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Thermodynamics of polysaccharide polyelectrolytes: Enthalpy changes in the conformational transition of κ-carrageenan

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ABSTRACT - *An approach, based entirely on Counterion Condensation (CC) polyelectrolyte theory and on thermodynamic consideration, is used to derive the ionic and the non-ionic energy contributions for processes involving the conformational transition of a linear polyelectrolyte. As an example of application, the conformational transition of the algal polysaccharide, κ-carrageenan, is analysed. This anionic polymer undergoes a thermoreversible disorder-order conformational transition which parallels specific binding of cations (as K^+, Cs^+,...) and, quite unusually, of the iodide anion. Experimental data on the conformational process and on the binding process will be compared with the theoretical prediction.*

INTRODUCTION

The effect of ionic strength on the behavior of a polyelectrolyte chain is the most striking and universally accepted one in the study of the interactions between linear charged biopolymeric species and ions in solution. However, the computational handling of the long-range electrostatic interactions in polymorphic macromolecular chains still remains difficult, although under some simplified circumstances it can be reasonably treated. The range of validity of such conditions has restricted the application of the theoretical results to cases in which the univocity of the chain structure and the absence of variations due to "specific" binding were considered as essential.
In the field of linear biopolymers (in particular, ionic polysaccharides), a self-consistent framework has been proposed for the proton binding process[1-2] on a combined basis of experimental solution thermodynamics together with an extensive use of molecular mechanics for the conformational analysis of structural moieties and with the application of polyelectrolyte theories to treat the long range electrostatic interactions.
Following these guidelines, an analysis of a thermodynamic model is outlined for an ion-induced conformational transition, with the aim of finding correlations between the calculated changes of thermodynamic quantities and the experimental results. In particular, the purpose of the present paper is to provide some understanding of compatibility between the conformational properties and the energetics of the κ-carrageenan molecules under different physical states.

THE κ-CARRAGEENAN POLYSACCHARIDE

Algal polysaccharides of the carrageenan family form thermally-reversible gels of industrial importance as a function of temperature, salt concentration and type of cation. The idealised pattern of κ-carrageenan is a dimeric alternating sequence of a (1-3)α-D-galactose-4-sulphate and of a (1-4)-3,6-anhydro-α-D-galactose residues. On decreasing the degree of sulphation from ι-carrageenan, through κ-carrageenan and furcellaran, to agarose, the gels formed increase in rigidity, are less elastic, more brittle, and require lower concentrations of polysaccharides for their formation. Agarose structure differs from that of carrageenan, in the (1-4)-3,6-anhydro-α-D-galactose residue being replaced by the L-enantiomer and being totally nonsulphated. A critical review of the different models proposed for the gelation process has been given by Morris[3]. To summarise some pertinent facts, the secondary structure of κ-carrageenan is easily recognised as being of random-coil type at high temperature and low salt concentration[4-6], while an ordered helical conformation is adopted at low temperature and/or high ionic strength[7-11]. A typical phase diagram

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showing the correlation between the temperature of transition (T_m) and the ionic strength of the solution (I_m) is shown for different salts in Figure 1. Most experimental data on the ordered phase are in favour of a dimeric structure. However, there are controversial interpretations whether this implies a double helix, i.e. coaxial duplex, or duplex of ordered single-strands, with no coincidence of chain axes. It has also been claimed that the ordered conformation involve double helical structures in agarose as well as the carrageenan-like polysaccharides, although for κ-carrageenan the evidence is less conclusive, and for ι-carrageenan stable single helical entities are observed. Optical rotation measurements as a function of the temperature on segments of κ-carrageenan show a concentration-dependent cooperative character, indeed the ordering process appears to be a pre-requisite for the subsequent aggregational step[11]. The marked hysteresis is also taken as evidence of the complexity of the degree of aggregation, and very recent electron microscopy studies have shown the different microstructure of gels formed with different cations[12]. The other peculiar characteristics of κ-carrageenan reside in the ability to bind anions, particularly iodide, giving rise to similar ordering phenomena as detected by optical rotation and NMR data[13], without observing an increase of molecular weight[5].

Despite all these intriguing conformational problems, only recently the study of statistically sequenced conformational states has revealed the bimodal distribution of the distances between nearest-neighbour sulphate groups[14]. These findings of different and bimodal topological properties of the chain may be of crucial interest to understand the ion binding exhibited by carrageenan and confirm the specific influence of the ionic strength (and ion type) on the configurational statistics of the carrageenan. From the same molecular mechanical study, regular helical conformations for κ-carrageenan can simply be derived from the two energy minima, b_1 and b_2. However, the helical parameters obtained either for a coiled helix with conformer of b_1 type (0.686 nm/disaccharide) or for the extended helix with conformer of b_2 type (0.956 nm) do not fit the helical conformation deduced from the x-ray fibre diffraction data (0.833 nm)[15]. An average value of 0.95 nm is obtained for the statistical random coil chain conformation (i.e., statistically averaged over all accessible conformational states), while a larger value of 1.03 nm is currently quoted in the literature, as deduced from a resemblance of the κ-carrageenan backbone with that of hyaluronan[4], and with support from the counterion activity coefficient data[10].

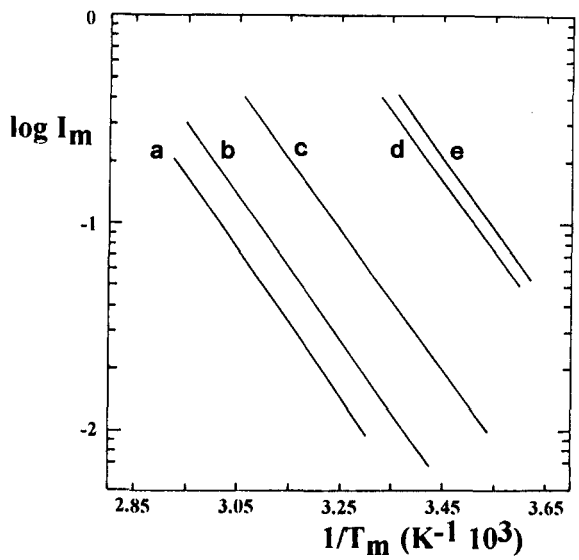

Figure 1. Counterion dependence of the relationship between the melting temperature, T_m, and the ionic strength, I_m, of κ-carrageenan for different counterions: (a) Rb^+, (b) K^+, Cs^+, (c) NH_4^+, (d) Na^+, $N^+(CH_3)_4$ and (e) Li^+. Reproduced with permission from ref. 10.

THE THERMODYNAMIC THEORY

The general approach. The great value of using a thermodynamic approach for handling the energetics of biopolymer phase transitions is in the conceptual simplicity of the formalism and the ease of manipulation of the analytical expressions. Let us consider the general case of a biopolyelectrolyte in aqueous solution which may undergo a conformational transition due to changes in temperature (T) and ionic strength (I). At mid-transition points (T_m,I_m), the total free energy change vanishes,

$$\Delta G^{tot}(T_m,I_m) = \Delta G^{ion}(T_m,I_m) + \Delta G^{ni}(T_mI_m) = 0 \qquad (1)$$

Equation (1) shows that the evaluation of nonionic term seems to be better affordable on the basis of

polyelectrolyte theories (i.e., from the ionic free energy term) than attempting the theoretical calculation of all proper interactions contributing to the non-ionic term. This approach has already been successfully applied when dealing with the charge-induced conformational transition of poly(α-L-glutamic acid)[16].

The problem of calculating the ionic free energy difference between the initial (i) and final (f) conformations, $\Delta G^{ion} = G_f^{ion} - G_i^{ion}$, for the appropriate set of T_m,I_m pairs can be done in the framework of different polyelectrolyte theories. Hereafter, the outline is given of the calculation of G^{ion} along the lines of the counterion condensation (CC) theory[17]. Although the CC theoretical treatments of the free energy and the enthalpy changes for a polyelectrolyte transition have been reported in different previous publications[16,18], it is considered convenient for clarity to report here some results. To justify the choice of the approach, it suffices to quote that the very marked theoretical differences between CC and other popular approaches (e.g., the Cell Model, CM) reside in the limiting conditions of the first and of the concentrate cell model (CM) of the second; both modelling approaches converge to the same quantitative results under some comditions. Application of CC theory has been made to many experimental cases, and in particular a correlation has been made between the theoretical predictions and the calorimetric data on the processes of protonation, dilution and mixing with ions[18].

In the CC approach, the reduced molar (rm) excess free energy of polyelectrolytic nature, $\mathbf{g}^{ion} = G^{ion}/RT$, is factorized into two main contributions: i) a purely electrostatic term, $\mathbf{g}^{el}$, and ii) a free energy of mixing of mobile species, $\mathbf{g}^{mix}$ (i.e., $\mathbf{g}^{ion} = \mathbf{g}^{el} + \mathbf{g}^{mix}$). The reference state is provided, as usual, by a solution at the so-called "iso-ionic" conditions.
The expression for $\mathbf{g}^{el}$ is given by:

$$\mathbf{g}^{el} = -\ \xi\ (q_{eff})^2\ \ln(1 - \exp(-\kappa b)) \qquad (2)$$

where q_{eff} is the net, "effective" charge on each ionized site on the polyelectrolyte and κ is the reciprocal of the Debye length. The polyelectrolytic system is characterized by the charge density parameter $\xi = l_b / b$, where l_b is the Bjerrum lenght and b is the projection of the intercharge distance onto the polyelectrolyte chain axis. In the case of monovalent ions, for all structural values of $\xi > 1$, a defined amount of counterions will "condense" from the solution into the domain of the polymer chain so as to reduce the "effective" value of ξ to unity. For water at 298 K, $\xi = 0.714/b$ (with b expressed in nm).

In the most general case of either mono- or pluri-valent ions ($z \geq 1$), whenever $\xi z > 1$, then condensation of counterions occurs. Therefore, the reduction of the effective charge of the ionized groups on the polyelectrolyte is given by $q_{eff} = q_{struct}\ (1 - r z)$, where q_{struct} is the value of the fixed charge on the polyion (assumed here to be = 1). The parameter r is the (molar) fraction of condensed counterions of valence z, which will effectively shield the fraction r z of fixed charge. The numerical value of r stems from the condition of stability of the system at infinite dilution (limiting law condition)[17]:

$$r = z^{-1}\ [1 - 1/(z\xi)] \qquad (3)$$

However, if condensation occurs, then $\mathbf{g}^{mix}$ has to be considered for the following free energy contributions of the different mobile species:

$$\mathbf{g}^{mix} = \mathbf{g}^{cond} + \mathbf{g}^{free} + \mathbf{g}^{coions} + \mathbf{g}^{solv} \qquad (4)$$

These terms are due to changes of the entropy of mixing of condensed counterions, of free counterions, of coions and of solvent molecules, respectively, and are given by:

$$\mathbf{g}^{cond} = r \ln [r / ((1+R_S)V_pC_p)] \qquad (5)$$

$$\mathbf{g}^{free} = (1+R_S - r) \ln [(1+R_S-r) / [(1+R_S)(1-V_pC_p)]] \qquad (6)$$

$$\mathbf{g}^{coions} = R_S \ln [1 / (1-V_pC_p)] \qquad (7)$$

$$\mathbf{g}^{solv} = r \qquad (8)$$

where R_S stands for the molar ratio between the concentration of the added salt and that of the polymer, C_p, and V_p is the molar volume of the condensed phase, expressed in liters per mole of fixed charge.
From the Gibbs free energy of electrostatic origin, as given by the equations (2) and (4)-(8), analytical expression for all other state functions can be easily derived. In fact, once the polyelectrolytic free energy

term has been defined for the conditions of the system, then the corresponding electrostatic (excess) enthalpy of the solution is calculated straightforward by proper derivatization.

$$H^{el}(T,I) = \left[d\left(G^{el}(T,I)/T\right)/d(1/T)\right]_{nj,b} = -\tfrac{1}{2}\, n_e RT\xi \left(1+\frac{d\ln D}{d\ln T}\right)\left[2\ln\left(1-e^{(-kb)}\right)+\frac{kb}{e^{kb}-1}\right] \quad (9)$$

The above equations have been always referred to the rather simple case in which the polyelectrolyte state functions may change by addition of solvent (dilution), of simple salt (mixing) or protons (protonation of a weak polyacid). In addition to the condensation phenomena, other physical responses may also occur simultaneously, which may mask the central statement of the CC theory that the onset of the critical value ξ constitutes a thermodynamic instability which must be compensated by counterion condensation. In fact, chain extension and/or chain disaggregation may occur upon the increasing of charge density, and the energetic instability actually becomes a function of the conformational state of the polyelectrolytic chain, i.e., the polyelectrolyte geometry is affected by ionic strength, charge density and temperature. From the standpoint of polyelectrolyte approach, conformational transitions resulting from intramolecular variation of torsional angles, as well as from intermolecular topological rearrangements resulting from side-by-side or intertwined alignment of chains, give in all case a change of the nominal charge density parameter, ξ_{max}. In a previous series of papers[16] the helix-coil transition of poly(α-L-glutamic acid) was modelled by the change of average charge distance from b = 0.15 nm (ξ_{max} = 4.76) to b = 0.35 nm (ξ_{max} = 2.04).

Analogously to the free energy derivation of one of such processes, the electrostatic enthalpy of transition is defined by the solution parameters and by the nominal charge density of the two conformational states of the polyelectrolyte, $\Delta_{tr}H^{el} = (H^{el})_f - (H^{el})_i$. The most common case is that of an ion-induced conformational transition with a final state characterized by a larger value of ξ. A throughout derivation of the electrostatic contribution to the conformational change, as it is obtained from the application of the above equations, has been reported in detail elsewhere[18]. When comparing these predictions with experimental results, the total value of the electrostatic enthalpy of transition contains however a contribution which arises from the net enthalpy effect on mixing a polyelectrolyte with salt. An equation of this effect has also been given[18].

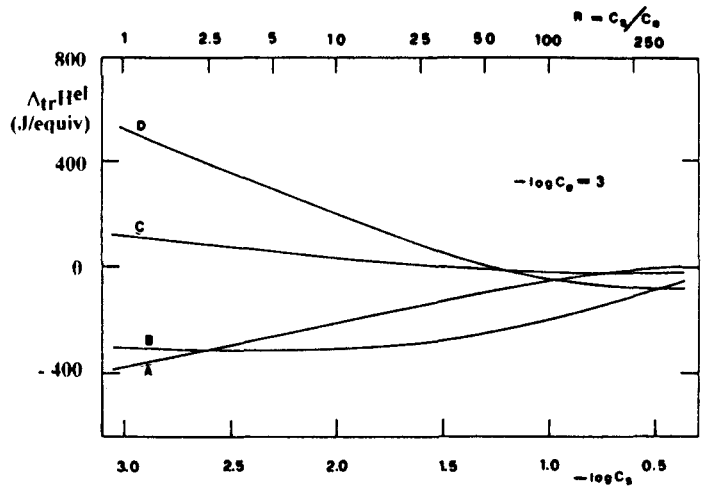

Figure 2. Dependence of the ionic contribution to the enthalpy of transition, $\Delta_{tr}H^{el}$, upon the concentration of added salt for the coil-to-helix (A) conformational transition of κ-carrageenan (ξ_i = 0.693, ξ_f = 0.870). Other curves refer to: (B) ξ_i = 0.693, ξ_f = 1.740; (C) ξ_i = 1.386, ξ_f = 1.647 and (D) ξ_i = 1.386, ξ_f = 3.293.

Figure 2 shows the dependence of the electrostatic contributions of $\Delta_{tr}H^{el}$ on the logarithm of the salt concentration for a conformational transition from ξ = 0.69 to ξ = 0.87 (corresponding to the coil to helix transition of *k*-carrageenan) and for other pertinent cases. It seems clear that all the enthalpic effects of electrostatic origin on mixing the polyelectrolyte with salts are small and endothermic. This result implies that the large change experimentally observed (of one order of magnitude larger, as schematically reported in Figure 3) can be safely ascribed to non-ionic contributions of conformational and/or binding origin.
Passing from the isothermal conditions to the thermally induced conformational transitions, the large and constant non-ionic enthalpy is in fact the basic assumption for the CC theory to predict a simple relationship

between the values of the melting temperatures, T_m, and the logarithm of the ionic strength, I_m:

$$d(\log I_m) / d(T_m^{-1}) = - \Delta_{tr}H / 9.575\, F(\xi) \qquad (10)$$

where $\Delta_{tr}H$ is the value of the enthalpy of transition (in J per mole of charged groups) determined by means of calorimetry. The linearity which is experimentally observed (Figure 1) implies, indeed, that the enthalpy change is essentially due to non-ionic contributions and largely independent of I. The function $F(\xi)$ depends on the charge density of both the final state (subscript f) and the initial state (subscript i), within the common condition that $(\xi)_f < (\xi)_i$, that is the final state is characterized by a smaller value of the charge density. The value of $F(\xi)$ is given in the three following cases:

if both $(\xi)_i$ and $(\xi)_f < 1$ then $F(\xi) = (\xi)_f - (\xi)_i$ (11)

if both $(\xi)_i$ and $(\xi)_f > 1$ $F(\xi) = (\xi)_f^{-1} - (\xi)_i^{-1}$ (12)

if $(\xi)_i > 1$ and $(\xi)_f < 1$ $F(\xi) = 2 - (\xi)_f - (\xi)_i^{-1}$ (13)

This relation has been successfully applied to the transition processes of different biopolymers, including DNA[19,20].

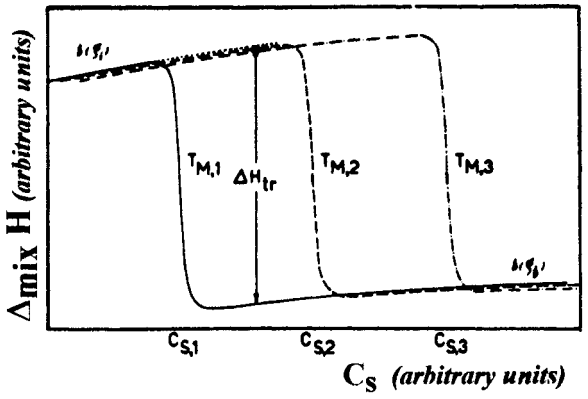

Figure 3. Schematic dependence of the isothermal enthalpy change upon mixing a simple salt (at final concentration C_S) with a biopolyelectrolyte undergoing a cooperative conformational transition between two states defined by ξ_i and ξ_f, respectively, at three different temperatures. The small dependence of the enthalpy of mixing for the initial and the final conformation is also shown.

RESULTS

On the basis of linear and parallel trend shown by the phase diagram (in Figure 1), the assumptions made in the thermodynamic treatment turn out to be appropriate. The change of ΔG^{ion} for the conformational transition (for the conformational states previously defined) can be calculated for different cations at each given temperature T_m (therefore, at different ionic strength). Given the constancy of the slope with the temperature, the stabilising (or destabilising) factor is of entropic origin, to be associated to the character of the individual counterions.

The isothermal enthalpy change on mixing the TMA salt of κ-carrageenan with CsCl solutions (corrected for the heats of dilution of both polymer and salt) is reported in Figure 4 as a function of the logarithm of the ionic strength for four different temperatures (279.4, 283.2, 298.2 and 308.2 K). The curves at lower temperatures clearly indicate the two-step process, which has been associated in the present model to the disorder-order transition and to the subsequent cooperative aggregation. With the thermodynamic hypotheses made above, the analysis of data of Figure 1 gives for the non-ionic enthalpy of transition a value of -5.86 kJ/mol (for the coil-helix transition) and of about -20 kJ/mol (for the transition with formation of dimers of helical structures). These values have been compared with isothermal calorimetric experiments reported in Figure 4, and with the range of values from -5.44 kJ/mol (at low ionic strength) to -18 kJ/mol (at high ionic strength) obtained by iso-ionic experiments carried out by means of differential scanning microcalorimetry. Other similar data obtained by other authors[21], as well as unpublished microcalorimetric data of this laboratory with a number of different ions[22], evidence the common enthalpic behaviour of κ-carrageenan undergoing the conformational transition. In fact, cations like Na^+ and NH_4^+, which do not show any specificity for the κ-carrageenan chain, do promote at high concentration a sigmoidal conformational transition. In addition to this, Tl^+ ions induce the same two-step process observed with K^+, thus confirming the original proposal of a complex phase diagram[23].

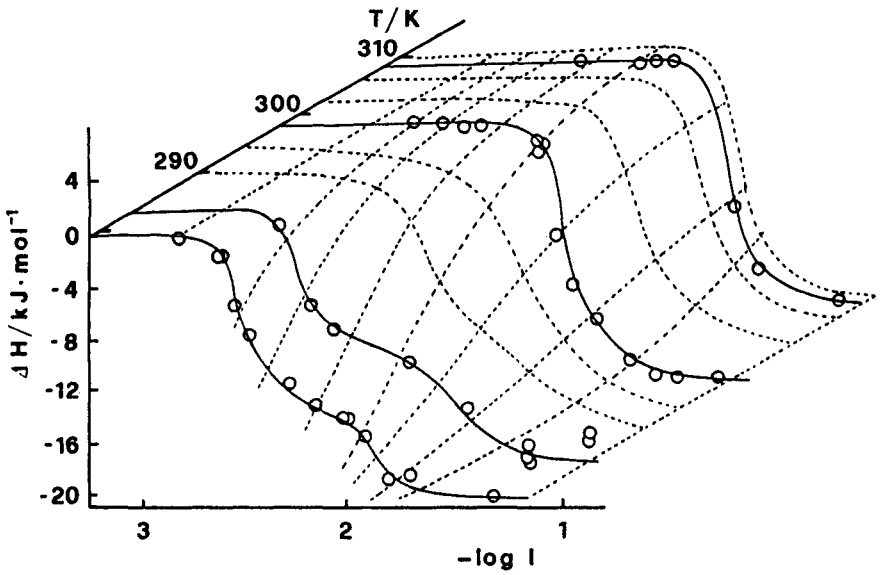

Figure 4. Isothermal microcalorimetric data of mixing κ-carrageenan (TMA salt, C_p= 10^{-3} mol/L) with CsCl as a function of logarithm of salt concentration (at four temperatures: 279.4, 283.2, 298.2 and 308.2 K). All data are corrected for the enthalpies of dilution of the two components.

The peculiar case of iodide ions is worth to mentioning. The addition of suitable amounts of lithium (or sodium, or tetramethylammonium) iodide (but not of the chloride forms at the same concentrations) to a κ-carrageenan solutions brings about the same increase of optical activity which is observed in the case of the conformational change that accompanies the gelation induced by KCl or CsCl. It is therefore straightforward to conclude that I^- is able to induce the same conformational ordering of the chain that is commonly accepted as the prerequisite for chain association.

However, probably the most extensive and accurate macromolecular investigations ever on κ-carrageenan, performed in those conditions using membrane osmometry[8,24] and multi-angle light scattering[5,24], have undisputedly shown that there is no change in the relative molar mass upon the iodide-induced conformational change. The transition is therefore intramolecular, from a disordered (wormlike?) coil to an ordered single helix.

That the peculiar behavior of the iodide anion toward the κ-carrageenan polymer was not mainly due to an unspecific "solvent effect" but, rather, to selective site-binding, it was proved by ^{127}I-NMR spectroscopy[13].

Anion binding to polyanions is not a common phenomenon, particularly so in view of the unfavourable change of the electrostatic (polyelectrolytic) free energy. For anion binding to happen, the most reasonable explanation was to assume a driving contribution from favourable energetic interaction terms (e.g. of the ion-dipole and/or dipole-dipole type), to be detected experimentally as negative enthalpy changes of interaction.

Having reasonably assessed that the enthalpy difference between the ordered single helix and the disordered coil of k-carrageenan is about -5.9 kJ/mol, one should then expect a significantly more negative value of ΔH upon helix formation by I^- binding. The experimental findings have not failed to support such an expectation, giving a value of about -13 kJ/mol in conditions of full conformational transformation[22], therefore accounting for the high stability of the I^- binding helix at low temperatures.

The intriguing problem of anion/polyanion binding have been also tackled theoretically, still within the frame and with the help of Manning's counterion condensation theory and with the aim at giving a complete thermodynamic description of the process of binding/conformational change.

A subtle combination of long-range physical interactions (i.e. the polyelectrolytic ones) and of specific chemical ones have been treated with some resemblance to the case of the (polyacid) proton dissociation process, to achieve a picture of the binding/ordering process which is consistent with the numerous bounds stemming from the experimental data[25].

EXPERIMENTAL

The polysaccharide, obtained from CECA S.A., (France), had more than 90% of κ-carrageenan-like sequences, according to ^{13}C NMR. The commercial sample was repeatedly precipitated with KCl and then exchanged through an anionic Dovex resin converting all cations with tetramethylamonium (TMA). The equivalent relative weight of the TMA-repeating unit has been assumed in all calculation as 495.
The polymer sample has been characterised by GPC-LALLS in aqueous 0.1 M TMA Cl, giving an Mw value of 355500 and a polydispersity index of 1.90. In the same solvent the intrinsic viscosity was extrapolated to be 4.51 dL/g at 25°C.
Calorimetric experiments were carried out with a LKB 10700-2 microcalorimeter thermostatted at the given temperature and located in a thermostatted room at nearly the same temperature. All experimental procedures and apparatus are described elsewhere[1].
The calorimetric experiments have been carried out by mixing the polysaccharide solution (TMA salt of k-carrageenan at concentration of 2 10^{-3} M with an equal volune of aqueous solution containg the salt (CsCl, KCl, TMACl, TMAI and $TlClO_4$) at a given concentration. The results are reported as the enthalpy changes of mixing, per mole of polysaccharide, as a function of the concentration ratio, R_S, of salt to polymer.

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REFERENCES

1. A. Cesàro, F. Delben and S. Paoletti, *Int. J. Biol. Macromol.*, **12**, 170-176 (1990).
2. A. Cesàro and M. C. Sagui, *Makromol. Chem., Macromol. Symp.*, **58**, 39-61 (1992).
3. V. J. Morris, *in Funcional Properties of Food Macromolecules, J. R. Mitchell and D. A. Ledwards (Eds), Elsevier Appl. Sci. Pub., London (1986), p. 131-143.*
4. H. J. Vreeman, T. H. M. Snoeren and T. A. J. Payens, *Biopolymers*, **19**, 1357-1374 (1980).
5. D. Slootmaekers, C. De Jonghe, H. Reynaers, F. A. Varkevisser and C. J. Bloys van Treslong, *Int.J. Biol. Macromol.*, **10**, 160-168 (1988).
6. D. Slootmaekers, M. Mandel and H. Reynaers, *Int. J. Biol. Macromol.*, **13**, 17-25 (1991).
7. S.T. Bayley, *Biochim. Biophys. Acta*, **17**, 194-205 (1955).
8. O. Smidsrod, I.-L. Andersen, H. Grasdalen, B. Larsen, and T. Painter, *Carbohydr. Res.*, **80**, C11-C16 (1980).
9. E. R. Morris, D. A. Rees and G. Robinson, *J. Mol. Biol.*, **138**, 349-362 (1980).
10. C. Rochas and M. Rinaudo, *Biopolymers*, **19**, 1675-1687 (1980).
11. S. Paoletti, F. Delben, A. Cesàro and H. Grasdalen, *Macromolecules*, **18**, 1834-1841 (1985).
12. A.-M. Hermansson, *Carbohydrate Polymers*, **10**, 163-181 (1989).
13. H. Grasdalen and O. Smidsrod, *Macromolecules*, **14**, 1842-1845 (1981).
14. R. Urbani, A. Diblas and A. Cesàro, *Int. J. Biol. Macromol.*, (in press).
15. R. P. Millane, E. U. Nzewi and S. Arnott, in *R. P. Millane, J. N. BeMiller and R. Chandrasekaran (Eds.), Frontiers in Carbohydrate Research-1, Elsevier Applied Science, London (1989), p. 104.*
16. S. Paoletti, A. Cesàro, C. Arce Samper and J.C. Benegas, *Biophys. Chem.*, **34**, 301-309 (1989).
A. Cesàro, S. Paoletti and J.C. Benegas, *Biophys. Chem.*, **39**, 1-8 (1991).
A. Cesàro, S. Paoletti, S. Guidugli and J.C. Benegas, *Biophys. Chem.*, **39**, 9-16 (1991).
17. G. S. Manning , *Account Chem. Res.*, **12**, 443-449 (1979).
18. S. Paoletti, A. Cesàro, F. Delben, V. Crescenzi and R. Rizzo, in *Microdomains in Polymer Solutions, P. Dubin (Ed.), Plenum Press, New York (1985), p.159.*
19. G. S. Manning, *Q. Rev. Biophys.* **11**, 179-246 (1978).
20. M. T. Record, C. F. Anderson and T. M. Lohman, *Quart. Rev. Biophys.*, **11**, 103-178 (1978).
21. C. Rochas and J. Mazet, *Biopolymers*, **19**, 2825-2833 (1984).
22. A. Beltrame, *Doctoral Thesis, University of Trieste*, (1986).
23. S. Paoletti, O. Smidsrod and H. Gradsladen, *Biopolymers*, **23**, 1771-1794 (1984).
24. O. Smidsrod and H. Gradsladen, *Hydrobiologia*, **116/117**, 19-28 (1984).
25. S. Paoletti, J. C. Benegas, A. Cesàro and R. Rizzo, *in preparation.*