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Cation specificity and cation binding to low sulfated carrageenans

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A low-sulfated carrageenan from *Eucheuma gelatinae* and a partially desulfated (by chemical means) furcellaran were studied by polarimetry and Cs-133 NMR. The polarimetric studies show that the cation specificity of the coil-helix transition remains a strong effect even for very low-sulfated carrageenans, whereas the sensitivity to inert salt is considerably weakened compared to unmodified furcellaran. Cs-133 NMR shifts and linewidths reveal a binding of Cs^+ to the helical conformation, but not to the random coil. The cation specificity is well predicted by the theoretical model previously used for unmodified furcellaran and kappa-carrageenan, if it is assumed that the intrinsic binding constant and the density of binding sites are independent of the degree of sulfation of the carrageenan helix.

INTRODUCTION

Carrageenans are a family of sulfated galactans extracted from red algae (Rees *et al.*, 1982). The wide-spread applications of carrageenan in industry depend on its good gel-forming properties. Gelation of carrageenan requires a conformational transition where ordered helices are formed. For the lower charged carrageenans, the conformational transition is influenced not only by the salt concentration, owing to the electrostatic interaction, but also by the identity of the salt. Thus, a dramatic cation specificity has been found for kappa-carrageenan (Rochas & Rinaudo, 1980) and furcellaran (Zhang *et al.*, 1991), but not for iota-carrageenan uncontaminated by kappa-carrageenan (Piculell *et al.*, 1987). Except for the half-ester sulphate content, iota-carrageenan, kappa-carrageenan and furcellaran (Bjerre-Petersen *et al.*, 1973) have identical repeating disaccharides units (Fig. 1). Counterion NMR studies have revealed a binding of Cs^+, K^+ and Rb^+ ions to the helices, but not to the coils, of kappa-carrageenan (Grasdalen & Smidsrød, 1981; Smidsrød & Grasdalen, 1984; Belton *et al.*, 1985) and furcellaran (Tanner *et al.*, 1990; Zhang *et al.*, 1991). Two of the present authors (Nilsson & Piculell, 1991) were able to analyse the cation specificity of the conformational transition of kappa-carrageenan by the Poisson–Boltzmann Cell Model (PBCM) and deduced an intrinsic equilibrium constant of cation binding to kappa-carrageenan helices which was of the order of a few M^{-1}. Subsequently, Zhang *et al.* (1991) could semi-quantitatively predict the

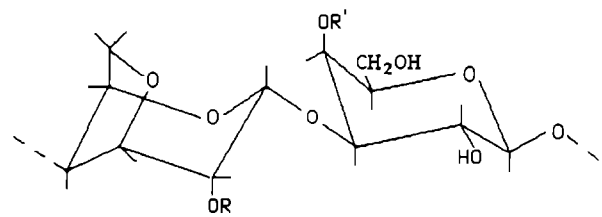

Fig. 1. Repeating disaccharide units of iota-carrageenan ($R = R' = SO_3^-$), kappa-carrageenan ($R = H$, $R' = SO_3^-$) and furcellaran ($R = H$, $R' = 0{\cdot}6\ SO_3^-$ and 0·4H).

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cation specificity of the conformational transitions of furcellaran by using the same model and the same cation binding constant as used by Nilsson and Piculell for kappa-carrageenan.

The fact that certain cations bind to kappa-carrageenan and furcellaran helices now seems well established. However, the nature of binding site(s) is not known. Early results from IR spectroscopy (Norton *et al.*, 1983; Belton *et al.*, 1986) were taken to suggest that the half-ester sulfate group was involved in the binding site, but a later analysis (Belton *et al.*, 1989) indicated that the difference in the spectra was caused by the helix formation, induced by the specific ions, rather than by the cation binding in itself. Moreover, there is no obvious reason to believe that the sulfate group is involved in the binding site, since iota-carrageenan, with two half-ester sulfate groups per disaccharide unit, does not show any cation specificity. An alternative proposal (Nilsson & Piculell, 1991) is that, in the double helix of the lower sulfated carrageenans, oxygen functions from the two carrageenan chains are located so as to form a size-specific ion binding site not involving the sulfate. In principle, spectroscopic methods should provide information as to the nature of the binding site, but the study of aggregated helical carrageenans is difficult in practice. An alternative route, taken here, is to decrease the sulfate content of carrageenan and monitor the concomitant changes in the cation specificity. Apart from serving as a further check of the cation-binding model used previously (Nilsson & Piculell, 1991; Zhang *et al.*, 1991), the results should, in principle, be different for the two alternative types of binding sites considered. If it is assumed that the binding constants of the remaining sites are not influenced by desulfation, we have the following possibilities. If the sulfate group is involved in the binding site, the density of binding sites decreases with the sulfate content and the cation specificity should thus become weak at a low degrees of sulfation. If, on the other hand, the sulfate is not involved in the site, the density of binding sites (presumably one site per repeating unit of a helix, i.e. two disaccharide groups) should be unaltered by desulfation. Hence, the cation specificity should remain a strong effect, although weakened by the decreased electrostatic potential on the lower charged helix.

EXPERIMENTAL

Materials

A commercial sample of carrageenan from *Eucheuma gelatinae* (EG) was obtained as an ethanol precipitate in KCl. The carrageenan had been produced at Hainan Island, China by boiling the algae with water after a previous treatment with a hot alkali solution. This carrageenan sample was purified by milling, washing with hot 70% ethanol saturated with NaCl, hot 70% ethanol, dialysis against neutral water and freeze drying. Analysis by proton NMR showed little methyl and no signals from precursors or starch. Kappa-carrageenan was obtained from Sigma Chemical Co. and furcellaran from *Furcellaria lumbricalis* was a generous gift from Litex A/S, Denmark. A partially desulfated sample (DF) was prepared using the method described by Dolan & Rees (1965). In order to overcome the disadvantage of severe depolymerization during desulfation, the carrageenan sample was prepared in the gel phase (Knutsen, 1992). Analysis by gel permeation chromatography showed no signs of any low-molecular fraction present in the DF. The fraction of kappa-carrageenan units, f_{kappa}, in the samples was determined by proton NMR (Usov, 1984; Knutsen *et al.*, 1990), and was found to be 0·20, 0·29 and 0·60 for DF, EG and furcellaran, respectively (Knutsen & Grasdalen, 1987, 1992; Zhang *et al.*, 1991).

Methods

The helix onset temperature, T_o, was determined by optical rotation at 435 nm in a jacketed cell with 5-cm pathlength on a Jasco DIP-360 polarimeter. Cs-133 NMR spectra were obtained at 47·45 MHz for samples in 5 mm tubes on a Nicolet NIC-360 spectrometer. 4K data points were used and the spectral width was 10 kHz for carrageenan samples and 1 kHz for reference solutions with the same CsCl concentrations as the carrageenan samples. The observed relative chemical shift of Cs-133, $\Delta\delta$, is the chemical shift difference between the carrageenan sample and the reference solution.

RESULTS AND DISCUSSION

NMR evidence of cation binding

The signal intensities, relaxation rates and chemical shifts obtained in ion NMR studies provide sensitive information on specific interactions between ions and carrageenans. Cs-133 NMR studies show no evidence of specific Cs^+ interaction with random coils of kappa-carrageenan (Grasdalen & Smidsrød, 1981) or furcellaran (Zhang *et al.*, 1991), whereas, with the formation of helices, a significant linebroadening of the Cs-133 signals occurs, the peak changes from a single-Lorentzian to a multi-Lorentzian lineshape and changes in the chemical shift are observed, indicating a specific ion binding to the helix.

The salt concentration dependence of the Cs-133 chemical shift of EG carrageenan is shown in Fig. 2. The features are the same as those previously demonstrated in similar experiments on kappa-carrageenan (Grasdalen & Smidsrød, 1981; Smidsrød & Grasdalen, 1984) and furcellaran (Zhang *et al.*, 1991): with the addition of

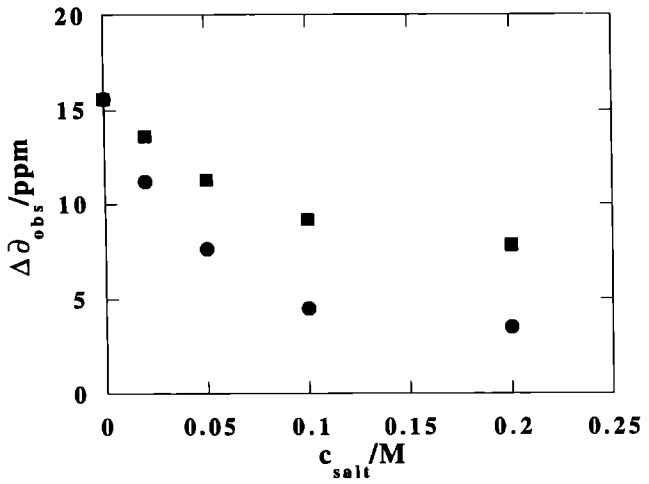


Fig. 2. Experimental variation of the Cs-133 chemical shift with the concentration of added salts to 4% Cs-EG carrageenan at 25°C. The symbols indicate addition of CsCl (●) and addition of NaCl(■).

CsCl, the chemical shift decreases due to a decrease in the fraction of bound Cs^+ ions although the site occupancy increases. With the addition of NaCl, the chemical shift also decreases, but to a lesser degree than for the addition of CsCl. This decrease originates from a decrease in the electrostatic potential at the polymer surface.

Chemical shift data for the DF and for unmodified furcellaran in aqueous solutions are compared in Fig. 3. Assuming that partially desulfated furcellaran samples have the same helical conformation as kappa-carrageenan, the PBCM was applied to the furcellaran system with the model parameters used previously (Nilsson *et al.*, 1989), but with the charge density obtained from f_{kappa}. Theoretical curves were calculated for two

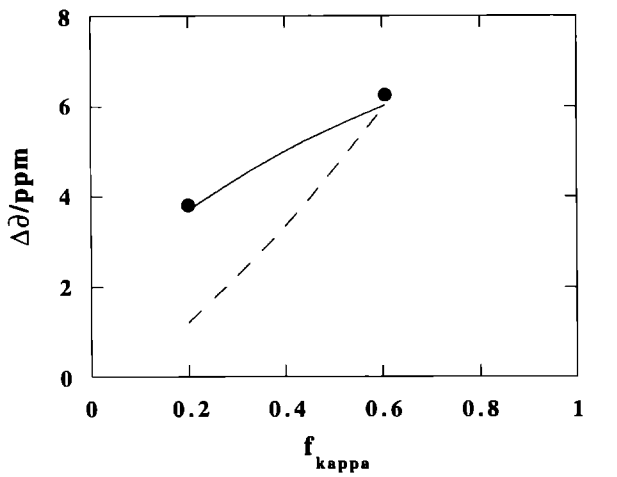


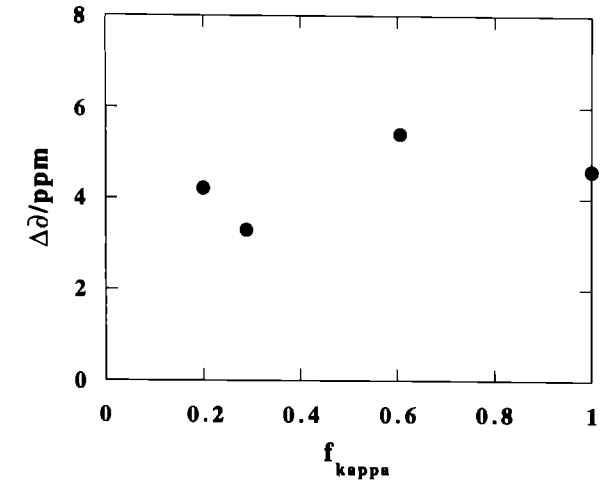
Fig. 3. Experimental (points) and theoretical (lines) variation of the Cs-133 chemical shift with the degree of sulfation in 46 mM Cs-furcellaran in 0·05 M aqueous CsCl at 25°C. The calculations were made with $n = 2$, $K_o(Cs) = 4{\cdot}1\ M^{-1}$ and $\Delta\delta_b = 41$ ppm (solid line) and with $n = 1/f_{kappa}$, $K_o(Cs) = 1{\cdot}6\ M^{-1}$ and $\Delta\delta_b = 51$ ppm (dashed line).

different assumptions regarding the density of binding sites, corresponding to one site per two disaccharides and one site per sulfate group ($n = 2$ and $1/f_{kappa}$ respectively; cf. below) using the appropriate binding constants previously deduced for furcellaran (Zhang *et al.*, 1991). The best agreement is found if a constant site density is assumed.

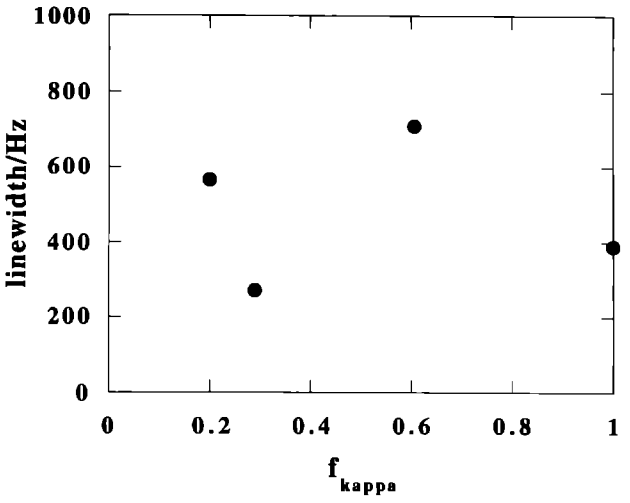
Unfortunately, the above evidence in favour of a constant density of the binding sites is not conclusive. First, there are difficulties in applying the PBCM to the lower sulfated samples (*vide infra*). Second, there are some quantitative uncertainties regarding the shift values. In aqueous solutions, the helices of lower charged carrageenans tend to associate into larger aggregates, especially in the presence of site-binding cations. This introduces a problem in the Cs-133 NMR studies in that the aggregation may inhibit the fast exchange (on the NMR time scale) between the free ions and bound ions trapped inside the aggregates (Zhang *et al.*, 1991). One way to attack this problem is to perform the measurements in formamide, where there is less helix aggregation (Rochas & Rinaudo, 1982). The results of such measurements on EG, DF, furcellaran and kappa-carrageenan at 15°C are shown in Fig. 4. The relative shifts in formamide are similar to those obtained in water, and the variations among the different samples are also very similar. Moreover, the variation in the NMR linewidths are consistent with the $\Delta\delta$ data. Therefore, it may be inferred that aggregation does not significantly prohibit the fast exchange between the bound and free ions in water. A serious problem for the interpretation is, however, that the NMR parameters do not vary smoothly with the degree of sulfation when carrageenans from different sources (EG, furcellaran and kappa-carrageenan) are compared. A trivial explanation of these variations would be that they reflect a similar variation in helical content (and, consequently, in the content of binding sites) among the samples. This was not confirmed, however, by optical rotation measurements, which showed that, if anything, the helical content of EG is higher than that of the DF under the same salt conditions (owing to the higher transition temperature of EG), although the NMR shift and linebroadening effects are larger for the DF. It is possible that more detailed knowledge on, for example, the distribution of the sulfate groups on the various samples might explain the pattern seen in Fig. 4. At present, however, the only safe conclusions from the NMR data are that all low-sulfated carrageenans display a similar cesium binding, and that the binding to a given carrageenan (furcellaran) decreases with partial removal of the sulfate groups.

Salt dependence of the conformational transition

Helix onset temperatures of the low-sulfated carrageenans in the presence of various concentrations of non-



(a)



(b)

Fig. 4. Experimental variation of the Cs-133 (a) chemical shift and (b) linewidth with the degree of sulfation for 46 mM Cs-carrageenans in 0·05 CsCl in formamide at 15°C.

specific (Na^+) or specific (Cs^+) cations are plotted in Fig. 5 and are compared to previous results (Zhang *et al.*, 1991) for unmodified furcellaran. Just as for furcellaran, the transition temperatures of DF and EG are much higher in CsCl than in NaCl. Thus, the cation specificity remains strong even when the degree of sulfation is low. The transition temperatures are higher, and the NaCl concentration dependence weaker for the low-sulfated samples, as expected, in view of the lower charge density. For EG, the transition temperature in 0·1 M CsCl solution does not change on addition of 0·3 M NaCl. This indicates that in the presence of high concentrations of salt, the electrostatic contributions to the transition are largely screened out.

The disappearance of electrostatic contributions greatly simplifies the theoretical analysis of the effects of ion binding on the transition. In previous analyses of ion effects on the transitions of carrageenans (Nilsson *et al.*, 1989; Nilson & Piculell, 1989, 1991; Zhang *et al.*, 1991) the starting point was to express the chemical potential difference between coil and helix (per disaccharide unit), $\Delta\mu_{tot}$, as a sum of three contributions due to electrostatic, ion binding, and all other interactions. The electrostatic contribution, $\Delta\mu_{el}$, was calculated within the PBCM, modelling the carrageenans as charged cylinders of uniform surface charge density. For the low-sulfated carrageenans studied here, however, a model with uniform charge density is expected to give a poor description, since the distance between charged groups on polysaccharide is not negligible compared to the electrostatic screening length. Therefore, we limit our analysis to the data where experiment indicates that $\Delta\mu_{el}$ may be neglected and write, accordingly

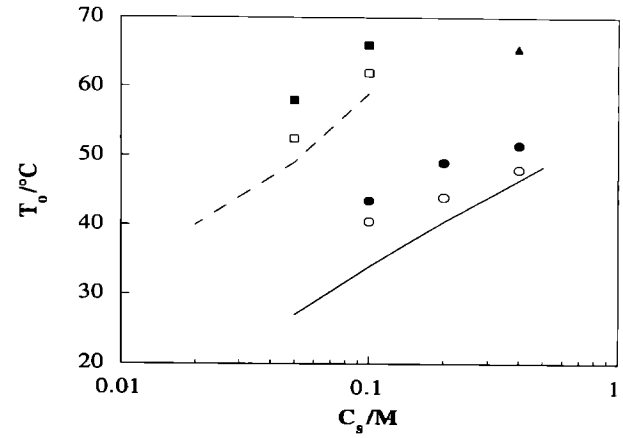


Fig. 5. Variation of helix onset temperatures with the concentration of added NaCl (circles) or CsCl (squares) in 0·4% EG (filled symbols) and 0·4% DF (open symbols). Lines indicate data for furcellaran (Zhang *et al.*, 1991) in NaCl (solid) and CsCl (dashed). The filled triangle corresponds to EG in 0·1 M CsCl + 0·3 M NaCl.

$$\Delta\mu_{tot} = \Delta\mu_{nonel} + \Delta\mu_{binding} \quad (1)$$

where

$$\Delta\mu_{nonel} = \Delta H_{nonel} - T\Delta S_{binding} \quad (2)$$

As usual, it is assumed that ΔH_{nonel} and ΔS_{nonel} are independent of temperature and salt concentration. For a given conformation, and neglecting electrostatic interactions, the binding free energy may be expressed as (Schellman, 1987; Nilsson & Zhang, 1990; Nilsson & Piculell, 1991).

$$\mu_{binding} = (kT/n)\ln(1 + K_o c_o) \quad (3)$$

where n represents the number of disaccharide units per binding site, K_o is the binding constant and c_o is the bulk concentration of binding ions. As there is no evidence of cation binding to carrageenan coils, $\mu_{binding} = 0$ for the coil conformation. At the helix onset temperature $\Delta\mu_{tot}/kT$ is a constant independent of the salt concentration and salt identity (Nilsson & Piculell,

Table 1. Calculated Cs^+ binding constants for low sulfated carrageenans at 65·5°C

Sample	$-\Delta H_{nonel}$(kJ/mol)	$K_o(M^{-1})$; $n = 2$	$K_o(M^{-1})$; $n = 1/f_{kappa}$
EG	8·1	2·9	5·6
EG	4·05	1·4	2·5
kappa	—	1·2	0·47

1989). Therefore, $\Delta\mu_{tot}/kT$ of EG in 0·4 M NaCl at 51·5°C has the same value as $\Delta\mu_{tot}/kT$ in 0·3 M NaCl + 0·1 M CsCl at 65·5°C. It is thus possible to deduce the cesium binding constants for the helical conformation from these data, once ΔH_{nonel} is known. Since calorimetric determinations of transition enthalpies for carrageenans also contain contributions due to ion binding and helix aggregation, it is difficult to obtain a reliable value of ΔH_{nonel} from experiment. Instead, we will base our estimate on $\Delta H_{nonel} = -8{\cdot}1$ kJ/(mol disaccharide), as previously deduced from a similar thermodynamic analysis of the transition of unmodified furcellaran, allowing for a 50% uncertainty in this value. In this way, we have calculated (Table 1) Cs^+ binding constants for EG corresponding to $n = 2$ and $n = 1/f_{kappa}$.

For comparison, we also include in Table 1 calculations, performed as described previously (Nilsson & Piculell, 1991), of binding constants for kappa-carrageenan at 65·5°C: The PBCM was applied on the experimental data of Rochas & Rinaudo (1980) to calculate $\Delta\mu_{el}$ and $\Delta\mu_{binding}$ at 65·5°C in CsCl and NaCl solutions individually, at the respective salt concentrations required for helix formation at the specified temperature. As above, the binding constant was obtained from the requirement $\Delta\mu_{tot}(NaCl) = \Delta\mu_{tot}(CsCl)$. Here $\Delta\mu_{nonel}$ cancels, however, since the calculations refer to the same temperature for both salts. Therefore, the binding constants calculated for kappa-carrageenan in Table 1 are independent of ΔH_{nonel}.

Table 1 shows that the assumption of a constant site density, independent of the degree of sulfation, explains the cesium specificity of EG by a binding constant which is almost the same as for kappa-carrageenan, while the assumption of $n = 1/f_{kappa}$ requires at least a five-fold increase in the binding constant for EG.

CONCLUSIONS

A strong cation specificity remains for low sulfated carrageenans. This specificity originates from the binding of cations to the carrageenan helices, as confirmed by Cs-133 NMR. Comparisons of chemical shift data from carrageenans of different origin indicate a variation in the chemical shifts of bound ions. The cation specificity of the conformational transitions of low sulfated carrageenans is well predicted by a thermodynamic model with the assumption that the density of the cation binding sites, as well as the binding constants, are the same as for furcellaran and kappa-carrageenan.

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