_{2,o}$. The Hg^{2+}-induced aquation reactions were carried out exactly as described earlier [26], the ionic strength being adjusted to 0.020 with $NaClO_4$. The Fe^{2+}-electron transfer reactions were carried out under N_2 in 3-ml quartz Thunberg cuvettes, in the presence of 1.0×10^{-3} M $HClO_4$ with sufficient $NaClO_4$ to give an ionic strength of 0.020. Table I gives the concentrations of metal salts and GAGs used in the temperature variation experiments. Similar reagent concentrations were used when GAG concentrations were varied at 25 °C. GAG concentrations throughout are expressed as molar concentrations of uronic acid, *i.e.* disaccharide subunits.

All reactions gave initial decreases in absorbance, providing initial rates free from significant product inhibition, which can be expected to arise from displacement of the divalent reactants from the polymer domain by trivalent products. Poisoning by Fe(III) is probably minimized by precipitation of ferric hydroxides upon formation [29]. Spontaneous hydrolysis of the Co complexes was corrected for by measuring the apparent rates in the absence of Hg(II) or Fe(II). Oxidation of Fe(II) was checked in the absence of Co species. None was observed over the longest reaction times (30 min) as indicated by a stable absorbance at 240 nm.

The GAG surface potentials and reaction annuli were calculated from the two-phase model of Morawetz and Schafer [30]. According to this model, an ion partitions between the bulk phase and polymer domain according to a Boltzmann term

$$x = \exp(-q\psi/kT) \quad (1)$$

where q is the charge on the electron, k is Boltzmann's constant, and T the absolute temperature. ψ is the average potential experienced by a univalent counter-ion in the polymer domain. The volume of the polyion phase is treated as that of a cylinder of radius r. If the structural radius, R, of the polymer is known, then the thickness of the reaction annulus, ρ, is

$$\rho = r - R \quad (2)$$

If the concentration of disaccharide repeating units of the GAG is given as the uronic acid concentration, [ua], in mol dm^{-3}, and L is the length of the disaccharide, then with all lengths in m (metres) the volume of the polyion phase is given by

$$\phi = \pi r^2 LN[\text{ua}] \times 10^3 \quad (3)$$

TABLE I. Reagent Concentrations Used in Temperature-variation Experiments[a]

[Fe(II)]	[Hg(II)]	[$Br(NH_3)_5Co(III)^{2+}$]	[ChS(A)]	[Heparin]
9.21×10^{-4}		2.93×10^{-5}	7.73×10^{-3}	
9.21×10^{-4}		2.93×10^{-5}		4.26×10^{-4}
	1.11×10^{-4}	7.44×10^{-6}	6.96×10^{-4}	
	1.11×10^{-4}	7.13×10^{-6}		2.56×10^{-5}

[a]All concentrations are molar. Those for ChS(A) and heparin are expressed as uronic acid residues.

where N is Avogadro's number. Morawetz and Schafer [30] derived expressions for ϕ in terms of x, and for k/k_0 in terms of ϕ. When the charge on both reactants is +2, these expressions reduce to (see Appendix A of ref. 25)

$$\phi_{max} = x^{-2} \quad (4)$$

and

$$(k/k_0)_{max} = x^2/4 \quad (5)$$

Equations (1) and (5) allow calculation of the effective potential from the observed rate acceleration, while eqns. (2)–(4) determine the proximity of the reacting ions to the polymer.

Results

The uncatalyzed rate of reduction of $Co(NH_3)_5 \cdot X^{2+}$ (X = Cl or Br) by Fe(II) is slow [$\log(k_{2,o}) = -2.43 \pm 0.11$ and -2.64 for X = Br and Cl, respectively, at $T = 30$ °C], as expected for reaction between like-charged species. The reaction is slower than the corresponding Hg-induced aquation of the Br complex [$\log(k_{2,o}) = 0.79 \pm 0.02$ at 30 °C]. All data reported here have been corrected for the rate of spontaneous hydrolysis of the Co(III) complexes, although this correction has a small effect on the aquation data, and on the reduction rates in the presence of GAG at higher temperatures. At temperatures below 25 °C, however, the rates of reduction by Fe(II) are not significantly greater than the rate of hydrolysis, and accurate values of $k_{2,o}$ cannot be determined.

The reduction of $Co(NH_3)_5 \cdot X^{2+}$ by Fe(II) is accelerated in the presence of either heparin or ChS(A). On increasing the concentration of heparin, the rate of reaction increases to a maximum before inhibition is seen at excess polymer concentrations. Sufficiently high concentrations of ChS(A) were not achieved to observe inhibition (Fig. 1). While this inhibition phenomenon is well documented in the presence of polyelectrolytes [25, 26, 29], these results are in contrast to our earlier study of Hg-induced aquation [26]. While the total reactant concentration is eight-fold higher in the present study (necessary to achieve reasonable rates of reaction), the onset of inhibition occurs at a 10^2-fold higher heparin concentration, at the same ionic strength.

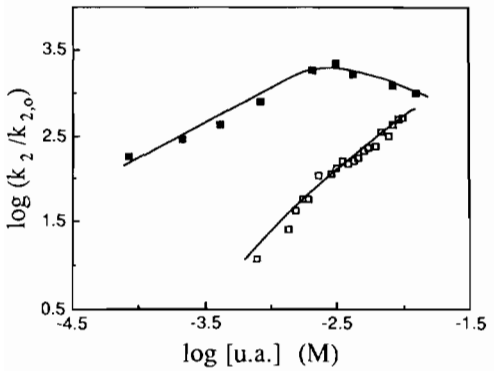

Fig. 1. Effect of added glycosaminoglycans on the rate of reduction of $Cl(NH_3)_5Co(III)^{2+}$ by Fe(II). Conditions are as described in 'Experimental', with ionic strength held constant at 0.020 and $T = 25.0 \pm 0.1$ °C; □, chondroitin-4-sulphate; ■, heparin.

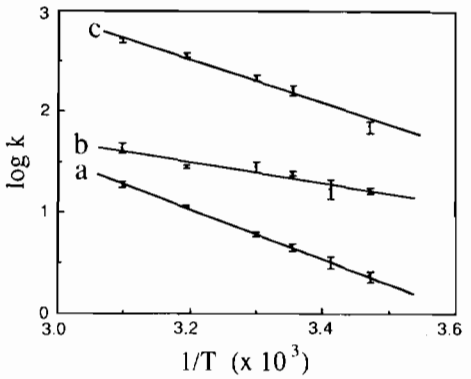

Fig. 2. Arrhenius plots of the Hg(II)-induced aquation of $Br(NH_3)_5Co(III)^{2+}$. Conditions are as described in 'Experimental': (a) No added polymer; (b) 2.56×10^{-5} M uronic acid, as heparin; (c) 6.96×10^{-4} M uronic acid, as chondroitin-4-sulphate. Error bars show standard deviations on the means of at least triplicate determinations.

TABLE II. Surface Potentials and Reaction Cylinders Calculated from the Two-phase Model, Based on Reaction of the Indicated Metal Ion with $Br(NH_3)_5Co(III)^{2+}$

GAG	Ion	x	Cylinder radius r (nm)	Reaction annulus ρ (nm)	ψ (mV)	Reference
Heparin	Hg^{++}	14.2	7.85	7.15	−68	26
	Fe^{++}	91.9	0.15		−116	present work
ChS(A)	Hg^{++}		1.4	0.94	−72	25
	Hg^{++}	11.0	1.76	1.06	−62	26
	Fe^{++}	>45	>51		<−98	present work

TABLE III. Activation Energies for the Reaction of M(II) with $(NH_3)_5Co(III)\cdot X^{2+}$, With or Without Added Glycosaminoglycan (GAG) Polyelectrolyte

GAG	M	X	$E^{\ddagger}$ (kJ/mol)
	Hg	Cl	82.0[a]
	Hg	Br	46.9
ChS(A)	Hg	Br	41.9
Heparin	Hg	Br	19.9
	Fe	Cl	60.3 ± 5.0[a]
	Fe	Cl	76.0
ChS(A)	Fe	Cl	71.0
	Fe	Br	21.4
ChS(A)	Fe	Br	85.4
Heparin	Fe	Br	58.2

[a]Data from ref. 29 are included for comparison.

Parameters calculated from the two-phase model are summarized in Table II. If a maximum occurs in the ChS(A)-accelerated Fe(II) reaction, it must be at $\log(k_2/k_{2,o})_{max} > 2.71$. As both the partitioning parameter, x, and polymer concentration increase, $|\psi|$ and r increase and decrease respectively. Therefore minimum and maximum limits for these quantities can be given for the latter reaction.

Representative Arrhenius plots for the Hg-induced aquations are given in Fig. 2. Activation energies derived from such plots for this and other reactions are summarized (Table III).

Discussion

Conditions are reported under which reduction of halopentammine–Co(III) complexes by Fe(II) can be accelerated by naturally occurring GAGs. As in previous reports of acceleration of aquation of these complexes [25, 26], this effect can be attributed to the polyelectrolyte nature of the GAGs, and greatly exceeds the expected contribution of non-specific salt effects [29, 31]. Local increases in concentrations of reactant counter-ions in the polymer domain account qualitatively for the rate accelerations, and for inhibition at higher polymer concentrations [29, 32]. However, quantitative prediction of maximum rates and optimum polymer concentrations for specific reactions are not possible. Taken together with our earlier report [26], the present results demonstrate that effects specific to a given system of polyion and counter-ions must be important. The optimal ratio of heparin to reactants is an order of magnitude greater for the Fe(II) reaction than for the Hg(II) reaction, with the same Co(III) complex as second reactant. Similar behaviour is indicated for the corresponding ChS(A) systems, although we did not extend our measurements to sufficiently high ChS(A) concentrations to observe inhibition of the Fe(II) reactions in the present study.

Several possible explanations for these differences in the Fe(II) and Hg(II) systems can be offered, all of which require some degree of counter-ion specificity in polymer associations. A degree of negative cooperativity is to be expected in counter-ion binding, since partial reduction of surface potential reduces the driving force for further binding. If this effect was greater in the Hg(II) system, counter-ion separation would be facilitated and inhibition due to decreasing local concentrations would be seen at lower GAG-to-metal ratios. An assymetric distribution of reactants in the Hg(II) system would have a similar effect, if for example binding of one reactant to a polymer molecule disfavoured binding of the other. Conversely, a degree of positive cooperativity in the Fe(II) system could be postulated, but would not be expected. We have previously suggested that a subset of reactants in the Hg(II) system could be bound in a manner not conducive to reaction, increasing the ratio of polymer to reactive species, and the work of Booij [25] has indicated a strong degree of covalency in the interaction of Hg(II) with polymer carboxyl groups. Such effects could be reduced or absent for Fe(II). In the framework of the Manning model, this could, for example, be due to a greater degree of site-specific binding for Hg(II), with Fe(II) favouring a territorial binding mode (see for example refs. 4, 33). Such explanations are at present speculative, and whether any of these effects, if they do occur, are of sufficient magnitude to explain the differences must await detailed metal-binding studies.

Another possibility which cannot be ruled out in the present study is that the higher counter-ion concentrations necessitated by the inherently slower Fe(II) reaction were sufficient to induce conformational changes in the polymers, which in turn decreased the affinity of the divalent reactants for the polymer domains. These changes would be independent of ionic strength, which was kept constant in all data compared herein, but could result from either site-specific binding of a counter-ion, or be due to the relaxation of conformational constraints in the polyion by non-specific charge compensation. The former has been observed, for example, in Cu(II)–hyaluronate interactions [12, 13], while the latter almost certainly plays a role in a number of GAG conformation-dependent phenomena. In either case, knowledge of the total ion concentration in the polymer domain would seem insufficient for prediction of acceleration/inhibition effects.

From the above discussion, it should be clear that a simple description of rate acceleration based on effective local concentrations is inadequate, and

it should not be surprising that an unmodified two-phase model does not yield sensible dimensions for the reaction annulus in most cases. An exception is the ChS(A)-accelerated Hg(II) reaction, for which our values are in good agreement with those of Booij [25] (*cf.* Table II). The relationship of GAG surface potential to the observed rate accelerations is perhaps of greater interest. Under conditions of the Hg(II) reaction, and considering only monovalent supporting electrolytes, we have calculated surface potentials of −115 and −92 mV, respectively, for the heparin and ChS(A) used in the present study (see ref. 26 for details). The corresponding values were −57 and −46 mV, respectively, when charge compensation by divalent reactants was included through the Grahame equation. It is tempting to note that the more negative values are in keeping with the effective potentials calculated for the Fe(II) reaction, while the lower ones are closer to the values found for aquation (Table II). This could arise, for example, if the Hg(II) reaction was accelerated by electrostatic effects, and so depended on the effective surface potential of the partially-compensated polymer, whereas acceleration of the Fe(II) reaction reflected the total amount of reactants drawn into the polymer domain, including those participating in charge compensation, and so depended more on structural surface potential. Such an explanation can be given some mechanistic rationale (*vide infra*), but until more details are known, it is more reasonable to attribute the effects of the polymers on both reactions to some manifestation of polymer charge, and then ask whether a higher potential is experienced by reactants in the Fe(II) system, or whether the reaction is inherently more susceptible to electrostatic effects.

The activation energy of the Hg(II)-induced aquation reaction is lowered by both GAGs. This reaction probably involves an intermediate of increased charge, such as $Co(NH_3)_5^{3+}$, and its formation would be stabilized in the negative field of the polymer. In keeping with this interpretation, heparin, with the greater structural charge density, is more effective in lowering the activation energy than ChS(A). In contrast, the Fe(II)-electron transfer reaction probably proceeds through a bridging halogen [29], with the trivalent species forming much later along the reaction coordinate. The observed increases in the activation energy by both GAGs may then indicate a less polar transition state, and clearly demonstrates that the polyion phenomenon is not catalytic. The observed rate acceleration for this reaction may well be due entirely to local concentration effects, while a decreased activation energy also contributes to Hg-induced aquation.

Whatever the detailed explanation for the rate accelerations caused by the GAGs, their opposite effects on the activation energies of Hg(II)-induced aquation and Fe(II) electron transfer reactions of halopentammine Co(III) complexes provide two distinct systems with which to probe further the polyelectrolyte properties of these polyanionic sugars. While such properties are of interest in elucidating the biological behaviour of these molecules, specific metal-binding interactions are also undoubtedly of functional importance. The large difference in optimal GAG-to-reactant concentration ratios for the two reactions may prove to be a sensitive indicator of different binding modes for the ferrous and mercuric ions.

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